The relationship between hyperactivated motility and protein tyrosine phosphorylation in two different IVF media for bull, boar and stallion spermatozoa.

Abstract

This study is an orientating study with the aim to provide a comprehensive basic characterization of hyperactivated motility patterns in species with functional IVF systems, i.e. cattle and pigs. It was also aimed to compare the observed motility changes in stallion spermatozoa which are subjected to bovine or porcine *in vitro* fertilization (IVF) conditions. The occurrence of tyrosine phosphorylation in the tail of viable spermatozoa under the respective incubation conditions was visualized by indirect immunofluorescence. Spermatozoa of boar, bull and stallion were incubated under established IVF conditions. For boar sperm the IVF medium modified tris buffered medium (mTBM) was used and for bull sperm a modified TALP medium was used. Stallion spermatozoa were incubated in both media. The motility patterns after 0.25, 1-6, 8 hours incubation were analyzed with a CASA system and statistically divided into clusters by hierarchical cluster analysis. The occurrence of tyrosine phosphorylation in the tail of viable spermatozoa under the respective incubation conditions was visualized by indirect immunofluorescence at selected time points. Live - dead staining was performed using Ethidium homodimer-1 and Hoechst 33342. The basic characterization of hyperactivated motility under successful IVF circumstances was not possible due to immediate agglutination of both boar and bull spermatozoa. Furthermore the development of hyperactivated motility and protein tyrosine phosphorylation in stallion spermatozoa seemed independent. The bovine IVF protocol appeared to have a higher efficiency in inducing protein tyrosine phosphorylation of the sperm tail of viable stallion sperm
In conclusion this thesis supports the idea that protein tyrosine phosphorylation of the sperm tail might not be relevant for fertilization.

Keywords: hyperactivated motility, protein tyrosine phosphorylation, stallion spermatozoa, IVF media.

Introduction

In vitro fertilization in horses

Co-incubating a mature oocyte with multiple capacitated spermatozoa in a petri dish can, under the right conditions, result in fertilization of the oocyte. This process is called *in vitro* fertilization (IVF) and is successfully applied in many mammals including man (1), farm animals and laboratory animals (2, 3). However conventional IVF does not seem to work using equine gametes. In 1991, a laboratory in France produced two foals using IVF techniques. They made use of spermatozoa which were treated with calcium ionophore (4). Remarkably, since then no laboratory succeeded in creating a reliable IVF protocol for equine gametes or even obtain again a single foal. Both the maturation of the oocyte as well as the capacitation of the spermatozoa could be the limiting factor why IVF is not working in horses. As seen in a study by Palmer *et al.* (4) IVF turned out to be equally unsuccessful with *in vivo* matured oocytes as with *in vitro* matured oocytes suggesting oocyte maturation is not the limiting factor. Moreover, oviductal transfer of *in vitro* matured oocytes into inseminated mares resulted in fertilization rates as high as those obtained by artificial insemination (5). Assuming the obstacle to equine IVF resides at the level of sperm capacitation more research has been conducted concerning these physiological events i.e. hyperactivated motility and protein tyrosine phosphorylation (6-9).

Capacitation of spermatozoa

Before spermatozoa are able to fertilize a mature oocyte they have to undergo several molecular and biochemical changes which are collectively called capacitation (10, 11). These changes consist at the level of the plasma membrane i.e. increased membrane fluidity, cholesterol depletion and lipid raft aggregation. Cytoplasmatic changes that occur are protein tyrosine phosphorylation in the sperm tail and elevation of the intracellular pH, cyclic AMP and Ca^{2+} concentration (12, 13). A suitable environment is needed for the spermatozoa to undergo such changes. Bicarbonate (HCO_3^-), Ca^{2+} and albumin are known factors to induce *in vitro* the changes in spermatozoa that are necessary for fertilization in many species such as man and pig (14, 15). Most species also have species-specific factors that are essential capacitation triggers, e.g. heparin for bovine spermatozoa (16). Unfortunately, these triggers have yet to be identified in equids.

Hyperactivation of spermatozoa

It is crucial for sperm capacitation to reach a state of hyperactivated motility to be able to get through the zona pellucida of the oocyte (11, 17). The primary second messenger that triggers hyperactivated motility is Ca^{2+} (18). A study by Ho *et al.* (19) showed that after hyperactivated motility was initiated in bull sperm the intracellular Ca^{2+} concentration increased from 50nM to 400nM. However, Loux *et al.* (20) reported that an increasing Ca^{2+} concentration did not induce hyperactivated motility in stallion spermatozoa. Alkalinization of the cytoplasm on the other hand did induce both a hyperactivated motility response and cytoplasmic Ca^{2+} rise. Moreover, hyperactivated motility of equine spermatozoa has been induced using procaine which acts independently to extracellular Ca^{2+} influx (20, 21). These findings make it likely that there is a species-specific relationship between Ca^{2+} sensitivity and hyperactivated motility in the horse.

In various species, e.g. the pig, hyperactivated motility is also associated with highly increased cAMP dependent tyrosine phosphorylation of the flagellar proteins like protein kinase A (22, 23). HCO_3^- and Ca^{2+} directly regulate soluble adenylyl cyclase which results in cAMP generation and therefore activation of protein kinase A (24, 25). Hence this demonstrates the importance for HCO_3^- and Ca^{2+} in a capacitation medium.

CATSPER channels, through which extracellular Ca^{2+} flows, are present in the sperm tail and need to be activated to induce hyperactivated motility in mammals such as cattle and mice. Creating a CATSPER gene knock-out mouse resulted in infertility due to the spermatozoa not being able to achieve hyperactivated motility (26, 27). The trigger for these CATSPER channels is alkaline depolarization resulting in an elevated pH (28, 29). In stallion sperm mRNA for CATSPER1 protein was identified however the pH-sensor region showed species-specific differences indicating that the mechanism of action is likely different in horses (20).

Multiple approaches have been tested to find out what may overcome these species-specific differences in horses. One of those approaches was the supplementation of procaine to a potential IVF medium. Procaine turned out to be a potent inducer of hyperactivated motility in stallion spermatozoa (13, 30). Using procaine in a potential IVF medium was reported to result in cleavage rates exceeding 60%. However, none of these oocytes developed to the blastocyst stage. Leemans *et al.* (13) demonstrated that procaine does not induce proper oocyte cleavage, but rather cytokinesis without proper replication of DNA.

Definition and quantification of hyperactivated motility

Hyperactivation of spermatozoa is not defined by a single description. Therefore this paragraph portrays multiple approaches on hyperactivated motility in different species. Hyperactivation in monkey spermatozoa is described by Ishijima (*et al.*) using a phase contrast microscope equipped with a high-speed camera. The motility patterns of the hyperactivated spermatozoa were characterized by highly asymmetrical or highly symmetrical and high-amplitude flagellar beating patterns of the sperm tail. It is suggested that the asymmetrical beating is the first stage of hyperactivation which is followed by the symmetrical beating. Ishijima (*et al.*) described that high Ca^{2+} concentrations resulted in asymmetrical bending and a high cAMP concentration resulted in symmetrical bending. Furthermore, a decrease in beat frequency of the tail was observed at a high cAMP concentration in comparison to the high Ca^{2+} concentration. *In vitro* this results in figure eight, zigzag or circular swimming motions as seen in **Figure 1** (31).

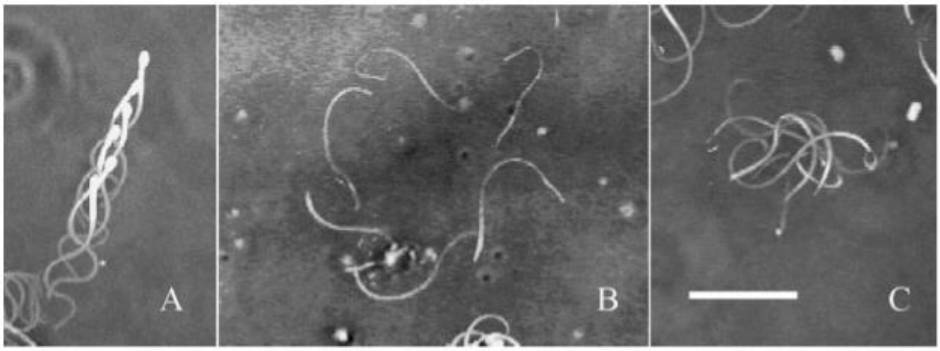


***Figure 1 Images of flagellar movement of activated and hyperactivated monkey spermatozoa**. **A:** activated spermatozoa **B:** hyperactivated spermatozoa with asymmetrical flagellar wave **C:** hyperactivated spermatozoa with symmetrical flagellar wave adjusted from Ishijima et al., 2006 (31).*

In a review written by Harayama, movement patterns of hyperactivated bull and boar spermatozoa are discussed. This review states that there are two types of hyperactivation: "nonfull type" and "full type". Full type hyperactivation of bull spermatozoa is characterized by twisting/figure eight-like movements. The non-full type hyperactivated bull spermatozoa show asymmetrical high amplitude beatings of the distal part of the sperm tail resulting in round counterclockwise circling motility patterns. Similar tail

beating pattern were observed in the boar spermatozoa whereas the boar spermatozoa showed a larger angle of bending of the proximal part of the sperm tail in comparison to the bull spermatozoa. These patterns were observed and classified by subjective visual inspection of movies divided into individual frames (32).
An alternative to subjective classification of sperm movement patterns is computer-assisted semen analysis (CASA). The CASA system can identify hyperactivated spermatozoa using preset thresholds for minimum curvilinear velocity (VCL), maximum path linearity (LIN) and for the amplitude of lateral head-displacement(ALH). The latter is used as an indirect measure of flagellar bend amplitude. A higher flagellar bend amplitude will lead to a higher lateral head displacement (18). There are no universally accepted criteria for the different hyperactivation settings. However, generally an increase in VCL and ALH and decrease in LIN in comparison to activated motility are considered to be characteristics for hyperactivated motility patterns (13, 20, 33).
Single-cell data obtained with the CASA system can be used for cluster analysis. Cluster analysis is done to acquire mean movement parameters from subgroups obtained after partitioning of the CASA data. By forming clusters using single cell data and their similarities no mean values are used as limit values for the different clusters. Furthermore the grouping of the cells is done objectively by giving the mean values of the different movement parameters. This provides a better understanding of the movement characteristics of a whole sample and for example of changes in movement pattern over time than general CASA analysis does (34).

Until today it is still unclear how to induce hyperactivation in stallion spermatozoa to enable them to penetrate the zona pellucida and achieve successful fertilization. At the same time, the reported means for the assessment of hyperactivated motility patterns are highly variable in literature. It ranges from subjective scoring of motility patterns to the assessment of changes in individual motility descriptors from computer-assisted semen analysis (CASA). Moreover, the tyrosine phosphorylation in the sperm tail that is considered as a hallmark of successful sperm *in vitro* capacitation and prerequisite for hyperactivated motility has never been demonstrated for viable spermatozoa. Therefore, the aims of this thesis are (a) to provide a comprehensive basic characterization of hyperactivated motility patterns in species with functional IVF systems, i.e. cattle and pigs, and (b) to compare the observed motility changes to changes in stallion spermatozoa which are subjected to bovine or porcine IVF conditions. The motility patterns will be recorded by means of CASA and analyzed by visual classification as well as by means of cluster analysis. The occurrence of tyrosine phosphorylation in the tail of viable spermatozoa under the respective incubation conditions will be visualized by indirect immunofluorescence.

Materials and methods

Reagents and media

All chemicals used were of analytical grade and purchased from Sigma Aldrich (Steinheim, Germany), unless stated otherwise. The porcine IVF medium was a modified Tris-buffered medium (mTBM) which consisted of 113.1 mM NaCl, 3.0 mM KCl, 20.0 mM Tris, 11.0 mM Glucose, 1.0 mM Caffeine (Fluka, 27600) and 7.5 mM CaCl2.2H2O. After the Osmolarity was adjusted to 275-290 mOsm, 0,1% of BSA (fatty acid free) was added and the medium was filter sterilized using a 0.45 µm filter (Merck Millipore, Darmstadt, Germany).

To modify the medium 1% Na Pyruvate and 1% Pen/Strep (Gibco 15140-148) was added. The medium should be modified and equilibrated 18 hours prior to the start of the experiment in the incubator on 38.5 °C and 5% CO_2. During this incubation the pH should stabilize from 10 to 7.6.

Bovine IVF medium was described by Parrish *et al.* (16) and consisted of modified versions of TALP medium in the article described as Sp-TALP and Fert-TALP. In this thesis SP-TALP is called Medium D and Fert-TALP medium E. Medium E is first prepared unmodified resulting in a minor alteration to the described Fert-TALP. Penicillamine, Hypotaurine, Epinephrine and Heparin are later added as described by Uhde *et al.* (35).

Experimental design
The highly diluted sperm concentration used in IVF protocols (2000 spermatozoa for porcine IVF and 1 x 10^6 spermatozoa for bovine IVF (16, 36)) are not compatible with the CASA system (SpermVision; Minitüb, Tiefenbach, Germany). At least 200 spermatozoa per sample should be detected to provide a reliable analysis (37). Therefore, a new concentration was devised. The first experiment consisted of a boar sperm concentration series to determine a suitable working concentration for the following experiments. For this experiment one boar was used. The second experiment was a time series from 15 min to 18 hours. This series was done to define timepoints, using the CASA system and cluster analysis, at which sperm from either pig or cattle show hyperactivated motility under IVF conditions. To compare the motility characteristics a redo of these time series was done using stallion sperm. The samples in this experiment were from 3 boars, 3 bulls and 6 stallions whereas 3 of the stallion samples were, after semen preparation, worked up following the porcine IVF protocol and 3 following the bovine IVF protocol. In the third experiment the reference points, determined from the second experiment, and a starting point were used to verify a change in tyrosine phosphorylation of the sperm flagella. From both bull and boar one sample was stained, from the stallions 2 samples were stained, one under porcine IVF condition and one under bovine IVF conditions.

Stallion semen collection and preparation
Stallion semen from seven mature stallions was collected at the Equine Reproduction Clinic of Utrecht University, the Netherlands. After filtration trough a gauze to get rid of excess debris, the semen was subjectively evaluated for motility at 100x magnification using phase contrast microscopy with a heated stage at 37°C. When the motility of the semen was ≥70%, the ejaculate was immediately transported to the laboratory for further processing. The semen concentration was analyzed using the Nucleocounter SP-100 (Chemotec, Allerod, Denmark) and the semen was diluted to the desired working concentration (30 x 10^6 cells/ml) in INRA96 extender (IMV Technologies, L'Aigle, France). Eight ml of sperm suspension was separated over a discontinuous Percoll gradient using four ml of 35% Percoll on top of two ml of 70% Percoll in a 15 ml centrifugation tube with conical bottom (falcon tube). Percoll suspensions were prepared in a saline medium (Composition:137 mM NaCl, 10 mM glucose, 2.5 mM ·KOH, 20 mM HEPES, pH of 7.40 at 20°C; 300 ± 5 mOsmol kg^{-1}) as described by Renaud and Nadeau (38). The tube was centrifuged on 300g for 10 minutes and immediately after on 750g for 10 minutes both at 25 °C in a swingout rotor centrifuge (Sigma 4k10, Salm en kipp bv., Breukelen, the Netherlands). After centrifugation, the excess fluid was removed making sure not to disturb the pellet and leaving approximately 150 µL of residual Percoll. The

concentration of the resuspended pellet was analyzed using the Nucleocounter SP-100 and then used for further processing.

Porcine semen preparation
Porcine semen from four boars of proven fertility was ordered at AIM Varkens KI Nederland (Vaassen, the Netherlands). The ordered semen is processed into insemination doses for pig artificial insemination by the company. The porcine semen was washed from the extender using PBS (buffered sodium solution). The washing contained two steps centrifuging for four minutes on 700g and 25 °C . After washing the concentration of the remaining pellet was analyzed using the Nucleocounter Sp-100. The pellet then was used for further processing.

Bovine semen preparation
Cryopreserved bovine semen from 4 bulls was obtained from the Utrecht University stock. On the day of the experiment semen was thawed at 37 °C for one minute.
In a 15 ml falcon tube one ml of semen was places on top of a 45/90 Percoll gradient using one ml of each. The falcon tube was centrifuged for 30 minutes on 700g and 27 °C. Excess fluid in the thawed straw was used for motility examination using a heated stage (37 °C) and light microscopy on 40x. After centrifugation the excess fluid was removed leaving 150 µL of fluid with an intact pellet. The concentration of the pellet was analyzed using the Nucleocounter SP-100 and the pellet was used for further processing.

Semen Hyperactivation
Bovine IVF protocol
The bovine and stallion ejaculates were processed as described in the semen preparation paragraph. Before the concentration was analyzed, 50 µL of medium D was added to the pellet. Afterwards 5 ml of medium E was "modified" using 200 µL of Heparine and 200 µL PHE (thawed just before use). The pellet was diluted to $15x10^6$ cells/ml using the modified medium E and 0,5 ml was placed in 8 post tubes. Each tube was labeled according to incubation time, i.e. "0,15, 1, 2, 3, 4, 5, 6, 18". The tubes were later used for CASA analysis using a Leja slide. The tubes were incubated at 38.5 °C and 5% CO_2. After 15 min incubation the first tube (0,15) was analyzed. The tubes were transported from the incubator to the CASA system using a 37 °C heated block and a cap was added. Each of the following 6 hours a new tube was analyzed. After 18 hours the final tube was analyzed using the CASA system.
Porcine IVF protocol
After the porcine and stallion ejaculates were processed as described in the semen preparation paragraphs they were placed in mTBM. The dilution of the pellet and further processing was identical to the bovine IVF protocol.
INRA Control
As a control of the sperm motility under incubation circumstances, one experiment with a non-capacitating medium (INRA 96) was conducted. The experimental procedure was identical to the porcine IVF protocol but instead of capacitation medium (mTBM), INRA 96 was used.

Computer-assisted semen motility assessment (CASA)
The CASA system was equipped with a 20-fold objective, a camera adaptor (U-PMTVC 4L02981; Olympus, Hamburg, Germany) and a camera with a resolution of 800x600 pixels (Accupixel TM6760CL; JAI, Glostrup, Denmark). The system was operated by SpermVision version 3.5 (Minitub).

For concentration analysis the porcine semen was prepared as described in porcine semen preparation. The concentration of the resulting pellet was adjusted using mTBM. Four different concentration samples of $5x10^6$cells/ml, $10x10^6$ cells/mL, $15x10^6$ cells/ml and $20x10^6$ cells/ml were made for CASA analysis. For each sample a four chamber slide (Leja, Nieuw Vennep, The Netherlands) with a chamber depth of 20 micrometer was prepared. The leja chamber was filled with approximately 3 µL of sperm suspension. The slide was prewarmed on a thermoplate (HT300, Minitüb) and maintained at 37 °C. The different concentrations were analyzed using the CASA system. After each incubation step in the time series the samples were analyzed identically.

For the assessment of porcine sperm 15 predefined microscopic fields in the center of the slide were scanned. For stallion and bovine sperm 12 predefined microscopic fields were scanned. The percentage of total motile and progressively motile spermatozoa was recorded. Furthermore, for the progressively motile cells the curvilinear velocity (VCL), average straight-line velocity (VSL), average path velocity (VAP), straightness (STR=VSL/VAP), linearity (LIN=VSL/VCL), wobble (WOB=VAP/VCL), beat cross frequency (BCF) and average amplitude of lateral head displacement (ALH) were assessed. Each analysis was performed twice making a new slide each time. In total 4 analyses per set of tubes were performed. The analysis was both visually and by means of cluster analysis assessed for different motility patterns. The CASA system settings for hypermotility of sperm consisted of VCL > 80 and LIN < 0.65 and ALH > 6,5 (Boar: cell area of 20 – 121 micrometer^2; Bull: cell area 23-85 micrometer^2; Stallion: cell area 14-85 micrometer^2).
Data were visually evaluated for the occurrence of hyperactivated motility tracks, but also evaluated by cluster analysis for an objective distinction of motility patterns and group forming. For statistical analysis, the values for each motility descriptor from all motile spermatozoa were exported.

Protein tyrosine phosphorylation
To compare the occurrence of protein tyrosine phosphorylation at set times different staining samples were prepared. The sperm of 1 bull, 1 boar and 2 stallions was processed as described in the sperm preparation section and thereafter partly incubated according to either the bovine or porcine IVF protocol (1 stallion for each protocol). The sperm processed according to the bovine IVF protocol was incubated for 15 min and 5 hours and the samples processed according to the porcine IVF protocol were incubated for 15 min and 3 hours prior to staining. As a control one sample without capacitation stimulus and one sample incubated under IVF conditions for 3 hours or 5 hours, respectively, were stained. The purpose of the 3/5 hour incubated sample was to confirm specific labeling of the secondary antibody and therefore no primary antibody was added to this sample (i.e. negative control). The control sample served to assess base levels of tyrosine phosphorylation in the respective semen samples.
Ethidium homodimer-1 (E1169, life technologies) was added to the control and 15 min samples with a concentration of 1 µL/ml and incubated for 15 min at 38 °C (38.5 °C and 5% CO_2 for the 15 min sample). The 3/5 hour samples were stained 15 minutes prior to the end of the incubation time, therefore not prolonging the incubation time in the capacitation medium. Ethidium homodimer was blocked using pre-warmed Salmon DNA (DNA, sodium salt from salmon testes. D1626, Sigma) with a concentration of 50 µL/ml. The samples were centrifuged for 5 min at 600g. The supernatant was removed, and the samples were washed using PBS (5 min at 600g). Afterwards the cells were fixated using a mild fixative to achieve a final formaldehyde concentration of 0.125%, Salmon DNA was added, and the samples were incubated for 10 min at room temperature. After

washing, the samples were resuspended in PBS with 0.1% Triton X-100 and 1% BSA (no specific BSA) and DNA was added. After 30 min incubation at room temperature the samples were pelleted, washed and 1 µL of primary antibody (anti-phosphotyrosine antibody, clone 4G10, 1 mg/ml, raised in mouse, 05-321X, Millipore) was added to each sample except the 3/5 Control. The samples were resuspended in 200 µL PBS with 1% BSA, Salmon DNA was added, and they were incubated overnight at 4 °C. The next day, the samples were pelleted and washed twice in PBS with 1% BSA and Salmon DNA (5 min at 600g) to remove unbound antibody. Five µL of the secondary antibody (goat anti mouse Alexa 488, 2 mg/ml, A11029, Life technologies) were added to the pellet and it was resuspended in 250 µL PBS with 1% BSA and Salmon DNA. The suspension was incubated at room temperature for 4 hours. After removing the unbound antibody, the final resuspension was in 100 to 1000 µL PBS. The volume depended on pellet size. Salmon DNA and 1 µL Hoechst 33342 (1.62 µM, Thermofisher) was added. For every sample, the immunolabeled spermatozoa were mounted on a glass slide using a 20x20 mm cover slip and sealed with clear nail polish. Samples were examined using a Leica DMR epifluorescence microscope with a mercury lamp and appropriate filters on 1000x magnification (oil emersion). Overlays were created from 3 images using the filters D (excitation 360/20 nm, DM 400 nm, 425 LP), I3 (excitation 490/45 nm, DM 510 nm, 515 LP) and N2.1 (excitation 515-560 nm, DM 580 nm, 590 LP). Exposure time, gain and gamma values were adjusted according to the samples needs. The samples were analyzed on staining pattern of the sperm flagella which where distinguished by completely stained and staining of the midpiece, principle piece and/or end piece (see **Supplemental figure 1** for an example). The proportion of sperm with different types of green coloration among the total sperm population as well as the live or dead status of the spermatozoa were determined by randomly scoring 200 spermatozoa. The viable cells appeared blue and the dead cells were red. Green coloration was seen when protein tyrosine phosphorylation occurred.

Statistical analysis

For analyzation of the data, Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA, USA) and Statistical Analysis Software (SAS, version 9.3, Cary, NC, USA) were used. CASA analysis defines eight motility descriptors (VCL, VSL, VAP, STR, LIN, WOB, BCF and ALH). Motility descriptors for every single, motile spermatozoon after the different incubation times (0.25h, 1h, 2h, 3h, 4h, 5h, 6h, 18h, 20h and 22h), different media (mTBM, Medium E). Cluster analysis was performed as described by Henning *et al*. (39). The aim was to create clusters that explained a large amount of the variance in the data set without formation of excessive major clusters. A Chi-squared test was performed to test whether the sperm distribution to the cluster differed between incubation periods (PROC FREQ). Cramer's V (ranging from 0 to 1) was used to measure the effect size that incubation time had on the different clusters. For interpretation the guidelines suggested by Cohen (40) were used: $V < 0.10$ = no effect, $0.10 < V \leq 0.30$ = slight effect, $0.30 < V \leq 0.50$ = moderate effect and $V > 0.50$ = strong effect.

Results

Determination of a working concentration

In order to determine a suitable working concentration for the motility assessments, a concentration series was performed. The goal was to determine which concentration led to at least 200 detectable spermatozoa without excessive agglutination. As seen in **Table 1** the number of recorded cells was the highest at a concentration of 20 x 10^6

spermatozoa/mL. However, between 5 and 15 x 10^6 spermatozoa/ml the increase in detected spermatozoa was 2.6 fold and between 5 and 20 x10^6 spermatozoa/ml only 2.9 fold. At the same time, a large number of agglutinated spermatozoa was observed at 20 x 10^6 spermatozoa/mL. Hence a working concentration of 15 x 10^6 was used for further experimentation.

Table 1 Number of detected boar spermatozoa by CASA with different working concentrations. *The amount of analyzed cells was based on a single CASA analysis per sample after placement in mTBM. Per analysis 15 predefined microscopic fields were scanned. For the determination of the amount of analyzed cells no distinction was made between non-motile and motile cells.*

Concentration (x 10^6)	5	10	15	20
boar 1	*111*	*240*	*319*	*306*
boar 2	*80*	*133*	*231*	*251*
boar 3	*75*	*128*	*142*	*212*
mean	*89*	*167*	*231*	*256*
Relative increase				
boar 1	*1*	*2.2*	*2.9*	*2.8*
boar 2	*1*	*1.7*	*2.9*	*3.1*
boar 3	*1*	*1.7*	*1.9*	*2.8*
mean	*1*	*1.8*	*2.6*	*2.9*

Motility pattern analysis

The CASA system was used to investigate the different motility patterns of boar, bull, and stallion spermatozoa under IVF conditions. However, due excessive agglutination of the boar and bull spermatozoa under their respective IVF conditions, only the motility patterns of the stallion spermatozoa in porcine or bovine IVF media were further investigated.

Visual analysis

Multiple articles describe hyperactivated motility as high speed circular sperm motions (13, 20, 30, 31). A visual interpretation of the CASA system analysis was done to distinguish the amount of “hyperactivated” cells from all highly active, progressive motile cells (**Figure 2**).

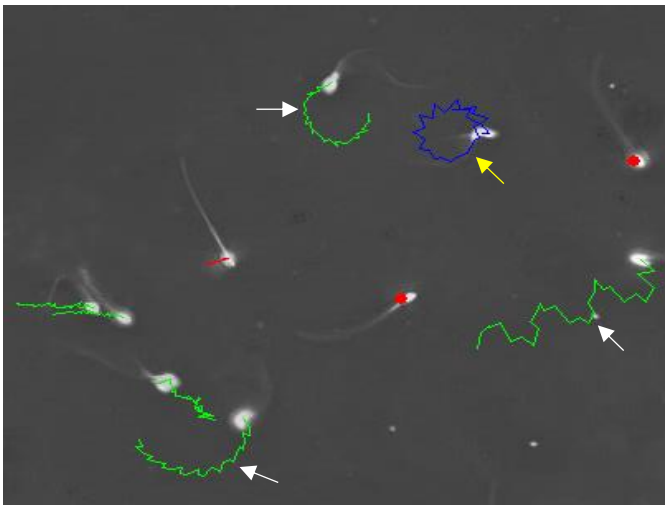


Figure 2 Distinction between hfighly active, progressive motile cells and hyperactivated spermatozoa on the CASA system for visual analysis. *The motility patterns pointed with a white arrow were considered highly active, progressive motile cells. The motility pattern pointed with a yellow arrow was distinguished as hyperactivated motility. The patterns who aren't pointed out were not included in the visual analysis.*

Results after 18 hours incubation were not included due to the lack of hyperactivated cells. Stallion 1 and 3 showed an overall higher number of hyperactivated cells (20.2% to 46.3%) than Stallion 2 (**Figure 3A**; 0.8% to 11.8%). In **Figure 3B** a similar difference is visible for Stallion 5 (20.4% to 41.9%) in comparison to Stallion 4 and 6 (0% to 18.3%). With 46.3% the highest amount of hyperactivated stallion spermatozoa was seen at 2 h incubation in mTBM for Stallion 3. The values for the individual stallions differ greatly from each other. A distinct peak of hyperactivated motility at a certain incubation time is not seen for either incubation in mTBM and medium E.

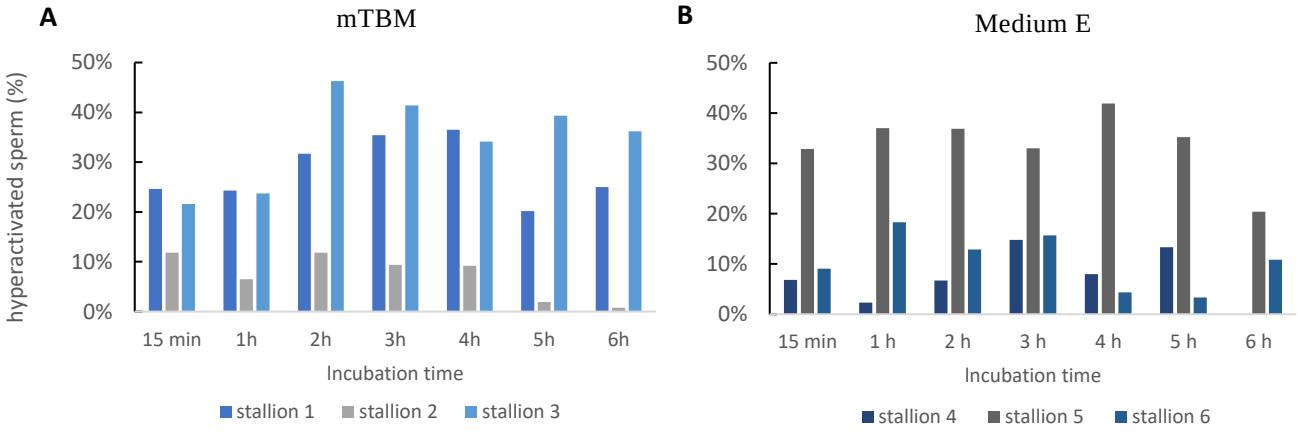


Figure 3: Hyperactivated motility of stallion spermatozoa under different IVF conditions. *The visually determined percentage of hyperactivated cells/highly active, progressive motile cells was based on CASA analysis. The stallion semen was prepared using different IVF protocols.* ***A,*** *Stallion 1-3 were incubated in mTBM. The pH of the mTBM medium ranged between 7.4 and 7.8 and the osmolarity was 303 Osm/L.* ***B,*** *Stallion 4-6 were incubated in Medium E. The pH of the medium E was 7.6 and the osmolarity was 278 Osm/L. The samples were incubated at 38.5 °C and 5% CO_2.*

Cluster analysis

For cluster formation a total of 11,123 sperm tracks from 56 measurements recorded after 15 min, 1 h, 2 h, 3 h, 4 h, 5h, 6 h, and 18 hours incubation in porcine IVF medium (n=3 stallions), bovine IVF medium (n=3 stallions) or INRA96 (n=1 stallion) were used. A solution with a total of 43 clusters was chosen, which explained 59% of variance in the

dataset. The 8 clusters which contained >5% of the spermatozoa on at least one of the incubation times are listed (**Table 2**). The number of tracks decreased over time and after 18 hours the least tracks were measured due to clump formation and large number of immotile spermatozoa. The ratio between the number of sperm tracks from spermatozoa incubated in mTBM, Medium E and INRA consisted of 38%/35%/27% respectively. The visual representation of the 8 selected clusters is portrayed in **Figure 4**.

Table 2: Parameters of the 8 selected clusters. *The values represent the details (mean + s.d.) of the motility patterns per cluster.*

Cluster no.	*VCL [µm/s]*	*LIN*	*ALH [µm]*	*BCF [Hz]*
1	56.1 ± 29.0	0.81 ± 0.14	0.9 ± 0.50	34.7 ± 7.1
2	32.3 ± 14.4	0.78 ± 0.12	0.71 ± 0.86	5.4 ± 7.7
4	133.2 ± 50.4	0.42 ± 0.11	2.29 ± 0.88	42.2 ± 8.3
5	65.2 ± 27.7	0.24 ± 0.12	1.55 ± 0.71	27.8 ± 10.0
6	163.3 ± 47.9	0.68 ± 0.11	2.33 ± 0.92	50.4 ± 6.2
7	292.9 ± 49.7	0.33 ± 0.11	4.84 ± 0.89	45.8 ± 7.2
8	199.1 ± 51.8	0.11 ± 0.06	3.42 ± 0.91	47.4 ± 7.5
10	75.8 ± 28.1	0.49 ± 0.16	2.9 ± 0.93	23.3 ± 8.7

VCL = curvilinear velocity, LIN = linearity (VSL/VCL), ALH = average amplitude of lateral head displacement, BCF = beat cross frequency

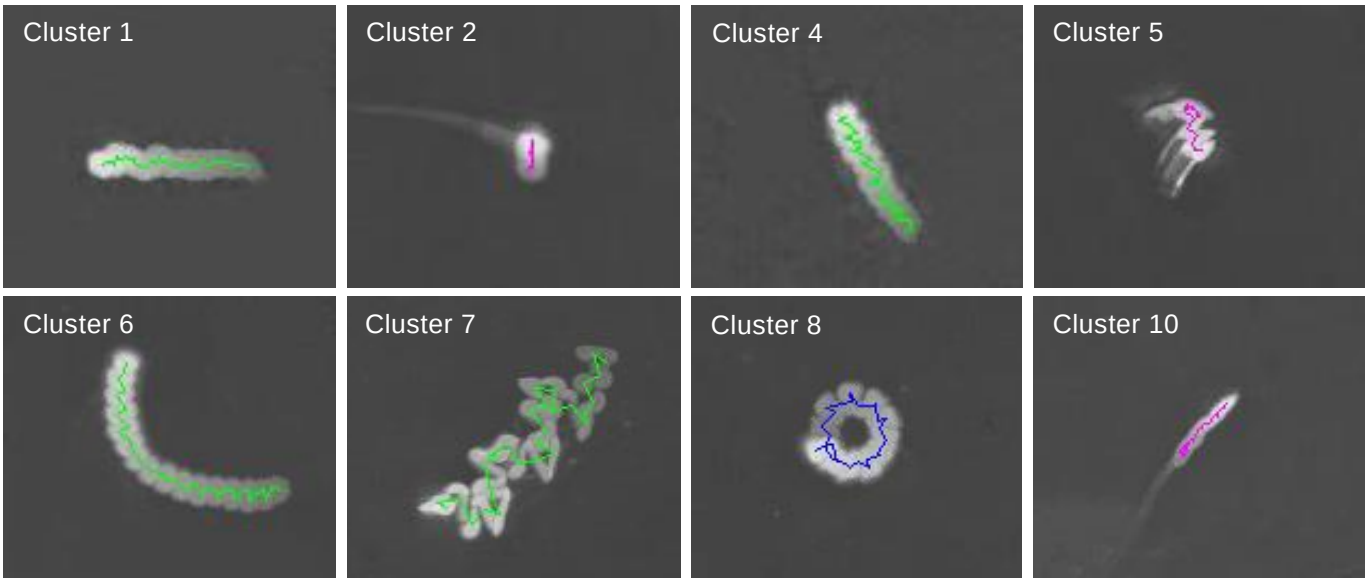


Figure 4 Representative CASA motility patterns per cluster as described in Table 2. *The motility patterns were obtained using the CASA system SpermVision version 3.5. The elapsed time was 0.5 seconds.*

To provide an overview of the motility patterns under different IVF conditions, multiple pie charts were designed. The segments represent the percentage of spermatozoa attributed to each cluster. Thus, making it possible to track the change of motility patterns (i.e. cluster) over time. The assessment after 15 min incubation was set as reference point. Thereafter, the incubation time with the most different cluster distribution compared to 15 min incubation was determined based on the Cramer's V, a measure for the effect size of incubation time (**Figure 5 and 6**). For stallion spermatozoa in Medium E the Cramer's V reaches a plateau value after 5 hours incubation. For spermatozoa in mTBM the Cramer's V shows little variation. After 3 hours incubation, the highest Cramer's V was already reached.

In all treatments most sperm tracks were allocated to cluster 4 which represented spermatozoa with relatively high velocity, moderate linearity, and a high beat cross frequency. **Figure 5** portrays that for spermatozoa incubated in medium E the clusters with the most difference between the two incubation times are cluster 5 (11% to 24%), 6 (14% to 5%) and 8 (14% to 7%). This implicates that the spermatozoa over time (5 hours) changed to a motility patter with declined VCL, LIN, ALH and BCF. Cluster 4 stays stable over the course of 5 hours with 40%. As seen in **Figure 4**, Cluster 8 is presumed to portray the hyperactivated motility pattern. This cluster declines over time from 14% to 7% in favor of a motility pattern with relatively low VCL and LIN. For spermatozoa incubated in mTBM (**Figure 6**) cluster 5 (7% to 16 %) and 6 (19% to 6%) showed the most variation over time. Motility patterns again changed over the course of 3 hours to a pattern with lower VCL, LIN, ALH and BCF.
When comparing the motility pattern of stallion spermatozoa in medium E to those in mTBM it is remarkable that cluster 7 shows a larger size (13% and 11% compared to 26% and 24%). Furthermore cluster 8 decreases over time in medium E (14% to 7%) and stays relatively the same in mTBM (10% to 11%). Overall, the spermatozoa in mTBM showed a higher percentage of cells with a high VCL, ALH and BCF and a low linearity (cluster 7 and 8). This percentage stayed relatively unchanged over time (1% decline in 3 hours). Spermatozoa incubated in INRA96 (**Figure 7**) clearly show 2 major motility patterns namely those of cluster 4 and 5. With 86% of sperm tracks being allocated to cluster 4 this is most seen motility pattern. There is a decrease of sperm tracks in cluster 4 from 86% at 15 min to 81% at 3 hours and 78% at 5 hours. This decrease is followed by an increase in cells in cluster 5 from 5% at 15 min to 14% at 3 hours and 20% at 5 hours. Cluster 5 portrays a motility pattern with a moderate velocity and BCF and low LIN and ALH.

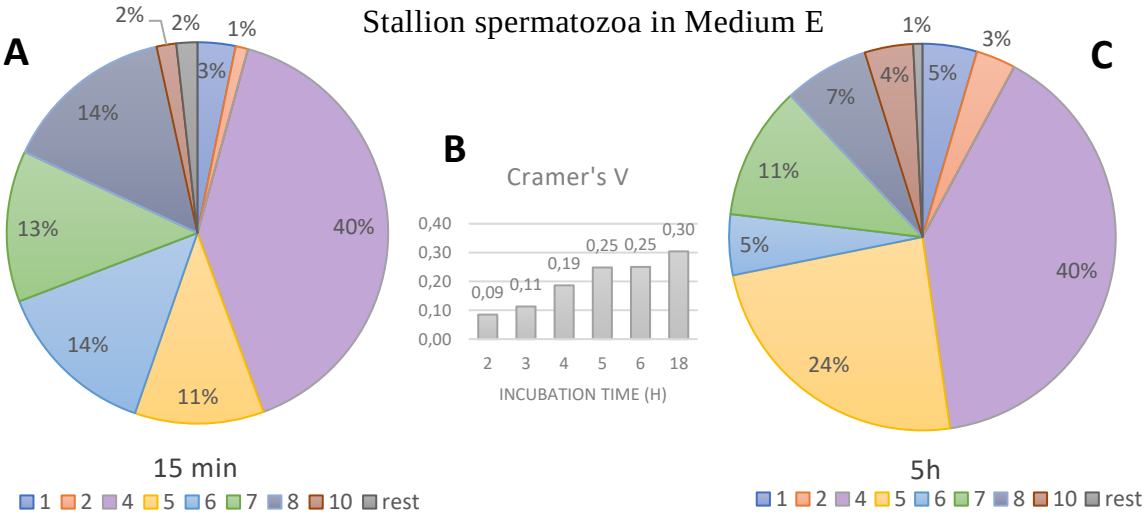


Figure 5: Influence of medium E on stallion sperm motility. *The spermatozoa were incubated according to the bovine IVF protocol at 38.5 degrees Celsius and 5% CO_2.* ***A,*** *Sperm tracks of 960 spermatozoa observed after 15 minutes incubation (n=3 stallions).* ***C,*** *Sperm tracks of 394 spermatozoa observed after 5 hours incubation (n=3 stallions). The sperm tracks were divided into 8 different clusters. Each cluster portrays a motility pattern as described in Table 1.* ***B,*** *The effect size of the incubation time on sperm motility is depicted as Cramer's V. The Cramer's V reaches a plateau value at 5 hours.*

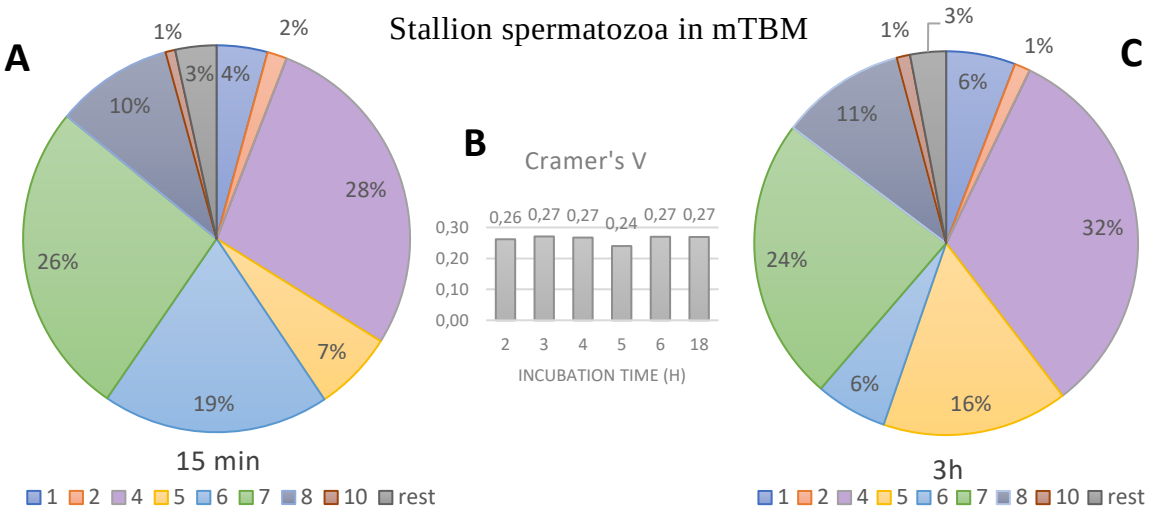


Figure 6: Influence of mTBM on stallion sperm motility. *The spermatozoa were incubated according to the porcine IVF protocol at 38.5 °C and 5% CO_2.* ***A,*** *Sperm tracks of 611 spermatozoa observed after 15 minutes incubation (n=3 stallions).* ***B,*** *Sperm tracks of 530 spermatozoa observed after 3 hours incubation (n=3 stallions). The sperm tracks were divided into 8 different clusters. Each cluster portrays a motility pattern as described in Table 1.* ***C,*** *The effect size of the incubation time on sperm motility is depicted as Cramer's V. The Cramer's V reaches a plateau value at 3 hours.*

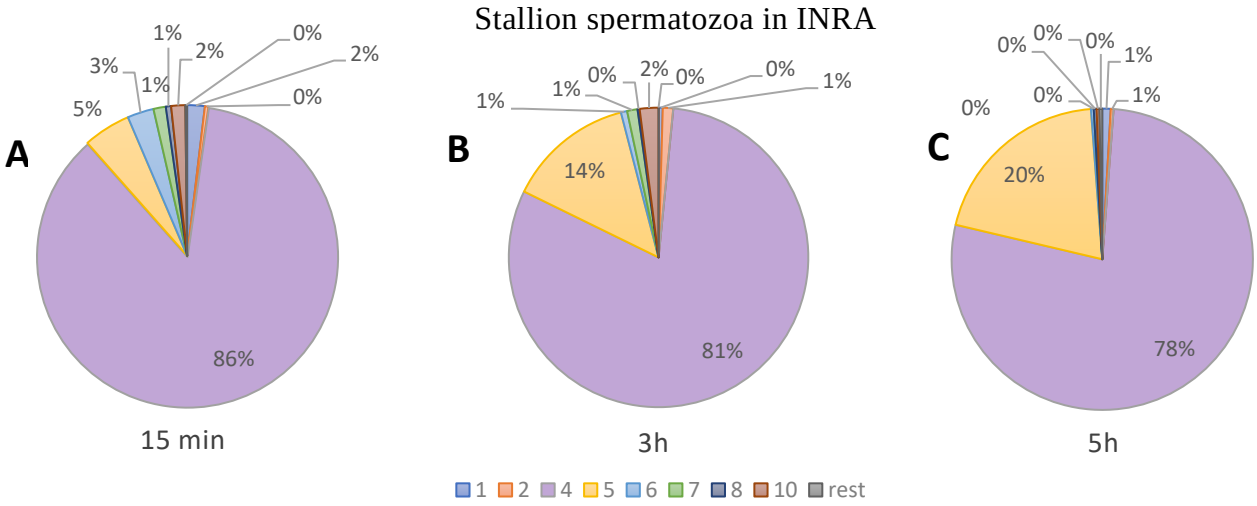


Figure 7: Stallion sperm motility in INRA storage medium. *The spermatozoa were incubated in INRA at 38.5 °C and 5% CO_2.* ***A,*** *Sperm tracks of 529 spermatozoa observed after 15 min incubation.* ***B,*** *Sperm tracks of 445 spermatozoa after 3h incubation.* ***C,*** *Sperm tracks of 333 spermatozoa after 5h of incubation.*

Protein tyrosine phosphorylation of equine, bovine and porcine spermatozoa

Spermatozoa of multiple species (boar n=1, bull n=1, stallion n=2) were immunolabeled to visualize the possible occurrence of protein tyrosine phosphorylation of the sperm flagellum. Furthermore, a visual comparison of said occurrence could be made between stallion, boar and bull spermatozoa. The chosen incubation times were dependent on the Cramer's V that is depicted in **Figure 5 and 6**. Overlays of the stained spermatozoa are presented in **Figures 8 and 9**. A negative control sample and fully stained sample are depicted in **Supplemental figure 2**. Visual scoring of 200 cells per sample gave an overview of the presence of various immunolabeled parts of the spermatozoa (**Supplemental figure 1**).

Stallion spermatozoa incubated in medium E showed variable amount of protein tyrosine phosphorylation in the sperm flagella. The following percentages are summarized in **Table 3**. In the control group, 26% of the counted cells showed a form of green staining in the sperm tail whereas 25% out of the 26% consisted of staining of the proximal midpiece of the sperm flagella. From all cells containing a flagellar staining, 71% appeared alive (blue) and 29% were dead (red). In the overall control sample the live - dead ratio was 1:0.35. Comparing these findings to the bovine spermatozoa in the control group it is remarkable that 85% of the bull spermatozoa showed green flagella. With 54% out of the 85%, the distal part of the principle piece and end piece of the bovine spermatozoa were most often stained. Furthermore 94% of these cells were alive (blue) and the live dead ratio of the overall sample was 1:0.14. After 15 min incubation only 11% of the stallion spermatozoa showed green stained sperm flagella with most of this coloration being in the head piece of the flagella. Interestingly, 43% of the flagella stained cells appeared dead (red) and the live dead ratio of the sample was 1:0.32. For bull spermatozoa, after 15 min incubation a total of 70% of the sperm tails performed protein tyrosine phosphorylation of which almost all were seen in the distal part of the principle piece and end piece of the sperm flagella. From these cells 91% appeared alive (blue) and the live dead ratio of the total sample was 1:0.12. Interestingly, after 5 hours incubation in medium E the number of sperm flagella showing protein tyrosine phosphorylation in the stallion sample consisted of 72% of which 46% consisted of illumination of the entire sperm tail with different degrees of brightness. From this 72%, 28% appeared to be dead (red). The overall live - dead ratio of the sample consisted of 1:0.49. Bull spermatozoa at 5h incubation demonstrated that 78% of all tails appeared stained with a 50/50 divide between complete illumination and illumination of the distal part of the principle piece and end piece. 80% of the bull spermatozoa with tail illumination were alive (blue) and the live dead ratio of the sample was 1:0.35.
Furthermore, in **Figure 8** it is seen that the acrosomal area of the bull spermatozoa was covered in green dots. The acrosomal area appeared to have lost its shape and a few acrosome-like structures were released from the spermatozoa.

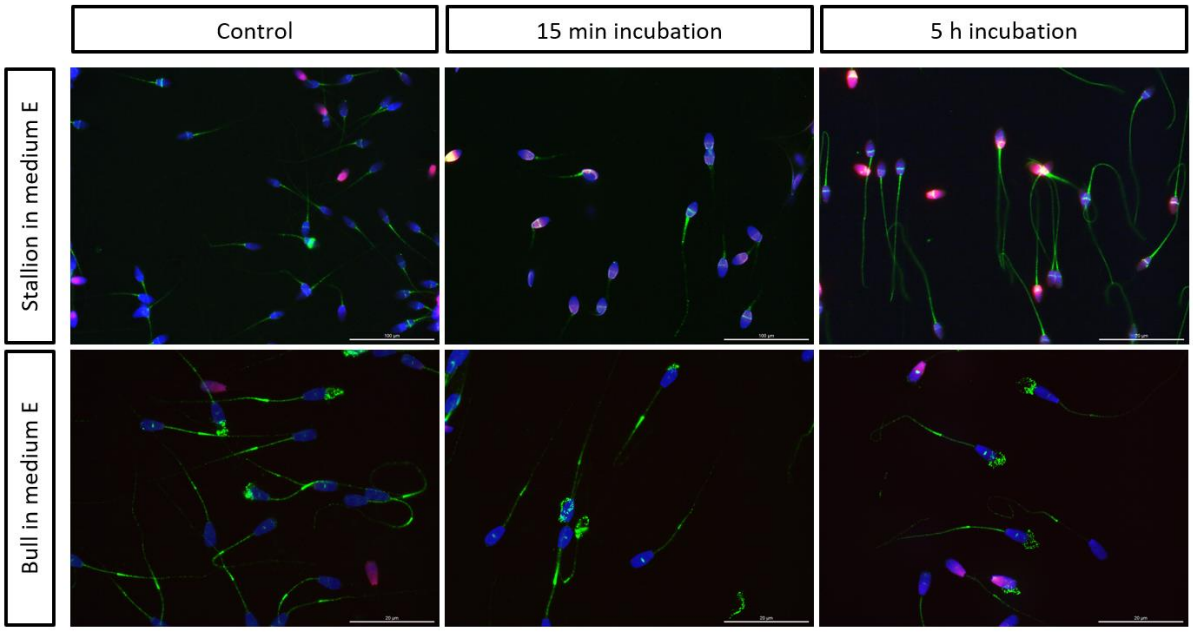


Figure 8: Protein tyrosine phosphorylation of the sperm flagella under bovine IVF conditions. *Detection of tyrosine phosphorylation in spermatozoa by immunofluorescence staining. Spermatozoa were probed by mouse anti-phosphotyrosine antibodies (clone 4G10) followed by goat anti mouse Alexa Fluor 488*

secondary antibody (green). Live and dead staining was performed using Ethidium homodimer-1 (red) and Hoechst 33342 (blue). Before staining of the stallion and bull spermatozoa, they underwent incubation in medium E at 38.5 °C and 5% CO_2 for given times. The control group did not receive any treatment. Images were made using a Leica DMR microscope with a mercury lamp and appropriate filters with 1000x magnification (oil emersion). Scalebar = 20 µm.

Table 3 Protein tyrosine phosphorylation of the sperm flagella. *Percentage of spermatozoa with a form of green coloration of the sperm flagellum in the control group and at 15 min and 5 h incubation in medium E. The live dead ratio of the flagellar stained spermatozoa is stated between the brackets. For representative staining pattern see Figure 8.*

	Control	*15 min*	*5 h*
Stallion in medium E	26% (1:0.41)	11% (1:0.75)	72% (1:0.39)
Bull in medium E	84% (1:0.06)	70% (1:0.1)	78% (1:0.25)

The stallion and boar spermatozoa in mTBM (**Figure 9**) showed another variety of staining patterns compared to the ones above (**Figure 8**). The stallion spermatozoa often showed a band of tyrosine phosphorylation amidst the sperm head whereas the boar spermatozoa showed high fluorescent equatorial segments and acrosomes.
When focusing on the appearance of tyrosine phosphorylation of the sperm tail (**Table 4**) it is seen that in the control group 3% of the stallion spermatozoa show green coloration of the midpiece of the sperm flagella. From these cells 67% appeared to be dead (red) and the live dead ratio of the sample was 1:0.39. During incubation in mTBM the percentage of stallion spermatozoa with immunofluorescent sperm tails went from 6% (50/50 dead/alive) at 15 min to 26% (24%/76% dead alive) at 3 h incubation. The 15 min incubation sample had a live - dead ratio of 1:0.52 and the 3h sample of 1:0.53.
In the control sample of the boar spermatozoa 20% of the sperm flagella were green. Most of the tails showed green coloration at the midpiece of the sperm tail. 8% of the green tailed spermatozoa appeared dead (red) and the live dead ratio was 1:0.20. At 15 min and 3h incubation showed a slight increase in protein tyrosine phosphorylating flagella from 30% (10% dead, 90% alive) to 32% (16% dead, 84% alive). Most of the tails at 15 min incubation were partially illuminated with the mid-, distal part of the principle- and end-piece showing the most coloration. At 5h incubation in mTBM the divide between complete and partial illumination was 50/50. The overall live dead ratio of the 15 min and 3 h incubation was 1:0.35 and 1:0.47 respectively.
Comparing the staining patterns of the stallion spermatozoa to the boar spermatozoa it was seen that the boar spermatozoa showed a distinct phosphorylated dot on the sperm flagella (as seen on the 3h incubation image of the boar spermatozoa in mTBM of **Figure 9** at the position of the Jensen ring). At 15 min incubation 10% of the boar spermatozoa showed this dot and at 3 h incubation 23% showed this dot. The dot seemed to appear independent of protein tyrosine phosphorylation of the tail. In the control group none of the sperm tails demonstrated this dot.
Comparing the stallion spermatozoa from the group incubated in medium E and mTBM it is obvious that medium E had a larger effect on the occurrence of protein tyrosine phosphorylation of the sperm flagellum than mTBM with 72% to 26% respectively.

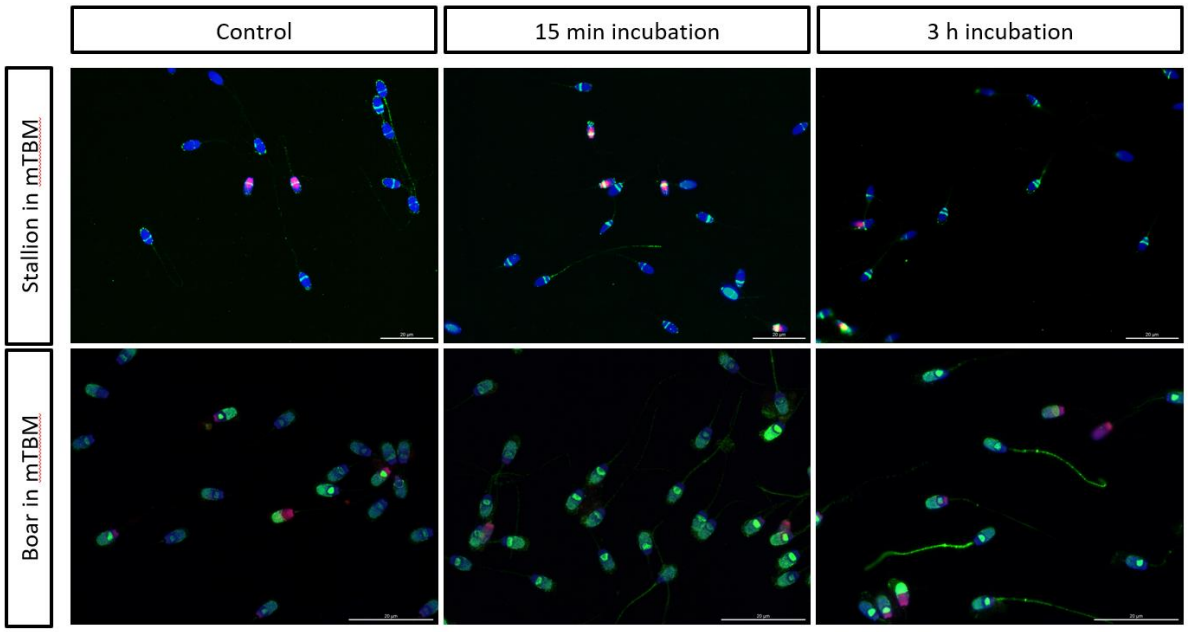


Figure 9: Protein tyrosine phosphorylation of the sperm flagella under porcine IVF conditions. *Detection of tyrosine phosphorylation in spermatozoa by immunofluorescence staining. Spermatozoa were probed by mouse anti phosphotyrosine antibodies (clone 4G10) followed by goat anti mouse Alexa Fluor 488 secondary antibody (green). Live and dead staining was performed using Ethidium homodimer-1 (red) and Hoechst 33342 (blue). Before staining of the stallion and boar spermatozoa, they underwent incubation in mTBM at 38.5 °C and 5% CO_2 for given times. The control group did not receive any treatment. Images were made using a Leica DMR with a mercury lamp and appropriate filters with 1000x magnification (oil emersion). Scalebar = 20 µm.*

Table 4 Protein tyrosine phosphorylation of sperm flagella. *Percentage of spermatozoa with a form of green coloration of the sperm flagella in the control group and at 15 min and 3 h incubation in mTBM. The live dead ratio of the flagellar stained spermatozoa is stated between the brackets. This table adds to Figure 9.*

	Control	*15 min*	*3 h*
Stallion in mTBM	3% (1:2)	6% (1:1)	26% (1:0.32)
Boar in mTBM	20% (1:0.1)	30% (1:0.11)	32% (1:0.19)

Discussion

This study is an orientating study with the aim to provide a comprehensive basic characterization of hyperactivated motility patterns in species with functional IVF systems, i.e. cattle and pigs. We also aimed to compare the observed motility changes in stallion spermatozoa which were subjected to bovine or porcine IVF conditions. The occurrence of tyrosine phosphorylation in the tail of viable spermatozoa under the respective incubation conditions was visualized by indirect immunofluorescence.

IVF medium composition is of vital importance for the success of fertilization. It is known that *in vivo* factors from the oviduct and/or oviduct fluid are needed to mediate fertilization. These factors can have effect on the sperm, the oocyte or both (5). The different media used in these experiments are composed of various (species-specific) capacitation triggers such as caffeine, heparin and albumin. Methylxanthines like caffeine inhibit phosphodiesterase activity which leads to an increase of intracellular cAMP levels (41). In various species including boar and bull, hyperactivated motility is associated with highly increased cAMP dependent tyrosine phosphorylation of the flagellar proteins like protein kinase A (22, 23, 42). The mTBM medium used in these experiments contained

caffeine. Interestingly, after only 15 min of incubation the stallion spermatozoa already presented a hyperactivation-like motility pattern which was also present after at least 3 hours (**Figure 6**). This suggests that caffeine may have an activating role for stallion spermatozoa, too. However, the occurrence of protein tyrosine phosphorylation in the stallion sperm flagellum after 15 minutes incubation did not seem in line with these results and neither is the conclusion of the honours thesis of Mr. Galati (43) which stated that no hyperactivation was seen after addition of caffeine. This might suggest that hyperactivated motility patterns occur independently of protein tyrosine phosphorylation. Another possibility would be that there are different states of hyperactivated motility. Protein tyrosine phosphorylation of the sperm tail could be a precursor for full fertilization-relevant hyperactivation. However, right now, it is unknown whether protein tyrosine phosphorylation of the sperm tail is fertilisation relevant or not. Experiments which focus on the occurrence of both hyperactivated motility and protein tyrosine phosphorylation could give more insight in this phenomenon. It is recommended that for these experiments both mTBM with and without caffeine are used to research the influence of caffeine. Furthermore, the same ejaculates should be used for both the motility analysis and the protein tyrosine staining due to possible ejaculate or stallion specific reactions to capacitation triggers or even stallion specific motility patterns.

An indication of the occurrence of stallion specific motility patterns is seen in **Figure 3**. Stallion 1, 3 and 5 appear to have a larger percentage of spermatozoa presenting a hyperactivated motility pattern. The actual fertility of the used stallions is unknown but according to a recent study by Suliman *et al.* (44) the VCL of fertile stallions was significantly higher than those of subfertile stallions. Furthermore, seasonal variation was investigated. Results showed an overall lower VAP (velocity of average path), VSL (velocity of the straight line) and BCF in the non-breading season. This suggests that variations in motility patterns between stallions are present.

Heparin is known to be a species specific capacitation trigger for bovine spermatozoa (16, 45, 46). Interestingly when bull spermatozoa were not incubated in IVF medium they already showed protein tyrosine phosphorylation of the sperm flagella in the majority of the spermatozoa (84%; **Table 3**). This was most likely due to the phenomenon cryo-capacitation. Thawing of cryopreserved spermatozoa can lead to the occurrence of capacitation like changes in the spermatozoa such as protein tyrosine phosphorylation and membrane fluidity changes. However the proteins that get phosphorylated do not contribute to hyperactivation nor fertilization. The cryo-capacitation phenomenon is seen in multiple mammals including bulls, boars and stallions (47, 48). This concludes that heparin is not necessary for the occurrence of protein tyrosine phosphorylation in bovine spermatozoa but might be obligatory for the phosphorylation of the right protein. For future research it is recommended to only use fresh sperm samples to avoid any bias from preservation techniques.

From the results in **Table 4** it is seen that when boar spermatozoa are incubated in mTBM only a slight increase in protein tyrosine phosphorylation is present (from 30% to 32%) while stallion spermatozoa under the same conditions show a significant increase in protein tyrosine phosphorylation from 6% to 26%. This same phenomenon is seen when looking at the medium E results. Bovine spermatozoa that are incubated in medium E show only a slight increase in protein tyrosine phosphorylation from 70% to 78% while with stallion spermatozoa protein tyrosine phosphorylation rises from 11% to 72%. It is known that both the IVF protocols show good results with the respective species and

media (16, 36). This might indicate that the protein tyrosine phosphorylation is not a hallmark for successful IVF.
Another result supporting this suggestion is that the rise in protein tyrosine phosphorylation over time (**Table 3**) is not coherent with the decline in hyperactivated motility of the stallion spermatozoa (cluster 7 and 8) during incubation in medium E as seen in **Figure 5**. However, it cannot be excluded that only a small fraction of spermatozoa shows the right combination of a fertilization-relevant motility pattern and degree of tyrosine phosphorylation in the tail.

For determining hyperactivated motility the CASA system was used. CASA systems show a great variety in how they are recognizing cells and how they classify the motility parameters given to each cell (49). Therefore two different CASA systems can give different outcome and cluster formation could be based on different parameters. Also, no strict parameters are universally accepted for hyperactivation which makes the pattern subjective. Overall an increased VCL, increased ALH and low linearity serve as very general descriptors and no reference point for these descriptors is universally accepted. However it is reasonable to assume that the trend would be the same for various CASA systems.

When inspecting the IVF medium composition it is seen that mTBM has to be buffered from a starting pH of 10.4 to a pH around 7.4. The trigger for the Ca^{2+} CATSPER channels is alkaline depolarization (28, 29). It is suggested that the mechanism of action of these channels might be different in stallions due to the pH-sensor region showing species-specific differences (20). However, alkalinization of the cytoplasm did induce both a hyperactivated motility response and cytoplasmic Ca^{2+} rise in stallions (20). The rise in Ca^{2+} in turn results in a higher cAMP concentration which ultimately induces protein tyrosine phosphorylation (24, 25). Thus, a pH > 8 of the used media could provoke "false" protein tyrosine phosphorylation. In these experiments the pH of the used IVF media ranged between 7.4 and 7.8. It is likely that elevated pH had very little influence on the results.

The inclusion of serum albumin in capacitation medium is considered to be obligatory. In order for capacitation to take place, cholesterol has to be removed from the plasma membrane of the spermatozoa. Adding serum albumin leads to fluidity changes that are accompanied by cholesterol removal and ion fluxes (50). As mentioned above, the Ca^{2+} and HCO_3^- -induced alterations result in protein tyrosine phosphorylation. Remarkably, the mTBM does not contain serum albumin. The results show that stallion spermatozoa incubated in mTBM showed significantly lower percentages of protein tyrosine phosphorylation of the sperm tail than the ones incubated in medium E (26% to 72%; **Table 3 and 4**). This suggests that the bovine IVF protocol had a higher efficiency in inducing protein tyrosine phosphorylation of the sperm tail of viable stallion spermatozoa. However Macías-García *et al.* reported that the removal of cholesterol by serum albumin does not apply to stallion spermatozoa (9).

No visualization of the motility patterns of bull and boar semen was possible due to massive agglutination of the spermatozoa. A recent study by Umezu *et al.* described that agglutination of bovine spermatozoa led to a longer viability and motility in culture than non-agglutinated spermatozoa (51). This may suggest that sperm-sperm agglutination is important for the survival of the spermatozoa and therefore the ability to fertilize, which is also seen in marsupials (52).

Investigating motility patterns of spermatozoa under successful IVF circumstances would give valuable information. A way to prevent agglutination of the sperm heads under bovine IVF conditions would be to leave out PHE and heparin from the fertilization medium (19). This results in an adjusted IVF protocol, however it could be possible that changes in the sperm flagellum are not related to changes in the sperm head. Another option would be to study direct sperm-oocyte interaction. The motility patterns of the bound and penetrating sperm could be observed.

Interestingly, the equine spermatozoa showed a low degree of agglutination and despite many attempts no IVF protocol has been successful for this species. The bovine IVF protocol seemed to induce the most protein tyrosine phosphorylation of the stallion sperm flagella. Assuming this is a hallmark for hyperactivation a similar protocol might have the best change at performing successful equine IVF.

Conclusion

The basic characterization of hyperactive motility under successful IVF circumstances was not possible due to immediate agglutination of both boar and bull spermatozoa. Furthermore, in stallion spermatozoa the development of hyperactivated motility and protein tyrosine phosphorylation seemed to be independent instead of coherent. In the different media there was a substantial difference in occurrence of protein tyrosine phosphorylation of the sperm tail. This might suggest that a high amount of protein tyrosine phosphorylation and or hyperactivated motility is not necessary for successful IFV. However, this study consisted of experiments with only low sample sizes. Therefore, it would be interesting to repeat the experiments with more ejaculates from different stallions. Preferably, one ejaculate would be processed for both hyperactive motility determination and protein tyrosine phosphorylation staining in parallel.

References

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978 Aug 12,;2(8085):366.

2. Betteridge KJ. Farm animal embryo technologies: Achievements and perspectives. Theriogenology. 2006 March 15,;65(5):905-13.

3. Galli C, Duchi R, Crotti G, Turini P, Ponderato N, Colleoni S, et al. Bovine embryo technologies. Theriogenology. 2003 January 15,;59(2):599-616.

4. Palmer E, Bézard J, Magistrini M, Duchamp G. In vitro fertilization in the horse. A retrospective study. J Reprod Fertil Suppl. 1991;44:375-84.

5. Hinrichs K, Love CC, Brinsko SP, Choi YH, Varner DD. In vitro fertilization of in vitro-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization in vivo after oviductal transfer. Biol Reprod. 2002 Jul;67(1):256-62.

6. Leemans B, Stout TAE, De Schauwer C, Heras S, Nelis H, Hoogewijs M, et al. Update on mammalian sperm capacitation : How much does the horse differ from other species? Reproduction. 2019;157(5):R181-97.

7. Morris LHA. The development of in vitro embryo production in the horse. Equine Vet J. 2018 Nov;50(6):712-20.

8. Leemans B, Gadella BM, Stout TAE, De Schauwer C, Nelis H, Hoogewijs M, et al. Why doesn't conventional IVF work in the horse? The equine oviduct as a microenvironment for capacitation/fertilization. Reproduction. 2016 12;152(6):R233-45.

9. Macías-García B, González-Fernández L, Loux SC, Rocha AM, Guimarães T, Peña FJ, et al. Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reproduction. 2015 Jan;149(1):87-99.

10. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature. 1951 Oct 20,;168(4277):697-8.

11. Cunningham JG, Klein BG. Cunningham's textbook of veterinary physiology. 5. ed. ed. St. Louis, Mo: Elsevier/Saunders; 2013.

12. Leemans B, Gadella BM, Sostaric E, Nelis H, Stout TAE, Hoogewijs M, et al. Oviduct binding and elevated environmental ph induce protein tyrosine phosphorylation in stallion spermatozoa. Biol Reprod. 2014 Jul;91(1):13.

13. Bart Leemans, Bart M. Gadella, Tom A.E. Stout, Sonia Heras, Katrien Smits, Minerva Ferrer-Buitrago, et al. Procaine Induces Cytokinesis in Horse Oocytes via a pH-Dependent Mechanism1. Biology of Reproduction. 2015;93(1).

14. Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J, Kopf GS. Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. Mol Hum Reprod. 1999 Nov;5(11):1017-26.

15. Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta. 2000 Nov 10,;1469(3):197-235.

16. Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod. 1988 Jun;38(5):1171-80.

17. Suarez SS, Ho H-. Hyperactivated motility in sperm. Reprod Domest Anim. 2003 Apr;38(2):119-24.

18. Suarez SS. Control of hyperactivation in sperm. Human Reproduction Update. 2008;14(6):647-57.

19. Ho H, Granish KA, Suarez SS. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca2+ and not cAMP. Dev Biol. 2002 Oct 01,;250(1):208-17.

20. Loux SC, Crawford KR, Ing NH, González-Fernández L, Macías-García B, Love CC, et al. CatSper and the relationship of hyperactivated motility to intracellular calcium and pH kinetics in equine sperm. Biol Reprod. 2013 Nov;89(5):123.

21. Loux SC, Macías-Garcia B, González-Fernández L, Canesin HD, Varner DD, Hinrichs K. Regulation of axonemal motility in demembranated equine sperm. Biol Reprod. 2014 Dec;91(6):152.

22. Flesch FM, Colenbrander B, van Golde LM, Gadella BM. Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. Biochem Biophys Res Commun. 1999 Sep 07,;262(3):787-92.

23. Harayama H, Noda T, Ishikawa S, Shidara O. Relationship between cyclic AMP-dependent protein tyrosine phosphorylation and extracellular calcium during hyperactivation of boar spermatozoa. Mol Reprod Dev. 2012 Oct;79(10):727-39.

24. Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, et al. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Dev Cell. 2005 Aug;9(2):249-59.

25. Liguori L, Rambotti MG, Bellezza I, Minelli A. Electron microscopic cytochemistry of adenylyl cyclase activity in mouse spermatozoa. J Histochem Cytochem. 2004 Jun;52(6):833-6.

26. Carlson AE, Quill TA, Westenbroek RE, Schuh SM, Hille B, Babcock DF. Identical phenotypes of CatSper1 and CatSper2 null sperm. J Biol Chem. 2005 Sep 16,;280(37):32238-44.

27. Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, et al. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc Natl Acad Sci U S A. 2007 Jan 23,;104(4):1219-23.

28. Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, et al. CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm. Proc Natl Acad Sci U S A. 2003 Dec 09,;100(25):14864-8.

29. Navarro B, Kirichok Y, Chung J, Clapham DE. Ion channels that control fertility in mammalian spermatozoa. Int J Dev Biol. 2008;52(5-6):607-13.

30. L. A. McPartlin, S. S. Suarez, C. A. Czaya, K. Hinrichs, S. J. Bedford-Guaus. Hyperactivation of Stallion Sperm Is Required for Successful In Vitro Fertilization of Equine Oocytes1. Biology of Reproduction. 2009;81(1):199-206.

31. Ishijima S, Mohri H, Overstreet JW, Yudin AI. Hyperactivation of monkey spermatozoa is triggered by Ca2+ and completed by cAMP. Mol Reprod Dev. 2006 Sep;73(9):1129-39.

32. Harayama H. Flagellar hyperactivation of bull and boar spermatozoa. Reproductive Medicine and Biology. 2018 October 1,;17(4):442-8.

33. McPartlin LA, Suarez SS, Czaya CA, Hinrichs K, Bedford-Guaus SJ. Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. Biol Reprod. 2009 Jul;81(1):199-206.

34. Martínez-Pastor F, Tizado EJ, Garde JJ, Anel L, de Paz P. Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. Theriogenology. 2011 Mar 15,;75(5):783-95.

35. Uhde K, van Tol, Helena T. A., Stout TAE, Roelen BAJ. MicroRNA Expression in Bovine Cumulus Cells in Relation to Oocyte Quality. Noncoding RNA. 2017 Mar 11,;3(1).

36. Gil MA, Abeydeera LR, Day BN, Vazquez JM, Roca J, Martinez EA. Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs. Theriogenology. 2003 Sep 01,;60(4):767-76.

37. Davis RO, Katz DF. Operational Standards for CASA Instruments. Journal of Andrology. 1993 September 10,;14(5):385-94.

38. Renaud V, Nadeau D. Adjustment of the osmolality of Percoll for the isopycnic separation of cells and cell organelles. Analytical Biochemistry. 1984 /09/01;141(2):322-8.

39. Henning H, Petrunkina AM, Harrison RAP, Waberski D. Cluster analysis reveals a binary effect of storage on boar sperm motility function. Reprod Fertil Dev. 2014 Jun;26(5):623-32.

40. Cohen J. Statistical power analysis for the behavioral sciences. Second edition ed. New York: Psychology Press; 1988.

41. VandeVoort CA, Tollner TL, Overstreet JW. Separate effects of caffeine and dbcAMP on macaque sperm motility and interaction with the zona pellucida. Mol Reprod Dev. 1994 Mar;37(3):299-304.

42. Galantino-Homer HL, Visconti PE, Kopf GS. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. Biol Reprod. 1997 Mar;56(3):707-19.

43. Galati M. The effect of caffeine, thimerosal and thapsigargin on stallion sperm hyperactivation [dissertation]. Cornell University; 2009.

44. Suliman Y, Becker F, Tuchscherer A, Wimmers K. Seasonal variations in quantitative and qualitative sperm characteristics in fertile and subfertile stallions. Arch Anim Breed. 2020 -5-14;63(1):145-54.

45. Chamberland A, Fournier V, Tardif S, Sirard MA, Sullivan R, Bailey JL. The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. Theriogenology. 2001 Feb 01,;55(3):823-35.

46. Parrish JJ. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology. 2014 Jan 01,;81(1):67-73.

47. Naresh S, Atreja SK. The protein tyrosine phosphorylation during in vitro capacitation and cryopreservation of mammalian spermatozoa. Cryobiology. 2015 Jun;70(3):211-6.

48. Thomas AD, Meyers SA, Ball BA. Capacitation-like changes in equine spermatozoa following cryopreservation. Theriogenology. 2006 May;65(8):1531-50.

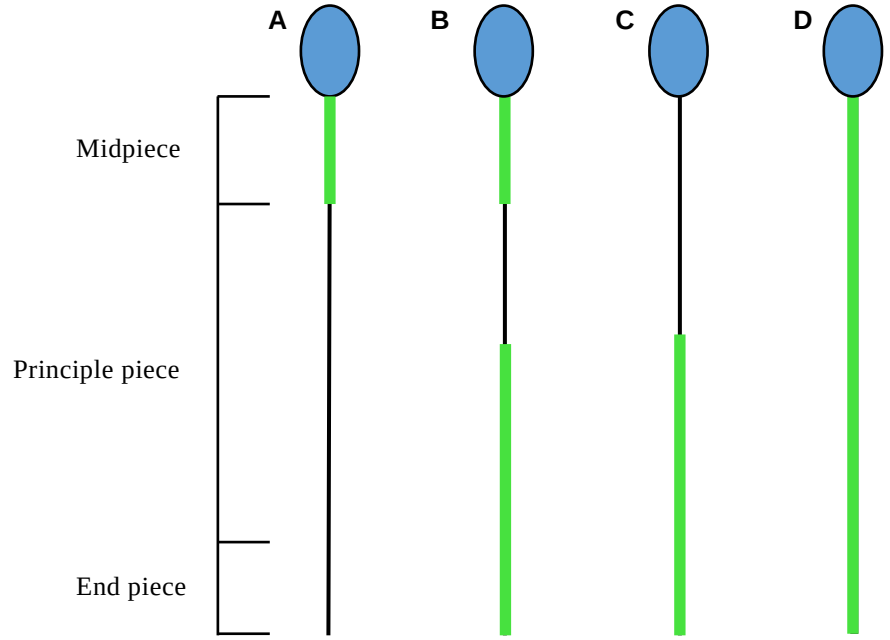
49. Boryshpolets S, Kowalski RK, Dietrich GJ, Dzyuba B, Ciereszko A. Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. Theriogenology. 2013 Oct 15,;80(7):758-65.

50. Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J Reprod Immunol. 2002 Jan;53(1-2):133-50.

51. Umezu K, Kurata S, Takamori H, Numabe T, Hiradate Y, Hara K, et al. Characteristics and Possible Role of Bovine Sperm Head-to-Head Agglutination. Cells (Basel, Switzerland). 2020 Aug 9,;9(8):1865.

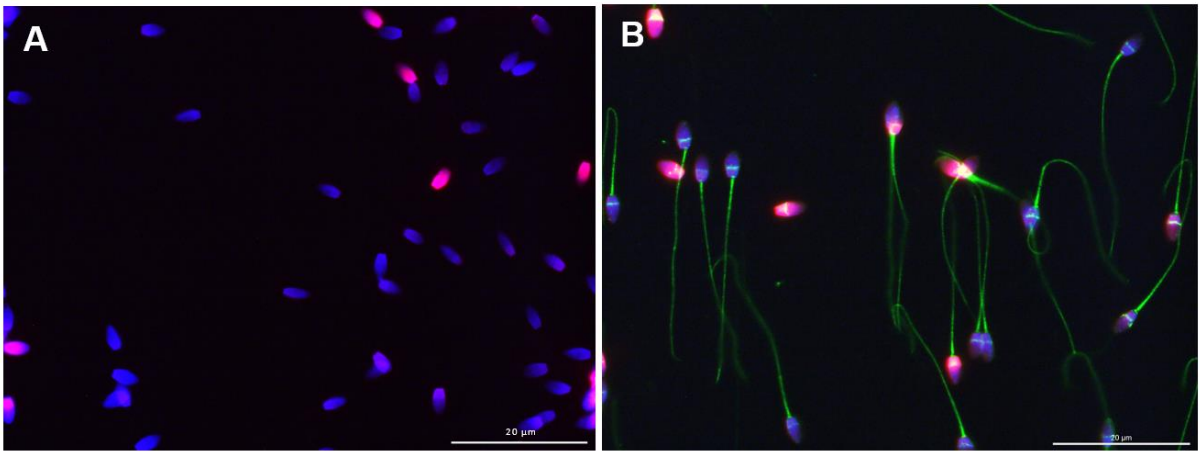
52. Monclus MA, Fornes MW. Sperm conjugation in mammal reproductive function: Different names for the same phenomenon? Mol Reprod Dev. 2016 10;83(10):884-96.

Supplemental figure 1: Different staining pattern of the sperm flagella



A; The midpiece of the flagellum showed protein tyrosine phosphorylation, B; The midpiece, distal principle piece and end piece showed PTP, C; The distal principle piece of the sperm flagellum showed PTP, D; The sperm tail was completely illuminated. No distinction in brightness was made.

Supplemental figure 2: Control of protein tyrosine phosphorylation staining



A; Negative control of protein tyrosine phosphorylation staining, B; Corresponding fully stained sample.