2017/2018

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September 2017 – August 2018

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Abstract

Conditions for successful *in vitro* capacitation of equine spermatozoa are not established. In other species, increased plasma membrane fluidity, externalization of phosphatidylethanolamine (PE) and phosphatidylserine, cholesterol efflux and hyperactivated motility in response to bicarbonate (HCO_3^-) are considered to indicate capacitation and preparation of spermatozoa for the acrosome reaction. We investigated whether extracellular bicarbonate (bic), bovine serum albumin (BSA) and calcium (Ca^{2+}) modulated membrane fluidity, PE exposure, cholesterol efflux, motility and acrosome integrity in equine spermatozoa. Semen was initially diluted in INRA96 ($30x10^6$ spermatozoa/mL). After Percoll® washing, spermatozoa were incubated at 37°C in Tyrode's medium without ($Tyr_{Control}$) or with bic ($Tyr_{Bic,}$ 5% CO_2 in air, 100% humidity). Simultaneous staining for viability (Hoechst 33258), acrosome integrity (PNA-FITC or PNA-AlexaFluor™647), membrane fluidity (merocyanine 540) or PE (duramycine-Cy5) was evaluated by flow cytometry after 15, 30 or 60 minute incubation. Results showed that high membrane fluidity can be induced in live acrosome intact spermatozoa in the presence of bicarbonate ($LAIMF_{high}$). The effect of Ca^{2+} (no addition or 2 mM) in the presence of 1 mg/mL BSA or 0.5 mg/mL each of PVA and PVP was investigated (n=6 stallions). After 60 min in Tyr_{bic}, the live MF_{high} sperm population was reduced by both BSA and Ca^{2+} ($p<0.05$, ANOVA for repeated measures) presumably because BSA and Ca^{2+} both simultaneously promoted ($p<0.05$) development of a live, acrosome-reacted sperm population. Viable spermatozoa with exposed PE were virtually absent in all treatments, whereas nearly all dead spermatozoa were PE positive. Confocal microscopy demonstrated that PE staining on dead sperm was confined to the mid-piece. No hyperactivated motility was induced by bicarbonate, BSA and calcium.

In conclusion, balanced bic, BSA and calcium concentrations can prime equine spermatozoa for a (spontaneous) acrosome reaction *in vitro*. PE exposure in equine spermatozoa is neither part of bicarbonate-stimulated activation *in vitro* nor under the control of cAMP/sAC/PKA.

Introduction

Nowadays, assisted reproductive technologies (ART) are commonly used in many different species. Classical *in vitro* fertilization is one of those well-established assisted reproductive techniques, which is successfully used in humans, dairy cattle and rodents (Wright et al, 2008). However, in the horse, only two foals have ever resulted from IVF, both born in France in the early 1990s (Palmer et al. 1991). Since then, research groups have struggled to establish a working IVF protocol, and no other reports have been published on live foal birth after classical *in vitro* fertilization. Therefore, equine IVF can still be considered a non-working technique.

IVF involves the fusion of a mature oocyte with a capacitated spermatozoon. To fertilize an oocyte, spermatozoa have to bind to and penetrate the zona pellucida. However, co-incubation of equine oocytes and spermatozoa *in vitro* results in very low fertilization rates (Leemans et al. 2016). It has been demonstrated that partial or total zona removal or zona drilling improves penetration rates (Choi et al. 1994; Li et al. 1995). The downside of zona removal or zona drilling is that it allows polyspermic fertilization. Polyspermy is incompatible with proper embryo development and thus zona removal is not suitable for practical embryo production.

Theoretically, either the inability to successfully mature oocytes, incomplete sperm activation or a combination of both, can be the reason for IVF failure. However, it has been demonstrated that *in vitro* matured oocytes transferred to the oviduct of an inseminated mare yield a similar percentage of embryos to spontaneous ovulation (Hinrichs et al. 2002). This suggests that *in vitro* matured oocytes are capable of being fertilized, and the problem may reside in the inability to capacitate stallion sperm *in vitro*. Tremoleda confirmed this speculation showing sperm failed to penetrate both *in vivo* and *in vitro* matured oocytes. Sperm were able to bind to the zona pellucida, but did not acrosome react. (Tremoleda et al. 2003).

Sperm need to capacitate, originally defined as the physiological changes that happen in spermatozoa in the female reproductive tract, leading to the ability of sperm to fertilize (Chang 1951; Austin 1952). After ejaculation, the spermatozoa within the seminal plasma are deposited in the uterus of the mare. The seminal fluid is considered to stabilize the sperm cells by coating the sperm surface with so called decapacitation factors, as they inhibit the fertilization potential of ejaculated sperm (Leahy and Gadella 2011; Bedford and Chang 1962). This stabilization allows the sperm to survive the passage through the female genital track.

However, during the process of fertilization, the sperm cell has to fuse with the oocyte. This implies that at time of fertilization the sperm plasma membrane must be in an unstable fusible state, so a preparatory step to destabilize the plasma membrane has to take place, namely, capacitation.

Once the sperm reaches the oviduct, spermatozoa have swum out of the seminal plasma and the adhered decapacitation factors are no longer present (Talevi and Gualtieri 2010). Spermatozoa interact with the ciliated cells from the oviduct epithelium and are released near ovulation (horse: Thomas et al. 1994; cattle: Sostaric et al. 2008; pig: Hunter 1981; rat: Shalgi and Kraicer 1978)

The oviduct environment primes the sperm for fertilization and once the sperm reaches the oocyte they have required fertilization abilities. Since new techniques like IVF have been introduced, more detailed studies into capacitation have been performed and the perception of capacitation has changed. It is now considered a consecutive activation of different signalling pathways inducing physiological and biochemical modifications which primes the sperm for fertilization *in vitro* (Visconti 2009; Gervasi and Visconti 2016). This enables the sperm to recognize and bind to the zona pellucida and undergo acrosome reaction, which is necessary to penetrate and fertilize an oocyte. The ability to undergo capacitation is thus a critical component of a sperm's fertilizing capacity. On the other hand, once capacitated, a sperm cell's longevity is reduced (Watson 1995).

However, until now it is not known how capacitation in equine sperm looks like. All knowledge about *in vitro* capacitation of equine sperm, has been gathered by comparing whether capacitation hallmarks that have been observed in other species, can be induced and detected in equine spermatozoa. Since *in vivo* capacitation happens in the oviduct, *in vitro* capacitation and IVF protocols try to mimic the oviduct environment. *In vitro* capacitation is well established in pigs, humans and rodents, and changes observed after *in vitro* capacitation or sperm exposure to IVF supporting media are considered capacitation-related changes (Gadella and van Gestel 2004). Although there are some species-specific differences, key stimulators for *in vitro* capacitation of mammalian sperm have been identified as bicarbonate, calcium and albumin.

In vivo, sperm are exposed to changes in extracellular bicarbonate concentrations. In the oviduct, we see relatively high bicarbonate levels (>20 mM), compared to the low levels of bicarbonate in the epididymis (<4mM) where the sperm is stored before ejaculation (Leese 1988; Pastor-Soler, Piétrement, and Breton 2005). When extracellular bicarbonate concentrations increase, the uptake via a bicarbonate-sodium co-transporter increases as well. Intracellular bicarbonate targets a soluble adenylyl cyclase which becomes activated once bound (Okamura et al. 1985). This results in an increase of intracellular cAMP levels, leading to protein kinase A activation, which induces membrane changes. (B. M Gadella and van Gestel 2004). This early capacitation response occurs within seconds to minutes after bicarbonate exposure showed by (Harrison et al. 1996), by detecting an increase in merocyanine 540 (M540) stainability. In the horse this response is also shown, although the responses in the horse might be slower (Rathi et al. 2001). M540 is amphipathic molecule, which stains cells with an enhanced fluidity in the plasma membrane. - This bicarbonate

induced alteration seemed to be concomitant with changes in membrane lipid distribution (Gadella and Harrison 2000). An altered lipid distribution in the bilayer of the sperm membrane may be responsible for this increased membrane fluidity. Normally, phosphatidylserine (PS) and to a lesser extent phosphatidylethanolamine (PE) are confined to the inner leaflet of the plasma membrane, whereas, sphingomyelin (SM) and to a lesser phosphatidylcholine (PC) are localized in the outer lipid leaflet of the plasma membrane (Gadella et al. 1999). When porcine sperm is incubated under capacitation stimulating conditions, bicarbonate changes this trans bilayer movements, resulting in detectable exposure of PE and PS in the outer leaflet of the membrane, indicating phospholipid scrambling (Gadella and Harrison 2002b). In porcine sperm, these early responses seem to be independent from albumin and Ca^{2+} (Harrison et al.1996). However, albumin is one of the key stimulators of *in vitro* capacitation of mammalian sperm. Albumin is thought to play a role in sterol and oxysterol removal from the surface of the plasma membrane during capacitation (Boerke et al. 2013). Addition of albumin to capacitating media causes cholesterol efflux in bicarbonate responsive cells (Flesch et al. 2001). Untill now, cholesterol efflux has not been proven in stallion sperm. However, there is substantial evidence that cholesterol controls the responsiveness to acrosome react (Cross 1998). The third key stimulator of *in vitro* capacitation is Ca^{2+}. Capacitation of mammalian sperm is only achieved in the presence of high Ca^{2+} levels (Stival et al. 2016; Miller et al. 2015). Ca^{2+} Calcium is in involved in several capacitation induced events, of which tyrosine phosphorylation, the acrosome reaction and hyper activated motility are the most important ones (Tardif et al. 2003). These three inducers of *in vitro* capacitation eventually lead to detectable membrane changes. These changes can be used to monitor capacitation. An overview of capacitation is shown in Figure 1.

Although at least bicarbonate is considered necessary to initiate capacitation in equine sperm, the composition of a medium which reliably supports sperm capacitation in equine species is currently still unclear. In the past years, various capacitating conditions to induce equine sperm capacitation have been investigated. An increase in phosphorylated tyrosine is considered to be a reliable hallmark of equine capacitation among researchers and is therefore often used in equine research (Macías-García et al. 2015). Tyrosine phosphorylation is only detectable after one or more hours after incubation, since it is a rather late response.

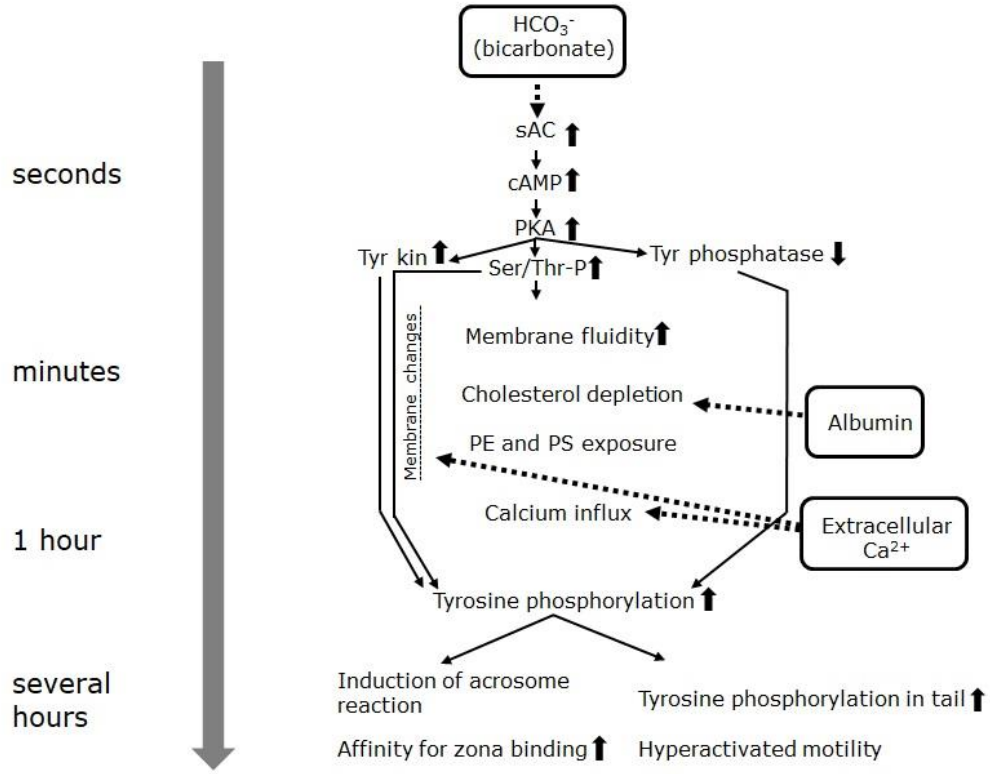

Figure 1; An overview of capacitation, modified from Boerke et al. 2008. Bicarbonate, Albumin and Ca^{2+} are known key stimulators for capacitation in mammalian sperm, causing capacitation-like events, like an increase in membrane fluidity, PE and PS exposure, cholesterol efflux and tyrosine phosphorylation. Eventually capacitation primes the sperm for fertilization as this process allows the following sperm priming events required for later fertilization: capacitated sperm gain higher affinity for the ZP of the oocyte, acquire hyperactive motility and induce the acrosome reaction which allows the spermatozoa to penetrate the ZP

In this study we tried to initiate and monitor early, mid-term and late stages of capacitation. We investigated whether extracellular bicarbonate, bovine serum albumin (BSA) and Ca^{2+} modulated membrane fluidity, PE exposure, motility patterns, cholesterol contents and acrosome integrity in equine sperm. A comparison with sperm from a species with established capacitation and IVF protocols, namely the pig, was made.

In comparison with important hallmarks in other species, the limiting step in successful activation of stallion sperm may be identified. If the three main triggers of mammalian capacitation are systematically balanced we might be able to get closer to a medium that successfully supports equine capacitation and therefore a medium that may also successfully support equine IVF.

Embedding in the faculty research program

This study is embedded in the 'Fertility and Reproduction' program of the faculty of Veterinary Medicine in Utrecht, which is embedded in the university research focus area "Growth and Differentiation". The key objective of the 'Fertility and Reproduction' program is to improve assisted reproduction technologies, by understanding the molecular, cellular and physiological mechanisms that are critical in reproduction: 1. "gamete development, maturation and preservation", 2. "gamete interaction and fertilization" and 3. "embryo development, storage and implantation" are key objectives in reproduction that this research group focuses on. This study fits in the second objective. The interaction of equine spermatozoa with their environment have been evaluated. *In vitro* fertilization is a commonly used assisted reproduction technologies. In this study we tried to activate equine spermatozoa *in vitro* in order to gain fertilizing abilities, since equine *in vitro* fertilization is still not established. This study might be a step in the right direction to a medium that successfully supports *in vitro* capacitation and therefore may also be able to support *in vitro* fertilization.

Material and methods

Chemicals and fluorescent probes

All chemicals were purchased from Merck Millipore (Amsterdam, the Netherlands) or Sigma-Aldrich (Zwijndrecht, the Netherlands) unless otherwise stated. Lectin from *Arachis hypogea* (peanut) conjugated to fluorescein isothiocyanate (PNA-FITC) was obtained from EY Laboratories (San Mateo, CA, USA), M540 was from Molecular Probes (Eugene, Oregon, USA), Hoechst3328 was from Sigma-Aldrich (Zwijndrecht, the Netherlands) and duramycin-cy5 from Molecular Targeting Technologies, Inc (West Chester, Pennsylvania, USA). The semen extender was a commercial milk-based product containing the antibiotics gentamycin and penicillin (INRA96, IMG technologies, l'Aigle, France). Gentamycin was from SERVA Electrophoresis GmbH (Heidelberg, Germany). A 10,000 Da PVA and a 40,000 Da PVP were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). A lyophilized powder of BSA (A6002-25G), essentially fatty acid free (≥96%, agarose gel electrophoresis) was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Semen collection and dilution

Semen was collected from stallions, attending the Faculty of Veterinary Medicine at Utrecht University for general semen evaluation by means of an artificial vagina (Hanover model) while stallions mounted either a mare or a dummy. Two stallions were owned by the faculty and are represented in multiple experiments. After collection, semen was filtered through gauze to remove gel fraction and large debris, and subsequently transported to the laboratory within ten minutes after ejaculation. A subjective motility check was performed in 8 µL drops using a pre-warmed microscope slide and cover slip by means of a phase contrast microscope with a heated stage (200x). Only samples with more or equal than 70% motile sperm where further processed. A seminal smear of raw semen with Aniline-Eosin was prepared for sperm morphology assessment. Concentration of the sample was determined by means of a Bürker Türk haemocytometer and sperm was diluted in INRA96 at room temperature to obtain a concentration of 30 x 10^6 spermatozoa/mL. A routine computer assisted sperm motility assessment (CASA) was performed to verify that the samples had still sufficiently motile sperm before experimentation. The average motility of samples processed for experimentation was 70.18 ±9.96%. (Motility data per experiment are shown in supplemental table 1). Diluted semen was kept at room temperature until further processing took place.

Sample preparation for flow cytometry, cell sorting and imaging

To separate the spermatozoa from the semen extender and seminal plasma, density gradient centrifugation was performed. Diluted semen (6 mL) was layered on top of a discontinuous gradient consisting of 2 mL of 70 % Percoll®-saline solution and 4 mL of 35% Percoll®-saline in a 15-mL centrifugation tube. Tubes were centrifuged for 20 minutes at room temperature, 10 minutes at 300g followed by 10 min at 750g, without stopping in between. After centrifugation the supernatant was removed using vacuum suction. The remaining pellet was resuspended in a residual 0.5 mL of 70% Percoll®. The concentration of the pellet was determined with a Bürker Türk haemocytometer and diluted with $Tyr_{control}$ to obtain a concentration of 30x 10^6sperm/mL. The sperm suspension was used within 30 min for experimentation.

Incubation media equine sperm

A control medium consisted of 111 mM NaCl, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 100 µg/mL gentamycin sulfate, 1.0 mM sodium pyruvate, 21.7 mM sodium DL-lactate. In the bicarbonate containing variants a varying amount of NaCl is replaced by 15mM, 30mM or 60 mM $NaHCO_3$, respectively. The pH was adjusted to 7.40 ± 0.05 at room temperature with NaOH and the osmolality was adjusted to 300 ± 5 mOsmol with NaCl. Media were passed through a syringe filter (PES membrane, pore size 0.22 µm; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration. The bicarbonate containing media were kept in an incubator with 5% CO_2 and 100% humidity at 37°C for equilibration prior to experimentation. Incubations of spermatozoa in control media were carried out in a metal heating block at 37°C. Incubations of spermatozoa in bicarbonate containing media took place in the same incubator used for equilibration. Variants of the media were prepared as stated in the description of the experiments.

Incubation media porcine sperm

A control medium consisted of 120 mM Nacl, 3,1 mM KCl, 5 mM glucose, 1 mM Na-pyruvate, 21.7 mM Na-lactate, 0.3 mM KH_2PO_4, 0.4 mM $MgSO_4$, 20 mM HEPES, 100 µg/mL gentamycin sulfate, 2 mM $CaCl_2$ and 3mg/mL BSA. In the capacitating medium 15 mM NaCl was replaced by 15 mM $NaHCO_3$. The pH was adjusted to 7.40 ± 0.05 at room temperature with NaOH and the osmolality was adjusted to 300 ± 5 mOsm. The IVF medium consisted of 113 mM NaCl, 3 mM KCl, 11 mM glucose, 5 mM Na-pyruvate, 20 mM TRIS, 7,5 mM $CaCl_2$, 10mg/mL BSA, 100 µg/mL penicillin/streptomycin and 1 mM caffeine. Osmolality was adjusted at 290 ± 5mOsmol/kg. The IVF medium was incubated for at least 18 hours in 5% CO_2 for pH to stabilize at 7.6. Media were passed through a syringe filter (PES membrane, pore size 0.22 µm; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration.

Flow cytometry

Flow cytometry was used to assess membrane changes. Ten microliter of the Percoll-washed sperm was added to pre-incubated FACS tubes containing 484 µL Tyrode's medium. All media contained 2 µL of each Hoechst 33258 (stock solution: 0.1 mg/mL in aqua dest) and PNA-FITC (stock solution: 0,25 mg/mL in aqua dest). One tube was prepared for each measurement. Samples were assessed after 15 min, 30 min and 60 min of incubation. 2 µL of M540 (stock solution: 750 mM in DMSO) was added 15 min before measurements took place, or 2 µL of duramycin-Cy5 (stock solution: 0.5 mg/mL in 1 % DMSO in aqua dest) was added 5 minutes before measurement took place. Before analysis on a FACSCalibur flow cytometer (BD Biosciences, Breda, The Netherlands), samples were briefly vortexed. Samples were kept on 37 °C and under CO_2 atmosphere (Tyr_{bic}) during transport from the incubator to the flow cytometer (less than 30 sec).

Excitation of Hoechst 33258 was achieved by a 405 nm laser (30 mW) and fluorescence was collected by a 450nm BP filter. For PNA-FITC a 488 nm laser (20 mW) was used and fluorescence was captured with a 520 nm BP filter. Excitation of M540 was achieved by using a 488 nm laser (20 mW). Fluorescence was collected by a 570 BP filter.

For each sample, data from 10,000 individual spermatozoa were acquired at medium speed . Compensation of spectral overlap between dyes was compensated post acquisition. Data were analyzed using FCS Express (version 3, De Novo Software, Glendale, CA, USA).

A general overview of the gating strategy is displayed in Figure 2. First, a forward and side scatter plot was used to the determine the single sperm population. These are the events in the oval (P1; Figure 2A). For further evaluation, only data from events in P1 were considered. Gated on this population, only viable, acrosome intact spermatozoa were taken into account. Thresholds for quadrants that distinguish between Hoechst 33258 positive (dead) sperm (P2 + P3) and Hoechst33258 negative (live) sperm with either low PNA-FITC signal (acrosome intact) or high PNA-FITC signal (acrosome defect/reacted) fluorescence intensity were set in the control medium ($Tyr_{Control}$; P4; Figure 2B). Similarly, the quadrants that distinguish between viable sperm with low M540 signal ($M540_{low}$; low membrane fluidity; P5) and high M540 signal ($M540_{High}$; high membrane fluidity; P6) (Figure 2C) were set. The quadrants were kept fixed to evaluate the shift in part of the live cell population from a live, acrosome intact, $M540_{low}$ state to a live, acrosome intact $M540_{high}$ state in the presence of bicarbonate (Tyr_{Bic}; Figure 2D; P7). For illustration purposes how the quadrants were defined, the dead spermatozoa are still depicted in Figure 2C and 2D. Data for PE exposure were evaluated in analogy to the date for increases in membrane fluidity.

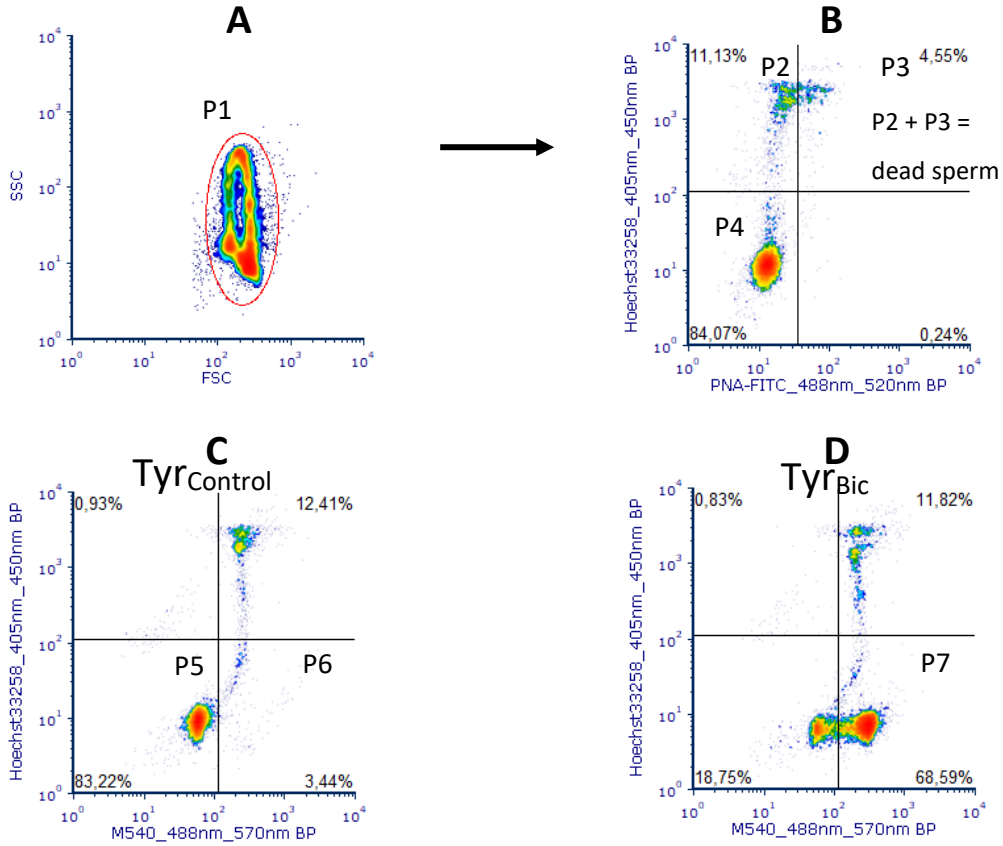

Figure 2: A general overview of the gating strategy. Forward scatter (FSC) and side scatter (SSC) are shown in figure 2A. P1 represents the single sperm population which were used for further analysis. Figure 2B shows quadrants that distinguish between Hoechst 33258 positive (dead) sperm (P2 + P3) and Hoechst33258 negative (live) sperm with either low PNA-FITC signal (acrosome intact) or high PNA-FITC signal (acrosome defect/reacted). Gated on this population, only viable, acrosome intact spermatozoa were taken into account (p4). Figure 2C shows the discrimination for viable sperm with low M540 signal ($M540_{low}$; low membrane fluidity; P5) and high M540 signal ($M540_{High}$; high membrane fluidity; P6). Thresholds were set in the control medium and kept fixed for evaluating bicarbonate effects. Figure 2D shows a clear shift from a live, acrosome intact, $M540_{low}$ state to a live, acrosome intact $M540_{high}$ state in the presence of bicarbonate (P7)

Sperm cell sorting

Sperm cell sorting and analysis were performed on a FACS Influx (Becton Dickinson, San Jose, Canada). A total of 10^7 spermatozoa were incubated in either 0.5 mL $Tyr_{Control}$ or Tyr_{Bic} supplemented with 1 mg/mL BSA and 2 mM Ca^{2+} for 60 minutes with 2 µl of each Hoechst 33258 (0,1mg/mL) and PNA-Alexa Fluor 488 (0.25 mg/mL). 2 µl of M540 (750 mM) was added 10 min before sorting.

The system was triggered on the forward light scatter signal (FSC). Hoechst 33258 was excited with a 405nm Laser. Emission was captured with a 460/50 nm filter. M540 was excited with a 561 nm laser, and emission was captured with a 585/42 nm filter. Sperm was analyzed at a rate between 8000 and 10,000 events per second. For each file, 10,000 events were stored in the computer for further analysis with FCS express. FSC and sideward light scatter (SSC) were recorded and only sperm cell specific events, were positively gated for further analysis. During sorting the sample-input tube on the FACS was kept at 38°C to maintain incubation temperature during the complete sorting procedure. Sperm cells were run through the machine using PBS as a sheath fluid. Two subpopulations were sorted: (1) sperm cell events that were not stained with Hoechst 33258 (viable) showing low M540 fluorescence; and (2) viable cells with high M540 fluorescence. Sorted cells were collected in precooled 15 mL tubes that were placed in a tube holder which was kept at -20°C. The concentration of the sorted sample was approximately 100.000 cells/mL. After sorting samples were kept at -20°C until lipid extraction took place.

Microscopy

A fluorescence microscope (Olympus BX60, Olympus Nederland BV, Leiderdorp, The Netherlands) and confocal laser scanning microscopy (LEICA SPE II DMI4000, Leica Microsystems, Wetzlar, Germany) were used for cell imaging. Samples were prepared as stated above and incubated for 60 minutes in either a control or a bicarbonate containing medium. Media were supplemented with 1 mg/mL BSA or 0.5 mg/mL of both PVA and PVP. 20 µl of the Percoll®-washed sperm was added to pre-incubated FACS tubes containing 468 µl $Tyr_{control}$ or Tyr_{Bic}. Tubes contained 2 µL of Hoechst 33258 (0.1 mg/mL) and 10 µl of PNA-Alexa Fluor 488 (0.25 mg/mL). In Experiment 5, 2 µL of duramycin-Cy5 (0.5 mg/mL) was added. On the fluorescence microscope, Hoechst33258 was excited and detected with filter A (BP350/20 nm for excitation, LP425 for detection) and PNA-FITC was excited and detected using filter I3 (BP490/45 for excitation and LP515 for detection). On the LEICA SPE II DMI4000 Hoechst33258 was excited with the 405 nm laser, PNA-FITC was excited with the 488 nm laser and duramycin-Cy5 was excited with the 635 nm laser. Tubes were briefly vortexed before 1.5 µL droplets were placed on a pre-warmed slide and covered with a coverslip to provide a thin fluid layer making it possible to image single cells.

Computer-assisted semen analysis

For the routine CASA assessment diluted semen samples (0.5 mL) were incubated for 15 minutes at 37°C before computer assisted sperm analysis was performed. In experiment 8, Percoll® washed-semen samples were incubated for 15, 30 or 60 minutes in either a bicarbonate containing medium or a control medium before CASA analysis was performed. Analysis was done with SpermVision® software (version 3.5, Minitube, Tiefenbach, Germany) at 200 times magnification. A microscope, equipped with a camera and an automatically moving, pre-warmed (38°C) microscope stage was used. Each sample consisted of 3 µL of the pre-incubated 0.5 mL aliquot, which was pipetted in a Leja-chamber slide of 20 µm in depth (Leja Products BV, Nieuw-Vennep, The Netherlands). Twelve fields at a rate of 60 Hz per field were analysed within one minute of filling the chamber. Spermatozoa were considered motile when they met either one of the following three combinations of criteria: I) Average head orientation change (AOC) > 7° and beat cross frequency (BCF) > 25 Hz, II) distance straight line (DSL) > 15 µm and straight line velocity (VSL) > 8 µm/s or III) average path velocity (VAP) > 15 µm/s. Spermatozoa were considered progressively motile when one of the following combinations of criteria was met: I) DSL > 15 µm, II) BCF > 40 Hz and radius > 5 µm, III) VSL > 20 µm/s, IV) DCL ≥ 20 µm, V) straightness (STR) > 0.3 and linearity (LIN) > 0.2, VI) Radius > 10 µm, VII) BCF > 30 Hz and amplitude of lateral head displacement (ALH) greater than 0.85 µm.

In vitro fertilization

Standard *in vitro* maturation procedures were performed as detailed in Fatehi et al. (2005)
The IVF was performed in either a capacitating medium or in a standard IVF medium. Thirteen matured oocytes were placed in each group. Oocytes were co-incubated with sperm for 24 hours before stained with Hoechst33342. Fertilization was evaluated as successful when two polar bodies were present and the formation of two pronuclei was detectable.

Experimental strategy

The aim in the first set of experiments was to identify a medium that is most likely to initiate capacitation in equine spermatozoa (Experiment 1 and 2). Flow cytometric assays using fluorescent stains were used to determine whether early milestones in capacitation, i.e. an increase in plasma membrane fluidity and PE exposure in the outer leaflet of the membrane in live spermatozoa, can be induced. Different concentrations of bicarbonate were compared (Experiment 1) and the effects of extracellular BSA and calcium were tested (Experiment 2). In order to provide a maximal stimulation for sperm to exhibit an increase in plasma membrane fluidity and PE exposure, direct and indirect stimulation of the sAC/cAMP/PKA pathway was tested (Experiment 3).

Not all hallmarks of early sperm capacitation could be induced in experiments 1 to 3, although spontaneous acrosome reactions in a subset of spermatozoa occurred. Therefore, boar spermatozoa were used in a control experiment to reproduce early hallmarks of capacitation in sperm from a species with established capacitation and IVF protocols (Experiment 4). Imaging of PE exposure and spontaneous acrosome reaction in equine spermatozoa was carried out in separate experiments in order to visualize staining patterns and verify the changes in fluorescence intensity which were observed in flow cytometry (Experiment 5 and 6).

The condition with the best results regarding the early hallmarks of sperm capacitation from Experiment 1 and 2 was used for assessing whether cholesterol efflux, a late event during capacitation, can be observed in equine sperm (Experiment 7). Capacitation is also supposed to influence sperm motility and induce a hyperactivated motility pattern. The effects of bicarbonate, BSA and calcium on sperm motility were evaluated in experiment 8.

Results from Experiments 1 to 6 raised the question whether changes in sperm observed in a capacitating medium represent fertilization relevant hallmarks of in vitro capacitation and would result in vitro fertilization when these sperm were co-incubated with mature oocytes. Therefore, a preliminary comparison was made whether porcine IVF is equally successful in a medium used for studying sperm capacitation and a medium used for routine porcine IVF (Experiment 9).

Experiment 1

The aim was to investigate the effect of 15, 30 or 60 mM bicarbonate on an increase in plasma membrane fluidity in live spermatozoa. A medium without bicarbonate served as control ($Tyr_{Control}$). In this experiment, media were supplemented with either 0.5 mg/mL each of polyvinylalcohol (PVA) and polyvinylpyrrolidone (PVP) or 1 mg/mL bovine serum albumin (BSA). Molecules like PVA, PVP, or BSA are commonly used to prevent spermatozoa from sticking to surfaces and diminish agglutinations in situations when semen storage is required. Percoll-washed semen samples (n=6) were incubated for 15, 30 and 60 minutes and analysed on a flow cytometer. Fluorescent dyes allowed for assessment of viability (Hoechst 33258), acrosome-integrity (PNA-FITC), plasma membrane fluidity (merocyanine 540, M540). The percentage of the live, acrosome intact spermatozoa with high plasma membrane fluidity (Hoechst 33258 and PNA-FITC negative, M540 high) were evaluated.

Experiment 2

In the context of *in vitro* capacitation with density gradient selected spermatozoa, BSA has been described to promote an increase in the live, M540high population in porcine spermatozoa in comparison to a mixture of PVA and PVP (Harrison et al. 1996). BSA and Ca^{2+} are known as key stimulators for in vitro capacitation in other species (Gadella and Luna 2014). The aim was to investigate the influence of bovine serum albumin and extracellular calcium on an increase in plasma membrane fluidity and phosphatidylethanolamine (PE) exposure in live spermatozoa. Percoll-washed semen samples (n=6) were incubated for 15, 30 and 60 minutes and analysed on a flow cytometer. Media were supplemented with either 0.5 mg/mL PVA and PVP or 1mg/mL BSA. Addition of 2 mM Ca^{2+} to half of the tubes allowed us to investigate its effects on membrane fluidity and PE exposure in live spermatozoa. Fluorescent dyes were used as stated in Experiment 1. For assessment of PE exposure, M540 was replaced by duramycin-Cy5. The percentage of live, acrosome intact spermatozoa with high plasma membrane fluidity and the percentage of live, acrosome intact spermatozoa positive for PE (Hoechst 33258 and PNA-FITC negative, duramycin-Cy5 positive) were evaluated.

Experiment 3

Bicarbonate is thought to be the key stimulator for in vitro capacitation (Gadella and van Gestel 2004). Bicarbonate is supposed to directly stimulate soluble adenylyl cyclase (sAC) and thus have its effect on PE exposure by stimulating the sAC/cAMP/PKA pathway (Gadella and Harrison 2002b). However, under previous conditions (Experiment 1 and 2) PE exposure in viable cells was virtually absent. The aim was to investigate the effect of sAC/cAMP/PKA pathway stimulators, namely dibutyryl-cAMP and caffeine, on an increase in plasma membrane fluidity and PE exposure in live spermatozoa. Dibutyryl-cAMP is a membrane permeable cAMP analogue directly increasing intracellular cAMP. Caffeine is a phosphodiesterase inhibitor, preventing degradation of endogenous cAMP, and thus increases cAMP in an indirect manner.
Samples (n=6) were incubated for 15, 30 and 60 minutes and analyzed on a flow cytometer. Media contained 1 mg/mL BSA and 2 mM calcium. A medium with and without 30 mM bicarbonate were used. 1 mM dibutyryl-cAMP, 1 mM caffeine or 1 mM of each were supplemented before incubation started. Bicarbonate-free and bicarbonate containing media without supplementation of dibutyryl-cAMP or caffeine served as controls. Fluorescent dyes were used as stated in Experiment 2. The percentage of live, acrosome intact spermatozoa with high plasma membrane fluidity and the percentage of live, acrosome intact spermatozoa with PE exposure were evaluated.

Experiment 4

The aim of this control experiment was to reproduce hallmarks of capacitation in porcine sperm, a species with established capacitation and IVF protocols. Hallmarks like high membrane fluidity and PE exposure have been induced and detected in capacitated porcine sperm in the past. Since capacitation should prime the sperm for fertilization, in this experiment porcine sperm was incubated in an IVF medium as a control condition and checked on membrane fluidity and PE exposure on the flow cytometer in the same way as performed in Experiment 2. Semen samples (n=2) were incubated for 15, 30 and 60 minutes in capacitating medium, IVF medium and a medium without bicarbonate. The fluorescent dyes PNA-FITC, Hoechst33258 and either M540 or duramycin-Cy5 were used and the percentage of live spermatozoa with high membrane fluidity and the percentage of live spermatozoa with PE exposure were evaluated.

Experiment 5

The aim was to visualize and verify the staining patterns for duramycin-cy5, which were observed in flow cytometry. Sperm was incubated for 60 minutes. Medium contained 1 mg/mL BSA, 2 mM calcium and 30 mM bicarbonate. A medium without bicarbonate was used as a control. Fluorescent dyes were used as stated in Experiment 2. Droplets of 1.5 µL were placed on a slide and covered with a coverslip to provide a thin fluid layer to trap the cells. Confocal laser scanning microscopy was used to image single spermatozoa.

Experiment 6

The aim was to visualize and verify changes in fluorescence intensity in live spermatozoa for PNA-FITC, which were observed in flow cytometry. Sperm was incubated for 60 minutes. Medium contained 1 mg/mL BSA, 2 mM Ca^{2+} calcium and 30 mM bicarbonate. Fluorescent dyes were used as stated in Experiment 1. Sample for microscopy were prepared as in Experiment 5. Fluorescence microscopy was used to image live spermatozoa, positive for PNA-FITC and investigate the sub-cellular localization of the PNA-FITC signal.

Experiment 7

Cholesterol efflux from the plasma membrane is thought to be one of the main events happening during in vitro capacitation. Therefore, the aim was to investigate the cholesterol content of subpopulations of sorted sperm. In this experiment, media were supplemented with 1 mg/mL BSA and 2 mM Ca^{2+}, i.e. conditions established in experiment 1 and 2 that provide a high degree of sperm activation. Fluorescent dyes were used to check for viability (Hoechst 33258), acrosome integrity (PNA-FITC) and membrane fluidity (M540). Sperm were incubated for 60 minutes in either a bicarbonate containing medium or a control medium without bicarbonate before flow cytometric cell sorting took place. Sperm were sorted based on viability and membrane fluidity, and collected at -20°C. Subpopulations of live M540 positive and live M540 negative spermatozoa were collected. Sorted samples from five stallions were used for lipid analyses (n=5). Lipid extraction took place before samples were analyzed on a mass spectrometer.

Experiment 8

Hyperactivated motility is thought to be a result of successful capacitation. The aim was to investigate the effect of bicarbonate, BSA and calcium on sperm motility. Computer-assisted sperm analysis was used to investigate motility patterns of incubated sperm samples (n=3). Percoll-washed sperm was incubated for 15, 30 and 60 minutes in either a bicarbonate containing medium or a control medium without bicarbonate. Media were supplemented with either 0.5 mg/mL PVA and 0.5 mg/mL PVP or 1 mg/mL BSA. Two millimolar calcium was supplemented to half of the tubes. At all measured time points, CASA assessment was performed. The percentage of motile sperm and the motility patterns were evaluated.

Experiment 9

Capacitation should prime sperm for fertilization. The aim was to investigate whether a commonly used capacitating medium facilitates porcine in vitro fertilization. Therefore, IVF was performed in a capacitating medium. A well-established porcine IVF medium was used as a control. One batch of oocytes was matured, and sperm was processed following IVF protocol. After maturation, oocytes were split into two groups. One group was used for routine IVF and in the other group IVF was performed in a capacitating medium. Oocytes and sperm were co-incubated for 48 hours and fertilization rates were evaluated.

Statistical analysis

Statistical analysis was performed, using SPSS statistics, version 25 (IBM analytics, Amsterdam, The Netherlands). Data were tested for normal distribution and compared with an multi-factorial ANOVA for repeated measures. Considered factors differed according to the experiment. They included time, bicarbonate concentration (control vs bicarbonate or in the first experiment 0 mM, 15 mM, 30 mM and 60 mM), macromolecule (BSA or PVA/PVP), presence of calcium and the presence of sAC/cAMP/PKA pathway stimulators. Paired Student's t-tests for dependent samples were performed to investigate differences between individual medium variants. Unless otherwise stated, data are presented as mean ± standard deviation (SD). Differences were considered to be significant at a significance level of 5 % ($p \leq 0.05$).

Results

Bicarbonate induces a population of equine spermatozoa with high membrane fluidity

In an attempt to determine the effect of different bicarbonate concentrations, in Experiment 1 Tyrode's media with either 0 mM (control), 15 mM, 30 mM or 60 mM bicarbonate was used in six stallions. To start with, the population of viable, acrosome intact spermatozoa on average was 69.07% of the cells in PVA/PVP containing media after 15 minutes. In BSA containing media 65.26% of the spermatozoa were viable and acrosome intact (supplemental table 2). The population of viable, acrosome intact cells decreased over time, due to an increase of dead spermatozoa. This decrease was more pronounced in BSA containing media ($p<0.05$). After 60 minutes, the viable acrosome intact sperm population consisted of 65.1 % in PVA/PVP containing media. In BSA containing media this population consisted of 58.6% of the spermatozoa. The bicarbonate concentration had no influence on the percentage of dead cells ($p<0.05$; supplemental table 3). An increase in plasma membrane fluidity occurred within 15 minutes after exposing spermatozoa to bicarbonate, as a clear shift from a live, $M540_{low}$ to a live $M540_{high}$ state indicated. High membrane fluidity could be induced in the majority of the live acrosome intact spermatozoa in the presence of all three concentrations of bicarbonate (Figure 2). This response could be induced regardless of whether BSA was present. The live, acrosome intact sperm population with high membrane fluidity ($LAIMF_{high}$) peaked after 30 minutes (69,9% ± 10,2 in 60 mM bicarbonate containing medium supplemented with both 0.5 mg/mL of PVA and PVP). However, incubation time had no significant influence of on the $LAIMF_{high}$ population ($p>0.05$). In general, the population of sperm responding to bicarbonate by an increase in membrane fluidity is significantly reduced by BSA in bicarbonate-containing conditions (Figure 3).

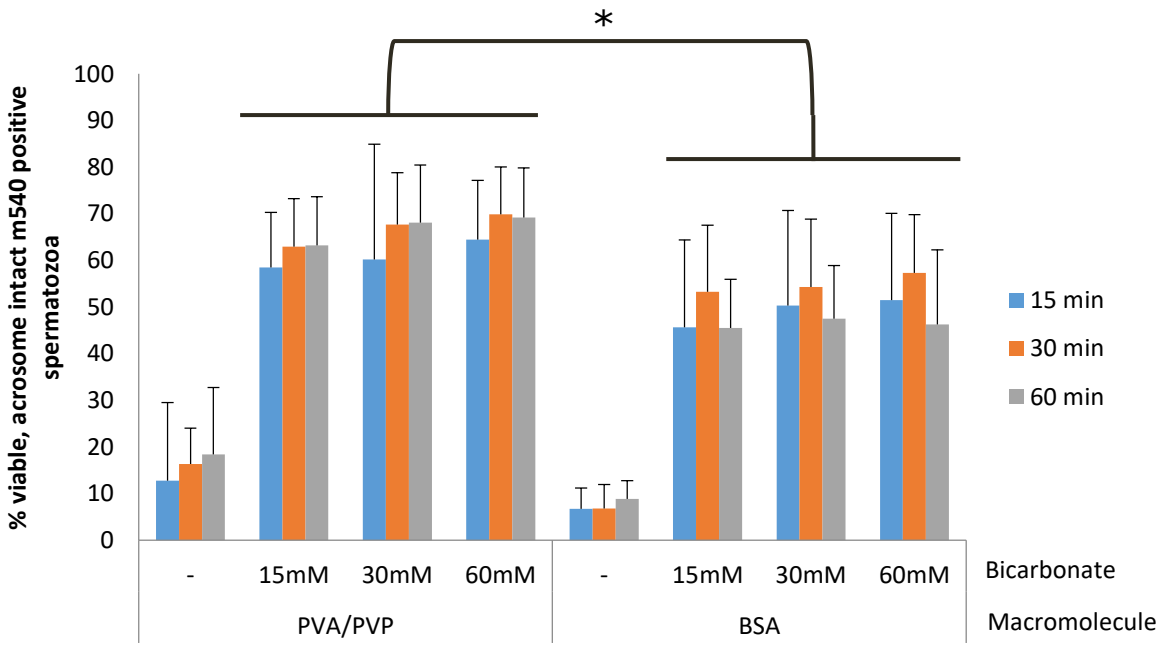

Figure 3; Bicarbonate induces an increase in membrane fluidity in viable, acrosome intact spermatozoa. The percentage of viable, acrosome intact spermatozoa with high membrane fluidity ($LAIMF_{high}$)is shown per measured time point. The concentration of bicarbonate has no significant influence on the percentage $LAIMF_{high}$. BSA reduced the $LAIMF_{high}$ in bicarbonate containing conditions (is $p<0.05$; n=6).*

The $LAIMF_{high}$ did not significantly differ between the different bicarbonate concentrations and the percentage of dead cells was also not influenced by bicarbonate concentrations. Thus, based on the afore-mentioned parameters, all bicarbonate concentration were equally suited to induce an early hallmark of in vitro capacitation.

The pH of the 60 mM bicarbonate containing plain medium drifted towards 8.0 during the time of the experiment (table 1). Therefore, 30 mM bicarbonate was used as set concentration in Tyr_{Bic} for further experimentation.

Bicarbonate concentration	PVA/PVP containing medium	BSA containing medium
15 mM	7.37	7.34
30 mM	7.45	7.48
60 mM	7.58	7.93

Table 1; The PH of the media was measured during experimentation. The PH of media containing 60 mM drifted towards 8.0, especially in the presence of BSA. The PH of the media with 15 mM and 30 mM stayed around the desired PH of 7.4

BSA and calcium modulate bicarbonate effects

In Experiment 2, the individual and combined effects of 1 mg/mL BSA and 2 mM extracelluar calcium on plasma membrane fluidity and PE exposure in equine spermatozoa were tested. In control medium supplemented with PVA/PVP a subpopulation of viable cells showed high membrane fluidity, in the absence of bicarbonate, when incubated for more than 15 minutes. After 30 minutes incubation in $Tyr_{control}$, 39.7 ± 23.8 % of the cells showed high membrane fluidity. The population $LAIMF_{high}$ in control media decreased to 8,1 ± 7.2 % and 8.5 ± 7.8 % of the cells, respectively, when 2 mM calcium or 1 mg/mL BSA were supplemented ($p<0.05$).

Adding BSA instead of PVA/PVP resulted in a decrease in the percentage of acrosome intact cells with high membrane fluidity in the presence of bicarbonate, as can be seen from Figure 2 ($p<0.05$). This effect was repeatable in the second experiment. Adding Ca^{2+} to bicarbonate-containing medias had the same effect (Figure 3; $p<0.05$). After 30 minutes incubation with PVA/PVP, 74.5 ±13.9% of the cells showed increased plasma membrane fluidity. When 2 mM Ca^{2+} was added to the medium the population of viable, acrosome intact spermatozoa with high membrane fluidity decreased to 63.6 ± 17.6%. The reduction of responding cells by calcium was more pronounced in media supplemented with BSA (Figure 4)

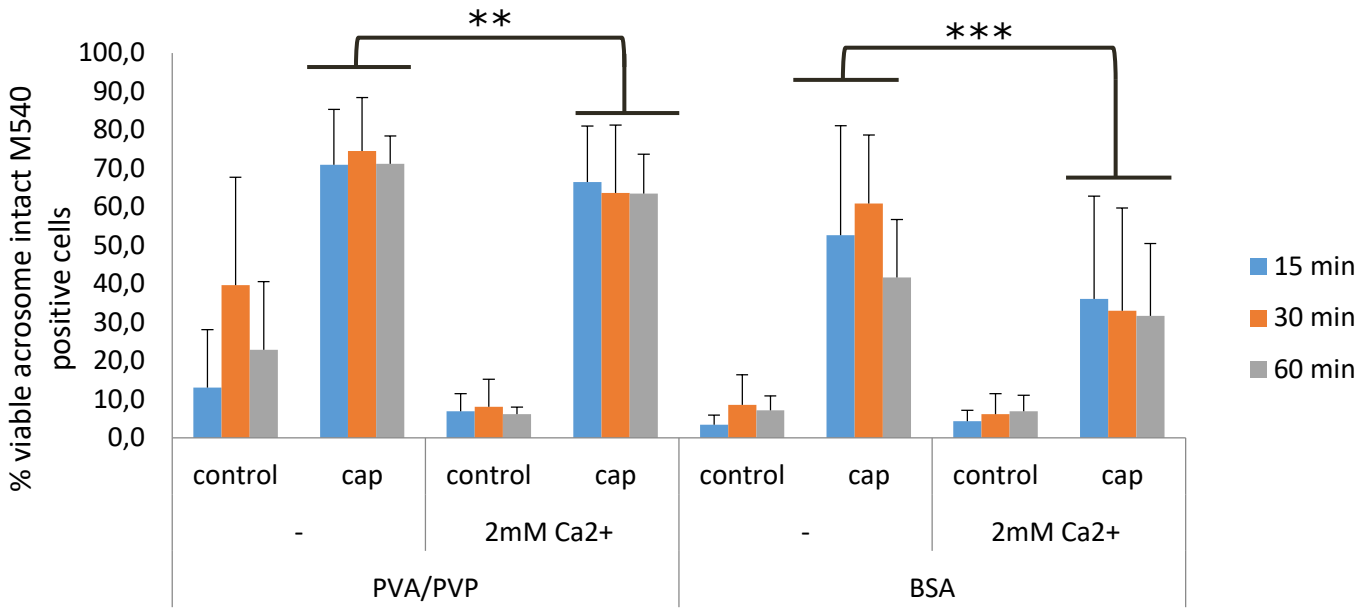

*Figure 4; The percentage of viable, acrosome intact spermatozoa with high membrane fluidity ($LAIMF_{high}$)is shown per measured time point. The $LAIMF_{high}$ in bicarbonate containing conditions (cap) is reduced by calcium (** is p<0.01, *** is p<0.001 n=6) and BSA (statistics not shown in this graph).*

BSA and calcium promote the development of a PNA-FITC positive subpopulation of equine spermatozoa

The percentage of viable acrosome intact cells with high membrane fluidity is reduced in presence of BSA and Ca^{2+}. This was based on the fact that the population of viable acrosome intact cells decreased in size as well. Although the percentage of dead cells significantly increased over time (p<0.05) (supplemental table XX) , this could not fully explain the decrease in viable acrosome intact cells. The decrease was due to the fact that the combined presence of BSA and calcium promoted the development of a viable population of PNA-FITC positive cells (Figure 5).

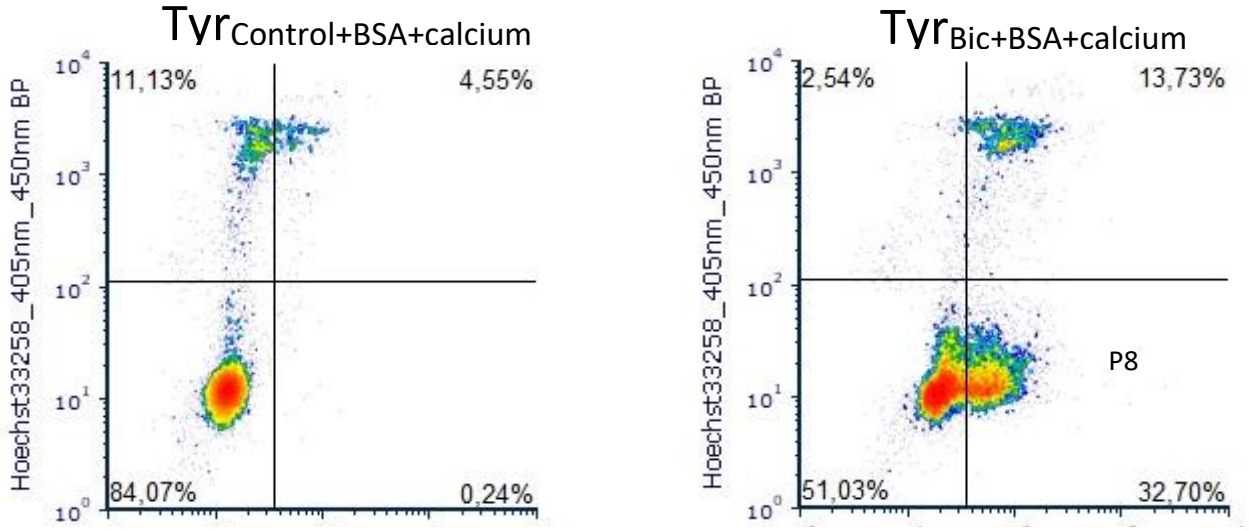

Figure 5; Scatterplots of Hoechst33258 vs PNA-FITC, representing viability and acrosome integrity, respectively. Incubation in the presence of bicarbonate, BSA and calcium promoted the development of a PNA-FITC positive subpopulation of spermatozoa (P8)

After 30 minutes incubation in $Tyr_{Bic+BSA+Calcium}$ a significant subpopulation of viable cells (18.7 ± 13.3%) became PNA-FITC positive. After 60 minutes the population of viable PNA-FITC positive cells had increased to 21.0 ± 18.8 % (Figure 6). A considerable variation between stallions was observed. Four out of six stallions showed the formation of PNA-FITC positive subpopulation of viable cells when both BSA and calcium were present in the bicarbonate containing medium.

Two stallions showed a formation of a PNA-FITC positive subpopulation of viable cells in the presence of bicarbonate and BSA but in the absence of calcium. However, the mean response was not significantly different from the condition with bicarbonate and PVA/PVP without calcium ($p>0.05$).

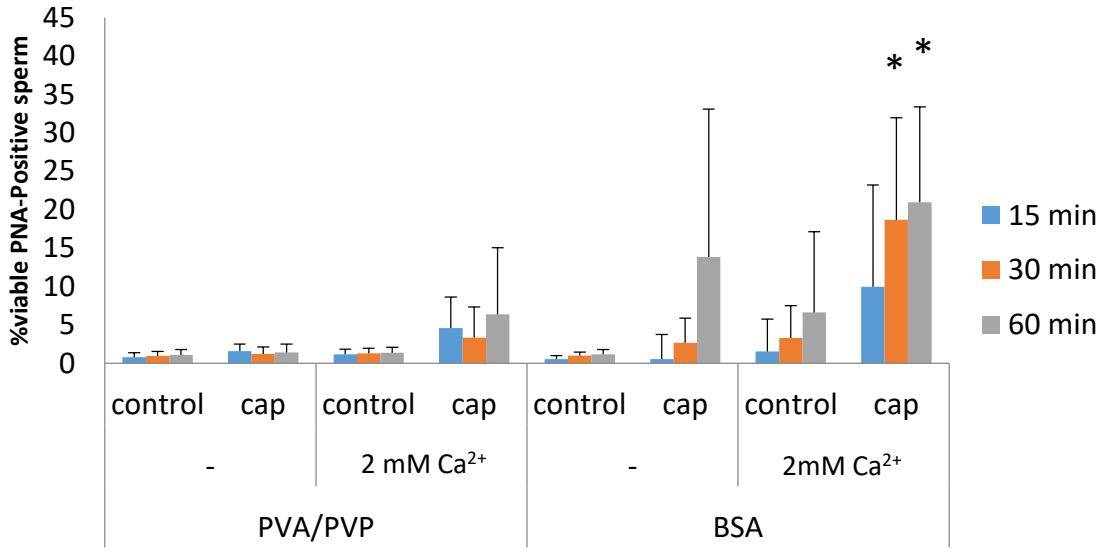

Figure 6; Bicarbonate, BSA and calcium promote the development of a subpopulation of viable PNA-FITC positive cells after 30 and 60 minutes incubation (is different from respective control sample, $p<0.05$, $n=6$). This subpopulation is not seen in control conditions or in the absence of either bicarbonate, BSA or calcium*

Influence of BSA and calcium on PE exposure

No PE exposure in the outer leaflet of the membrane could be detected in live, acrosome intact spermatozoa. BSA and calcium did not promote the development of a viable population with PE exposure in the presence or absence of bicarbonate (supplemental table 4). All dead cells were PE positive

Imaging of a live, PNA-positive sperm subpopulation

Intact equine spermatozoa in samples that had been incubated in control conditions ($Tyr_{control+BSA+calcium}$) did not show any PNA-FITC staining. However, in $Tyr_{Bic+BSA+calcium}$ some spermatozoa that were Hoechst 33258 negative (viable) and PNA-FITC positive could indeed be identified. It was difficult to find single viable spermatozoa, because the majority of the viable cells clustered together in the thin fluid layer, which made imaging challenging. A representative image is given in Figure 7. The PNA-FITC signal originates from the acrosomal region of the sperm head, and might be indicative of a spontaneous acrosome reaction. All viable PNA-FITC positive cells showed this pattern.

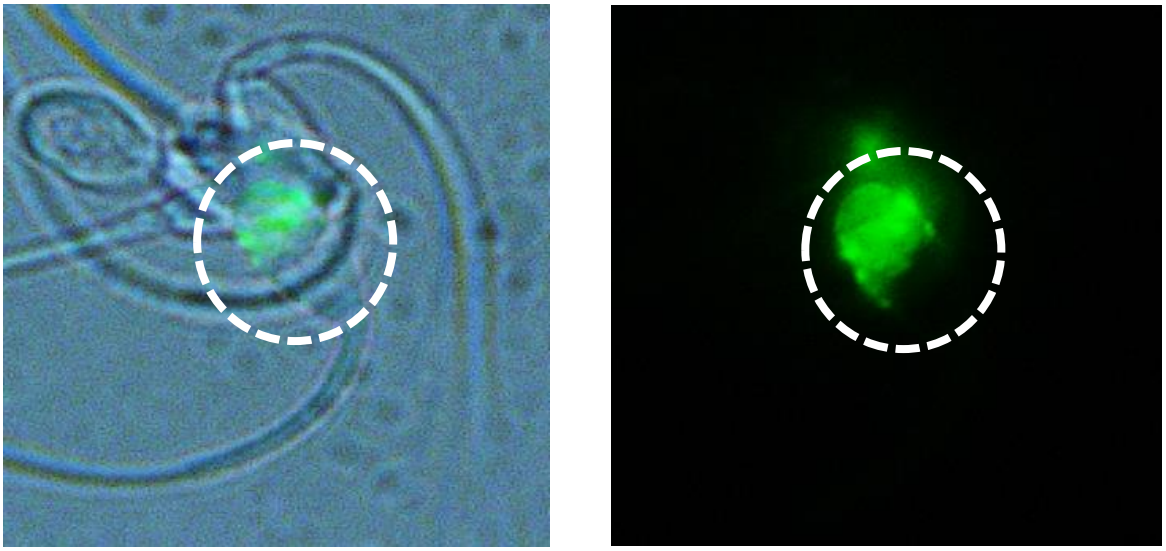

Figure 7; A clustered group of viable spermatozoa (1000X,oil immersion), out of which one cell is PNA-FITC positive. The PNA-FITC positive signal in viable spermatozoa originated from the acrosomal region of the sperm head (circle).

sAC/cAMP/PKA pathway stimulators have their effect predominantly in control conditions

In Experiment 3, the effect of stimulating the sAC/cAMP/PKA pathway in equine spermatozoa was tested. Since bicarbonate is thought to trigger this pathway, extra stimulation might add up to the effect of bicarbonate on membrane fluidity and especially PE exposure, since PE exposure in viable cells was absent in previous experiments. Dibutyryl-cAMP and caffeine were used for this purpose. When these substances are supplemented together, they increase the percentage of spermatozoa with high membrane fluidity in control conditions at all assessed time points (ANOVA for repeated measurement; $p<0.05$). When both 1 mM of each dibutyryl-cAMP and caffeine is present, their effect is already significant after 15 minutes incubation and increases over time ($p<0.05$, compared to $Tyr_{Control+BSA+calcium}$, no additions). Adding dibutyryl-cAMP alone has no additional effect on the responsive cell population regarding to membrane fluidity in control conditions, and an effect of caffeine alone was only detectable at 30 minutes incubation ($p<0.05$; Figure 8)

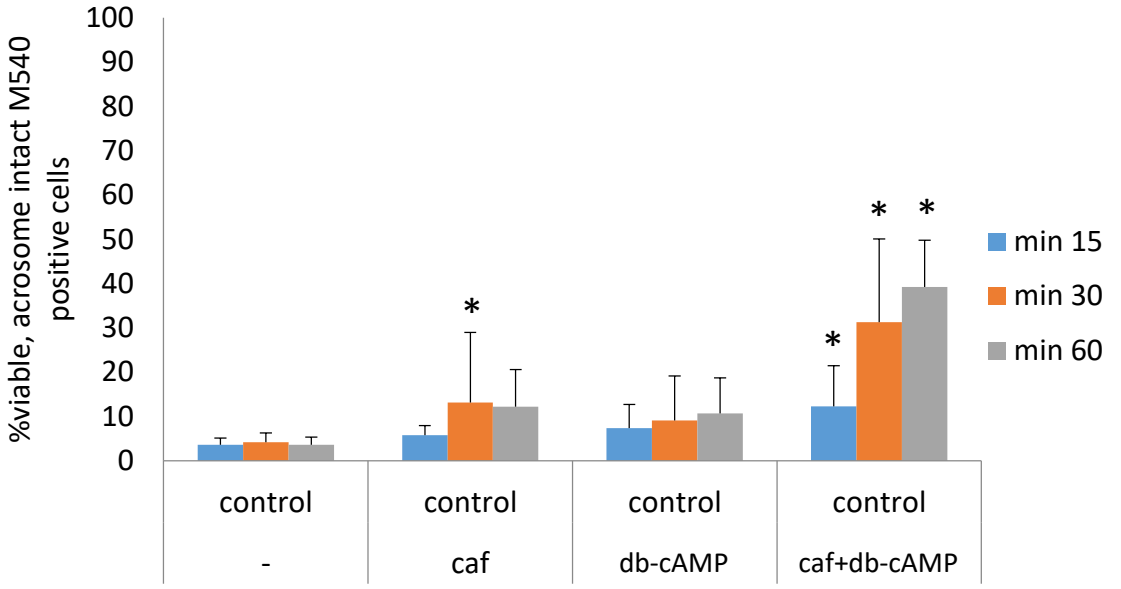

Figure 8; The population of viable, acrosome intact spermatozoa with high membrane fluidity ($LAIMF_{High}$) is increased by the sAC/cAMP/PKA pathway stimulators caffeine and dibutyryl-cAMP (db-cAMP) in control conditions (without bicarbonate) at all time points (is $p<0.05$) when compared to the non-supplemented medium. Caffeine (caf) alone increases $LAIMF_{High}$ after 30 minutes incubation.*

However, no significant difference in percentage of cells with high membrane fluidity between the bicarbonate containing medium without additions ($Tyr_{Bic+BSA+calcium}$) and the bicarbonate containing media ($Tyr_{Bic+BSA+calcium}$) supplemented with dibutyryl-cAMP or caffeine could be found (Figure 9).

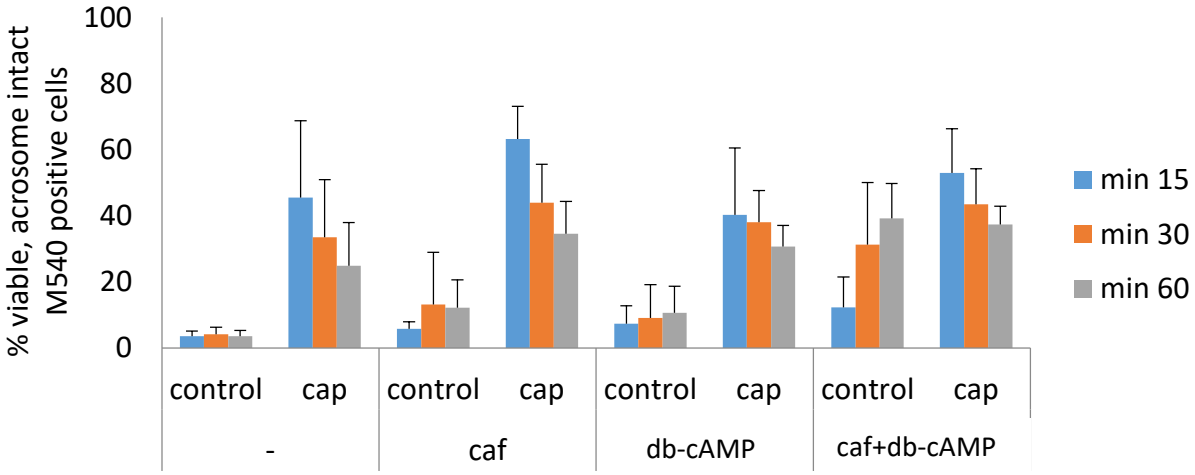

Figure 9; The population of viable, acrosome intact spermatozoa with high membrane fluidity ($LAIMF_{High}$) is not influenced by caffeine (caf) and dibutyryl-cAMP (db-cAMP) in bicarbonate containing conditions (n=6).

The effect of dibutyryl-cAMP and caffeine on the percentage of viable PNA-FITC positive cells was also investigated. In the control conditions without any bicarbonate, a very low percentage of cells (6.01 ± 6.13%) is both viable and PNA-FITC positive without any additions of sAC/cAMP/PKA pathway stimulators after 60 minute incubation. In the presence of either 1 mM caffeine, 1 mM dibutyryl-cAMP or 1 mM of both, the population of PNA-FITC positive cells is significantly increased (9.37 ± 7.98% in $Tyr_{control+caf}$, 7.53+6.22% in $Tyr_{control+cAMP}$ and 19.04 ± 11,83 % in $Tyr_{control+caf+cAMP}$; all $p<0.05$; n=6) (Figure 10).

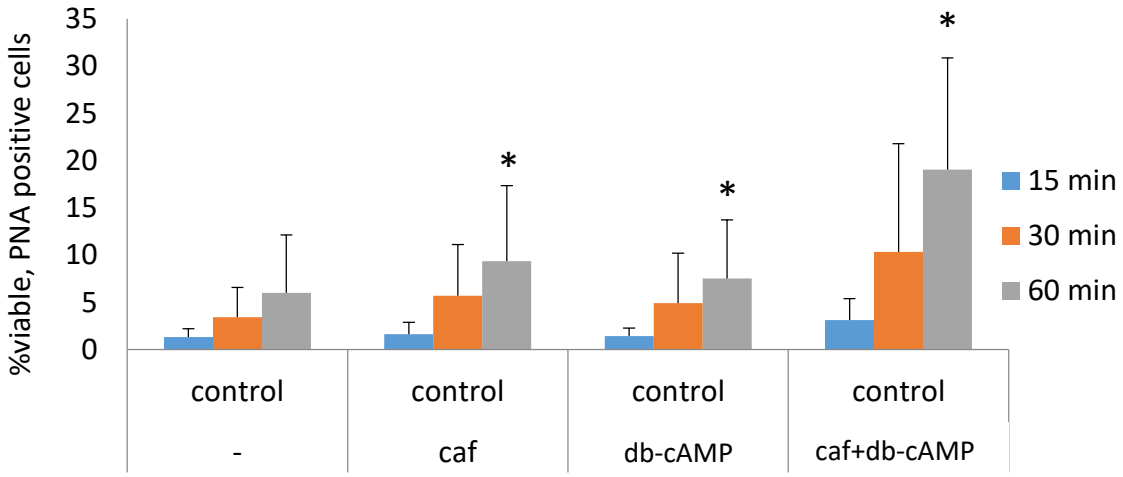

Figure 10; The percentage of viable PNA-FITC positive spermatozoa is increased by caffeine (caf) and dibutyryl-cAMP (db-cAMP) in control conditions (no bicarbonate) after 60 minutes incubation in comparison with a control condition without any additions (is different compared to control, no addition; p<0.05; n=6)*

An increase in viable PNA-FITC positive spermatozoa is also seen in bicarbonate containing media, after 15 and 30 minutes in media supplemented with both dibutyryl-cAMP and caffeine and after 30 minutes in media with dibutyryl-cAMP supplementation. Caffeine alone had no significant effect on the percentage of PNA-FITC positive cells (Figure 11).

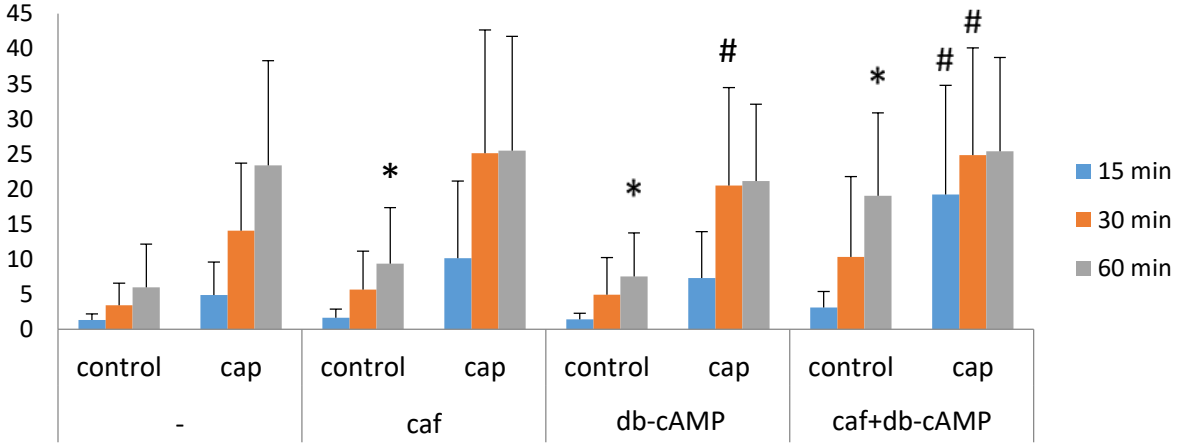

Figure 11; The percentage of viable PNA-FITC positive spermatozoa is increased by caffeine (caf) and dibutyryl-cAMP(db-cAMP) in bicarbonate containing conditions after 15 and 30 minutes incubation in comparison with a bicarbonate containing condition without any additions (# is different compared to cap, no addition; p<0.05; n=6)

Effect of dibutyryl-cAMP and caffeine on PE exposure

PE exposure in the outer leaflet of the membrane was virtually absent in live, acrosome intact spermatozoa (Table 2). The sAC/cAMP/PKA pathway stimulators caffeine and dibutyryl-cAMP could not promote the development of a viable subpopulation of spermatozoa with PE exposure.

Time (min)	Medium	sAC/cAMP/PKA stimulator	viable spermatozoa with PE exposure (%)
15	Control	-	1.23 ± 0.59
	Cap		0.84 ± 0.24
	Control	caf	0.74 ± 0.35
	Cap		0.60 ± 0.39
	Control	db-cAMP	0.99 ± 0.49
	Cap		0.71 ± 0.37
	Control	caf + db-cAMP	1.20 ± 0.30
	Cap		0.83 ± 0.02
30	Control	-	0.59 ± 0.11
	Cap		0.64 ± 0.47
	Control	Caf	0.42 ± 0.28
	Cap		0.68 ± 0.66
	Control	db-cAMP	0.57 ± 0.33
	Cap		1.08 ± 1.03
	Control	Caf+db-cAMP	1.03± 0.47
	Cap		1.05 ± 0.72
60	Control	-	0.39 ± 0.13
	Cap		0.75 ± 0.52
	Control	Caf	0.57 ± 0.30
	Cap		0.67 ± 0.38
	Control	db-cAMP	0.64 ± 0.28
	Cap		0.80 ± 0.39
	Control	Caf+ db-cAMP	1.61 ± 0.38
	Cap		0.97 ± 0.32

Table 2 the percentage of viable spermatozoa with PE exposure in the outer leaflet of the membrane per incubation condition per measuring time point(n=6 stallions)Caffeine (caf) and dibutyryl-cAMP (db-cAMP)

Microscopic detection of phosphatidylethanolamine staining

Viable, acrosome intact spermatozoa with PE exposure were virtually absent in both $Tyr_{control}$ and Tyr_{Bic}. However, all Hoechst33258 positive cells were duramycin-Cy5 positive (Figure 12). Staining was mainly localized in the region of the mid-piece of the spermatozoa, although the sperm tail and sperm head showed a weak duramycin-Cy5 signal as well.

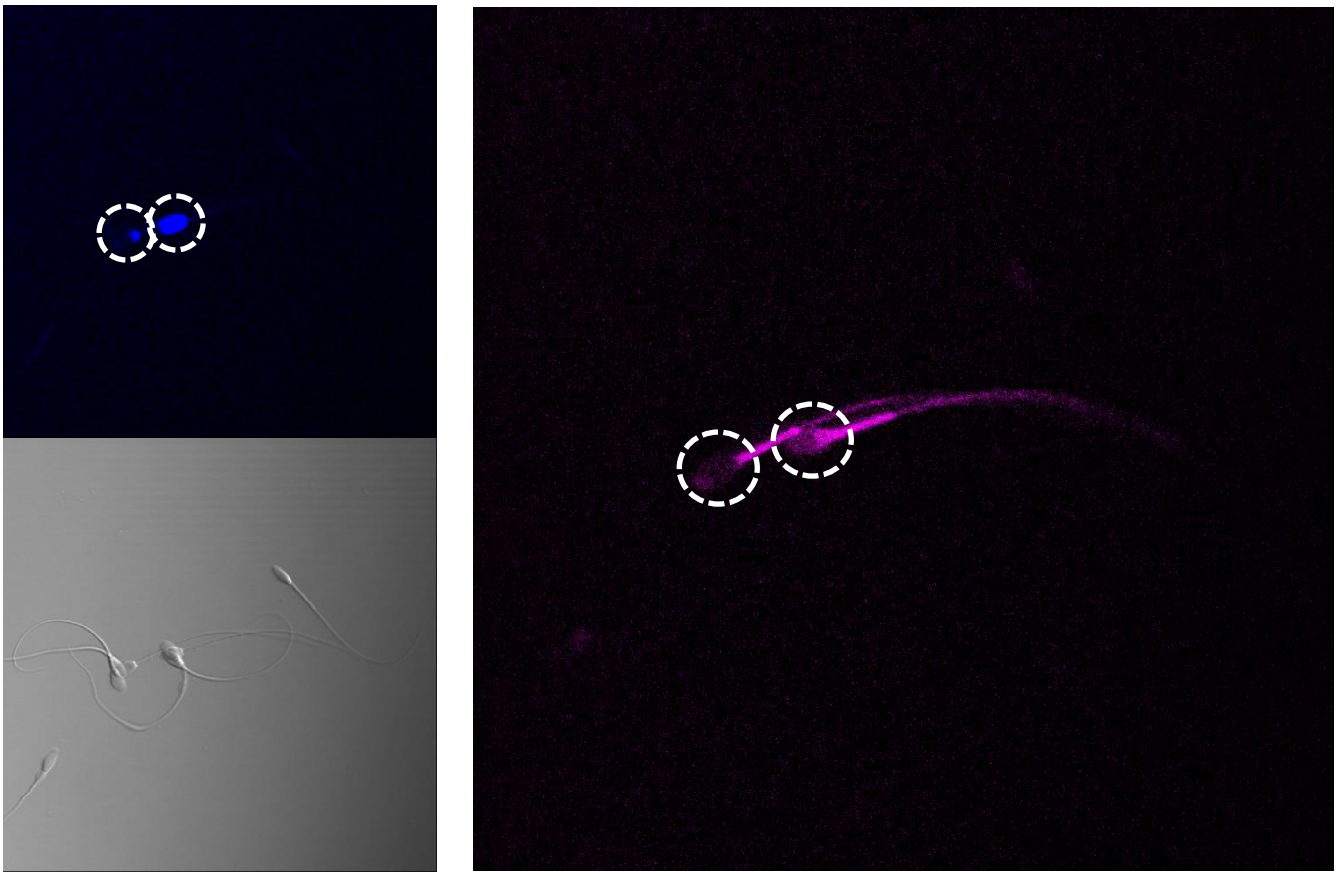

Figure 12; A group of spermatozoa is shown (630x), out of which two are Hoechst33258 positive (dead). Only these two dead spermatozoa are duramycin-cy5 positive. The positive signal predominantly originated from the midpiece of the spermatozoa.

PE exposure is not induced in porcine sperm in IVF medium

Since capacitation in boar sperm is well established and IVF has a working protocol, a capacitating and an IVF medium were used to investigate whether capacitation hallmarks like increased membrane fluidity and PE exposure in the outer leaflet of the plasma membrane can be induced in porcine sperm.

Increased membrane fluidity in viable spermatozoa was induced in the capacitating medium and the IVF medium, after 15 minutes incubation (Figure 13). In the control medium, less than 1% of the viable cells showed increased membrane fluidity. Over 95% (96.79 ± 0.62%) of the cells showed increased membrane fluidity after 15 minutes in the porcine IVF medium. In the capacitating medium, high membrane fluidity was also induced after 15 minutes in the majority of the population, although the response was present in a smaller subpopulation (61.1 ± 1.92%). The percentage of cells with high membrane fluidity cells in capacitating medium increased after 30 minutes (89.03 ± 1.17%) but decreased again after 60 minutes (48.64 ± 15.74%).

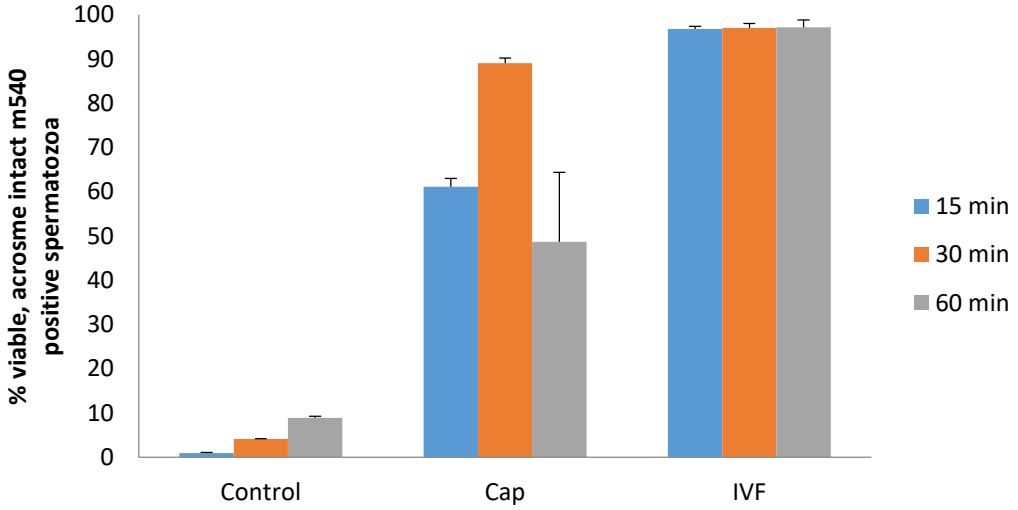

Figure 13; the percentage of viable, acrosome intact porcine spermatozoa with high membrane fluidity. The population of cells with high membrane fluidity increased in capacitating medium and IVF medium (n=2)

Although the percentage of cells with high membrane fluidity was extremely high especially in IVF medium, no PE exposure could be detected in viable spermatozoa. Viable spermatozoa with phosphatidylethanolamine exposure were virtually absent under all conditions at all measured time points (Table 3).

Time (min)	**Medium**	**viable spermatozoa with PE exposure (%)**
15	Control	0.52 ± 0.12
	IVF	1.03 ± 0.14
	Cap	0.68 ± 0.23
30	Control	0.08 ± 0.05
	IVF	0.18 ± 0.14
	Cap	0.16 ± 0.21
60	Control	0.11 ± 0.03
	IVF	0.12 ± 0.04
	Cap	0.13 ± 0.09

Table 3; percentage of viable spermatozoa with PE exposure. PE exposure was virtually absent in all tested conditions at all measured time points (n=2)

Capacitating medium does not support porcine IVF

In Experiment 9 we investigated whether capacitating medium facilitated fertilization. No fertilization (0/13) took place after 48 hours in capacitating medium. 12 out of 13 oocytes were degenerated, and no second polar body was formed. In the fertilization medium, using oocytes from the same batch, treated the same way, 84.6% of the oocytes was fertilized showing two polar bodies and 2 pronuclei . Sperm bound to the oocyte in both conditions, but in the capacitating medium spermatozoa were apparently unable to penetrate the zona pellucida and fertilize the oocyte (n=1).

Bicarbonate, BSA and Ca^{2+} do not induce hyperactivated motility in equine spermatozoa

Capacitation is supposed to prepare spermatozoa for hyperactivated motility. CASA was used in Experiment 8 to identify changes in motility patterns over time. Hyperactivated motility would be characterised by an increase in curvi-linear velocity (VCL), a decrease in straight line velocity (VSL) line) and linearity (LIN), and an increase of the amplitude of lateral head displacement (ALH). In this experiment, bicarbonate, BSA and calcium did not systematically change those parameters which indicates that no hyperactivated motility was induced in the majority of the motile spermatozoa (table 4 and 5). However, when inspecting the image sequences from the analyses, individual sperm with characteristic star-shaped motility tracks of hyperactivated sperm in aqueous solutions were visible (white circle in Figure 14 C, E, F, G; McPartlin et al. 2009). An enrichment of spermatozoa with these tracks in a specific medium variant was not evident.

Time (min)	Medium (0.5 mg/mL PVA & PVP)		Total motility (%)	Progressive motility (%)	VCL (µm/s)	VSL (µm/s)	LIN	ALH (µm)
	Bicarbonate (30 mM)	calcium (2 mM)						
15			49.6 ± 18.0	37.9 ± 19.3	161.1 ± 17.6	94.2 ± 5.9	0.60 ± 0.01	2.68 ± 0.08
	X		45.7 ± 22.5	35.2 ± 23.0	159.5 ± 19.0	93.9 ± 8.2	0.57 ± 0.02	2.70 ± 0.12
		X	54.4 ± 19.8	41.1 ± 20.6	165.2 ± 13.8	96.2 ± 3.7	0.58 ± 0.00	2.83 ± 0.22
	X	X	54.6 ± 17.8	43.0 ± 20.0	159.9 ± 19.2	97.4 ± 4.3	0.58 ± 0.08	2.97 ± 0.23
30			47.2 ± 19.4	36.3 ± 19.8	158.8 ± 14.9	94.0 ± 4.4	0.56 ± 0.06	2.90 ± 0.24
	X		53.0 ± 22.2	37.8 ± 22.7	161.5 ± 8.3	87.1 ± 2.1	0.53 ± 0.03	3.20 ± 0.33
		X	54.5 ± 16.4	39.4 ± 16.5	161.8 ± 15.9	99.5 ± 8.8	0.60 ± 0.09	2.86 ± 0.53
	X	X	52.3 ± 13.9	40.5 ± 14.5	162.9 ± 18.3	100.6 ± 8.0	0.59 ± 0.06	2.87 ± 0.07
60			47.4 ± 16.6	32.4 ± 17.1	162.3 ± 16.5	90.1 ± 0.9	0.52 ± 0.01	2.97 ± 0.08
	X		42.3 ± 15.9	21.4 ± 8.8	163.0 ± 17.4	81.7 ± 7.1	0.53 ± 0.06	3.12 ± 0.13
		X	50.9 ± 17.1	36.3 ± 21.2	162.7 ± 14.9	88.3 ± 7.8	0.55 ± 0.04	2.97 ± 0.19
	X	X	51.5 ± 17.7	38.7 ± 17.9	166.0 ± 9.9	99.1 ± 8.0	0.56 ± 0.06	2.95 ± 0.09

Table 4; CASA parameters after incubation of sperm in the presence of either bicarbonate, calcium or a combination of both (n=3).

Time (min)	Medium (1 mg/mL BSA)		Total motility (%)	Progressive motility (%)	VCL (µm/s)	VSL (µm/s)	LIN	ALH (µm)
	Bicarbonate (30 mM)	calcium (2 mM)						
15			49.4 ± 18.7	37.3 ± 20.6	161.8 ± 16.7	96.4 ± 4.9	0.60 ± 0.02	2.60 ± 0.04
	X		48.2 ± 23.1	37.1 ± 23.1	153.3 ± 10.4	98.9 ± 2.1	0.60 ± 0.04	2.50 ± 0.28
		X	46.9 ± 19.5	35.1 ± 16.6	169.4 ± 17.8	101.1 ± 2.0	0.63 ± 0.02	2.45 ± 0.21
	X	X	47.9 ± 21.3	39.0 ± 21.7	155.2 ± 17.8	99.3 ± 5.7	0.60 ± 0.06	2.45 ± 0.34
30			46.3 ± 25.3	32.6 ± 19.9	157.2 ± 9.2	91.5 ± 3.9	0.59 ± 0.03	2.59 ± 0.12
	X		48.7 ± 22.7	36.0 ± 22.2	158.5 ± 13.2	94.8 ± 5.1	0.55 ± 0.03	2.73 ± 0.16
		X	48.9 ± 17.6	33.6 ± 13.0	159.8 ± 6.2	95.4 ± 2.5	0.58 ± 0.01	2.63 ± 0.06
	X	X	45.7 ± 15.0	34.1 ± 17.7	154.5 ± 13.0	96.7 ± 10.1	0.57 ± 0.33	2.79 ± 1.64
60			47.1 ± 13.9	30.4 ± 11.9	164.0 ± 10.3	91.8 ± 3.2	0.56 ± 0.05	2.92 ± 0.17
	X		44.7 ± 16.5	37.3 ± 20.6	161.8 ± 16.7	96.4 ± 4.9	0.56 ± 0.02	2.98 ± 0.39
		X	49.5 ± 10.1	37.1 ± 23.1	153.3 ± 10.4	98.9 ± 2.1	0.55 ± 0.03	3.02 ± 0.19
	X	X	44.5 ± 8.9	35.1 ± 16.6	169.4 ± 17.8	101.1 ± 2.0	0.59 ± 0.04	2.76 ± 0.09

Table 5; CASA parameters after incubation of sperm in the presence of either bicarbonate, calcium or a combination of both (n=3).

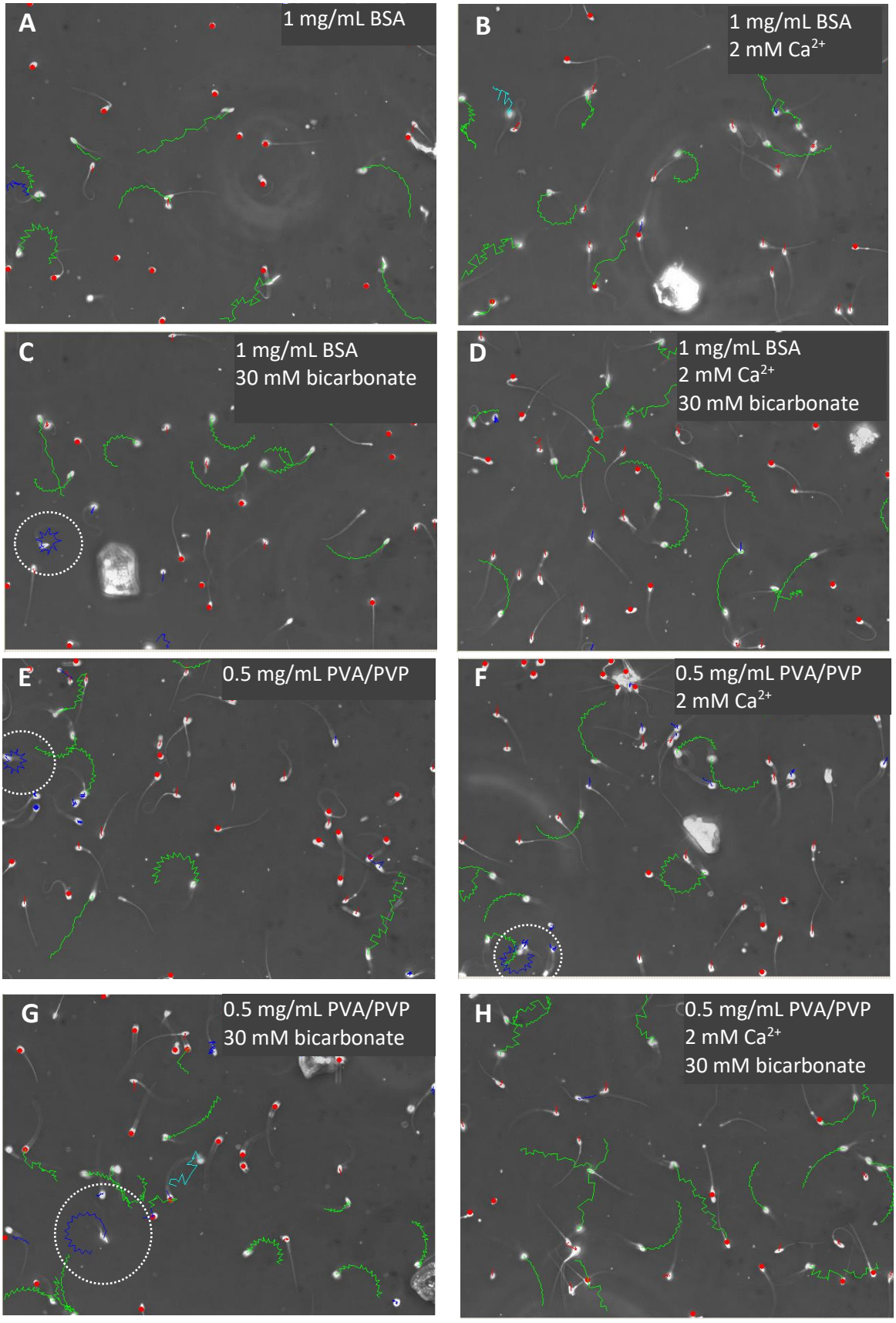

Figure 14; image sequences from the CASA analyses. Individual sperm with characteristic star-shaped motility tracks of hyperactivated sperm in aqueous solutions were visible (white circle in Figure 14 C, E, F, G). An enrichment of spermatozoa with these tracks in a specific medium variant was not evident.

Discussion

Equine sperm capacitation is still a poorly understood process. A condition that successfully supports this sperm activation for *in vitro* fertilization is still lacking. Therefore, the aim of the current study was to design a defined medium that is most likely to reliably induce early and late stage hall marks of the capacitation process.

Bicarbonate induces early changes in equine spermatozoa

The main stimulators for *in vitro* capacitation in other mammalian species are bicarbonate, calcium and albumin. Bicarbonate is considered to be the molecule that initiates capacitation (Gadella and Visconti 2006). Since there is no working IVF protocol as a control to prove successful capacitation, studies on equine *in vitro* capacitation mostly rely on the observations of changes in spermatozoa induced by bicarbonate.

Classical media for studying capacitation in pigs contain 15 mM of bicarbonate (Flesch et al. 2001; Gadella and Harrison 2002a; Harrison et al. 1996). In the equine species usually 25 mM bicarbonate is used (McPartlin et al. 2008; Macías-García et al. 2015). Consequently, in this study a concentration series of 15, 30 and 60 mM bicarbonate was tested. All bicarbonate concentrations were sufficient to induce an increase in membrane fluidity, an early indicator of capacitation, in the majority of the live, acrosome intact, equine spermatozoa within 15 minutes, as indicated by a clear detectable shift from a live, $M540_{low}$ to a live $M540_{high}$ state. This serves as an indication that bicarbonate indeed initiates capacitation in stallion spermatozoa. The early response is maintained in a large population of viable spermatozoa, thus corresponding to other publications reporting on membrane fluidity changes after one or two hours incubation (Ortgies et al. 2012; Rathi et al. 2001). In our study, the bicarbonate concentration for inducing a response seemed to be of low influence. Although the pH of the media differed due to the buffering capacities of bicarbonate, the percentage of viable, acrosome intact spermatozoa with high membrane fluidity did not differ between tested concentrations.

In boar spermatozoa, the percentage of viable spermatozoa with high membrane fluidity follows a logarithmic increase with increasing bicarbonate concentrations (Harrison and Miller 2000). The bicarbonate concentration to induce a response in 50% of the responsive cells, i.e. the half-maximal response, has been reported to be between 1.5 and 5.3 mM for individual animals. (Harrison et al. 1996). Our bicarbonate concentrations induced a response in more than 90% of the live spermatozoa in medium supplemented with PVA/PVP. This indicates that 15 mM bicarbonate is higher than the bicarbonate concentration required for half-maximal response levels in equine spermatozoa. In order to figure out a similar dose-response effect and evaluate the sensitivity of equine sperm to bicarbonate, a concentration series below 15 mM should be tested.

PE Exposure

Increased plasma membrane fluidity, as measured by increased merocyanine binding, is a very rapid and early capacitation-related response. This increase in membrane fluidity might be accompanied by lipid scrambling. Lipid scrambling or phospholipid translocation is of great importance in somatic cells for the maintenance of membrane asymmetry in normal cells and destruction of this asymmetry at events like cell activation, coagulation and apoptosis. Two different classes of membrane proteins are responsible for these translocations: ATP dependent and ATP independent phospholipid translocators. In somatic cells, the bilayer of the cell membrane knows an asymmetrical distribution of lipids, maintained by ATP dependent amino phospholipid translocases (APTs) and multidrug resistance (MDR) transporters. Rises in intracellular calcium concentrations deactivate these APTs, but scramblases, ATP independent translocases, are activated by such a calcium increase (Sahu et al. 2007). These scramblases destroy the asymmetry of the plasma membrane, leading to exposure of PE and PS in the outer leaflet of the membrane. It is suggested that these scramblases are responsible for the change in lipid re-organization in the plasma membrane during sperm capacitation. The presence of scramblase transporters in spermatozoa has been demonstrated in both human and stallion spermatozoa (de Vries et al. 2003; Thomas et al. 2006). However, proof for their activity is lacking in stallion sperm.

In this study the probe duramycin-Cy5 was used to monitor PE exposure on the cell surface of equine spermatozoa. Duramycin is a 19-amino acid tetracyclic peptide that belongs to the lantibiotic group, with high affinity for binding phosphatidylethanolamine (Hullin-Matsuda et al. 2016). It has been shown in porcine sperm that incubation in the presence of bicarbonate, BSA and calcium indeed induces PE exposure in the outer leaflet of the plasma membrane (Gadella and Harrison 2002a). This exposure took place within 5 minutes and the proportion of PE exposing cells very closely matched the population of cells with high membrane fluidity. In contrast, in our study we were not able to detect PE exposure in viable stallion spermatozoa in the outer leaflet of the membrane using duramycin in all tested conditions. However, the use of the probe duramycin in sperm has not been reported so far. (Gadella and Harrison 2002a) used a comparable molecule, cinnamycin, to detect PE exposure. Duramycin and cinnamycin only differ by a single amino acid in position 2: lysine for duramycin and arginine for cinnamycin (Hullin-Matsuda et al. 2016). Another difference was that Gadella and Harrson (2002a) used fluorescein-conjugated streptavidin/biotinylated cinnamycin conjugate while in our approach a dyramycyn-Cy5 conjugate was used. Thus it is possible that both probes have other binding characterics. At any rate from our flow cytometry data and cell imaging we can conclude, duramycin-Cy5 does not recognize PE in live sperm. Due to the fact that only deteriorated sperm did show duramycin-Cy5 staining we concluded that PE exposure did not take place in living sperm cells. Note that our duramycin-Cy5 staining patterns of dead spermatozoa

matched the cinnamycin staining patterns found in dead porcine spermatozoa (Gadella and Harrison 2002a) and deteriorated duramycin stained sperm were readily identified by flow cytometry. Additional experiments should be performed to check whether phosphatidylserine (PS) exposure can be detected in equine spermatozoa after incubation with Annexin V. Whether phosphatidylethanolamine and phosphatidylserine exposure are indeed hallmarks of capacitation is debateable, since controversial studies have been published on this subject (de Vries et al. 2003; B. M. Gadella and Harrison 2002a; Kurz et al. 2005). Our results suggest that PE exposure is either not a part of equine *in vitro* capacitation, or the lack of PE exposure indicates that under the tested conditions capacitation does not proceed beyond the very early stage.

Late capacitation responses and the influence of calcium and BSA

BSA and calcium are considered key molecules to support capacitation in mammalian sperm beyond the early stages. BSA is supposed to facilitate the process of increased membrane fluidity, as shown in porcine sperm (Harrison, Ashworth, and Miller 1996). However, BSA and calcium were not required to induce an increase in membrane fluidity in equine spermatozoa. Moreover, the viable sperm population with high plasma membrane fluidity in bicarbonate containing media supplemented with BSA decreased, compared to PVA/PVP containing conditions. BSA may have mediated this response in several ways. BSA promotes sperm agglutination. Therefore, a skewed population of cells might be analysed. Another option is that BSA may have interacted with the plasma membrane of the spermatozoa. The interaction might have had a stabilizing effect on the lipid structure of the sperm plasma membrane, resulting in a lower percentage of viable, acrosome intact spermatozoa with high membrane fluidity. This has been described in porcine sperm (Medrano et al. 2012). BSA could also have interfered with our M540 staining by binding a considerable amount of M540 (Banerjee et al 2012) which may have led to a reduced availability of merocyanine for the spermatozoa, resulting in a lower fluorescent intensity. However, our results show no differences in fluorescence intensity in both the M540 low and M540 high stained subpopulations of sperm in BSA containing media versus PVA/PVP containing media. Thus the BSA does not interfere in the M540 staining directly but apparently affects the sperm surface and thus its M540 staining responsiveness indirectly.

The decrease in percentage of viable, acrosome intact spermatozoa with high membrane fluidity was more pronounced when both calcium and BSA were supplemented. This was due to the fact that bicarbonate, BSA and Ca^{2+} promoted a spontaneous acrosome reaction in a subpopulation of spermatozoa in the majority of tested stallions. This has not been reported before. PNA-FITC was used to monitor the acrosome integrity. PNA-FITC exclusively binds to the outer acrosomal

membrane of stallion spermatozoa and can be used as a reliable probe for the evaluation of the status of the acrosome in stallion spermatozoa (Cheng et al. 1996). The PNA-FITC signal we detected, indeed originated from the acrosomal region of viable cells. This indicates, since the plasma membrane of the spermatozoa must be intact, that Ca^{2+} and BSA in combination promote spontaneous acrosome reaction. The acrosome reaction is a late capacitation response. During this exocytotic event the sperm plasma membrane fuses with the outer acrosomal membrane, resulting in the release of the acrosomal contents and exposure of the inner acrosomal membrane. This response seems at least to be cAMP dependent, since it only occurred in bicarbonate containing conditions, or in the presence of dibutyryl-cAMP and caffeine. The response is less abundant when compared to the percentage of cells showing increased membrane fluidity, but it can be systematically induced under controlled circumstances in the majority of the stallions.

A lot of variation between stallions has been observed with respect to a subpopulation of spontaneously acrosome reacting spermatozoa. 2 out of 6 stallions not only developed this subpopulation of spermatozoa in the presence of bicarbonate, BSA and calcium, but also in the presence of bicarbonate and BSA alone, although the response is only seen after 60 minutes. Due to the small amount of tested animals and the resulting high standard deviations, a significant effect of BSA alone on the occurrence of spontaneous acrosome reactions could not be confirmed. So, according to this study, both the presence of BSA and calcium are required in bicarbonate containing media to induce the development of a population showing spontaneous acrosome reaction. It should be tested whether sperm are indeed primed to acrosome react by these established conditions by adding progesterone. Progesterone has been shown to be a natural inducer of acrosome reaction in stallion spermatozoa (Meyers et al. 1995; Cheng et al. 1998; Rathi et al. 2003). By adding progesterone, it can also be tested whether an acrosome reaction can be provoked by progesterone in stallions that did not develop a subpopulation of sperm with spontaneous acrosome reaction under our conditions.

Hyperactivated motility is a Ca^{2+} driven late capacitation response. In mice, it has been shown that fertilization is not possible in the absence of this hyperactivated motility (Quill et al. 2003). Although both the acrosome reaction and hyperactivated motility require Ca^{2+}, the pathways regulating these events are considered to be independent due to the unique compartmentalization of the spermatozoa (McPartlin et al. 2009). Although the late response of (spontaneous) acrosome reaction was induced by our tested conditions, no hyper activated motility could be observed. Additional incubation time or factors such as progesterone may be required to induce hyperactivated motility as demonstrated in hamster spermatozoa (Kon et al. 2014).

sAC/cAMP/PKA pathway in equine sperm

Both an increase in membrane fluidity and PE exposure in the outer leaflet of the membrane are linked to the sAC/cAMP/PKA axis (Harrison and Miller 2000; Gadella and Harrison 2002a). Bicarbonate is thought to activate the sAC/cAMP/PKA axis by binding a soluble sAC (Okamura et al. 1985), resulting in increased levels of cAMP, which in turn activate a protein kinase A dependent phosphorylation cascade leading to alterations in the lipid architecture of the plasma membrane (Harrison and Miller 2000)

It has been reported that cAMP analogues and phosphodiesterase inhibitors like caffeine and pentoxyfilline induce capacitation-like responses in stallion sperm (Thomas et al. 2006; Ortgies et al. 2012). cAMP analogues raise the intracellular cAMP levels directly whereas caffeine inhibits the enzyme that degenerates cAMP and thus raises the cAMP levels indirectly. Theoretically, addition of both stimulators should raise the intracellular cAMP levels, and maintain these levels for a prolonged time. However, in our study no artificial raising of cAMP levels was required. The formation of a population of viable, acrosome intact spermatozoa with high membrane fluidity was induced by the presence of bicarbonate alone. It has been shown in porcine sperm that bicarbonate raises intracellular cAMP levels, within 1 minute after exposure. (Harrison and Miller 2000). In this study, the tested concentrations of bicarbonate seem to promote the sAC/cAMP/PKA axis in such a way that any additional rise in intracellular cAMP levels caused by caffeine or dibutyryl-cAMP does not increase the percentage of viable spermatozoa with high membrane fluidity in the presence of bicarbonate. Noteworthy is, that in control conditions, when the sAC/cAMP/PKA axis is not stimulated by bicarbonate, the increase in $LAIMF_{high}$ was low to absent, when either caffeine or dibutyryl-cAMP was added. Only the combined use of caffeine and dibutyryl-cAMP increased the population of viable, acrosome intact M540 positive cells, with a response that was equal to the effect of bicarbonate itself. However, where the response to bicarbonate is already present within 15 minutes, caffeine and dibutyryl-cAMP only manage to reach a similar level of responding cells after 60 minutes. It has been shown in porcine sperm that the addition of phosphodiesterase inhibitors induces M540-detectable membrane changes indistinguishable from those induced by bicarbonate within 10 minutes after exposure (Harrison et al. 996) Our tested concentration of caffeine and dibutyryl-cAMP might have been too low to mimic the effect induced by bicarbonate in equine spermatozoa at an early timepoint, although even lower concentrations have been used and proven their abilities to increase membrane fluidity in porcine sperm (Harrison et al. 1996; Harrison and Miller 2000). To confirm this hypothesis, a concentration series of dibutyryl-cAMP and caffeine should be performed. Other phosphodiesterase inhibitors with relatively higher inhibitory abilities should also be tested. (Harrison et al. 1996) showed that different phosphodiesterase inhibitors have

different potency. The ability to induce an increase in membrane fluidity was comparable with their reported inhibitory activities (Kemp and Huang 1971; Beavo 1995). A concentration series or use of different phosphodiesterase inhibitors should elucidate whether the increased membrane fluidity induced by bicarbonate, is due to rising intracellular cAMP levels. It could be that the detectable changes in -binding are actually not only affected by cAMP levels, but also by an effect of bicarbonate itself. Bicarbonate has been shown to have a direct stimulatory effect on a special kind of adenylyl cyclase founded in sperm (Okamura et al. 1985). However, bicarbonate could also have had its effect by raising intracellular pH, since bicarbonate is a alkaline molecule. To check whether bicarbonate indeed raises levels of intracellular cAMP in equine spermatozoa, assessments of cAMP would be needed. In porcine sperm this has been performed and it has been confirmed that bicarbonate indeed raises intracellular cAMP levels, within 1 minute after exposure. However, this rapid rise is followed by a dip. Levels of cAMP rise again after 15 minutes. (Harrison and Miller 2000). Keeping a stable bicarbonate concentration seems to be very important to keep cAMP levels high, after the first dip. Incubating in an atmosphere with sufficient CO_2 concentration should provide for sufficient bicarbonate levels in the media. When bicarbonate containing media are kept in air, like it has been proposed for equine sperm capacitation (Macías-García et al. 2015), CO_2 will be gassing out, resulting in a lower concentration of bicarbonate. The leaking of CO_2, will in turn cause a further dissociation of bicarbonate into CO_2 and water. This causes an even further decrease in bicarbonate concentration ($H^+ + HCO_3^- \leftrightarrow CO_2 + H_2O$).

In contrast to the changes in membrane fluidity, PE exposure is apparently not under the control of the sAC/cAMP/PKA pathway in equine spermatozoa. Addition of caffeine and dibutyryl-cAMP did not promote PE exposure in the outer leaflet of the membrane in equine spermatozoa. However, in boar sperm it has been shown that with 100 fold higher activator/inhibitor concentrations, after 2 hour incubation, there may be an effect of cAMP on PE exposure in the plasma membrane (Gadella and Harrison 2002). This emphasizes the need for further concentration series with cAMP analogues and a variety of phosphodiesterase inhibitors. Longer incubation times should also be tested, since two hours incubation time has been used in the previously cited study. However, the current data strongly suggest that PE exposure in equine sperm is not controlled by the sAC/cAMP/PKA axis.

A surprising observation was that addition of dibutyryl-cAMP and the combinational use of caffeine and dibutyryl-cAMP enhanced the occurrence of spontaneous acrosome reaction (after 15 and 30 min incubation) which suggests that the cAMP levels may have a vital role in this late capacitation-related event.

Porcine capacitation and IVF as control

Although some early and late capacitation-related changes could be detected in stallion spermatozoa, we were not able to identify all that have been proposed in literature. Since IVF is the ultimate control for *in vitro* sperm activation, a comparison of capacitation hallmarks with sperm from a species with well-established *in vitro* capacitation and *in vitro* fertilization protocols was made. The proportion of porcine spermatozoa acquiring high M540-binding ability was extremely high in fertilization media (>96%) within 15 minutes, indicating that an increase in membrane fluidity is indeed an early indicator of sperm activation before fertilization. When taken a closer look at the recipe for the IVF medium, it might be striking to find no bicarbonate in there. However, the medium has equilibrated in an incubator with 5% CO_2 atmosphere for 18 hours before use. Carbon dioxide will have dissolved in the aqueous solution and thus bicarbonate is present in the medium due ($H^+ + HCO_3^- \leftrightarrow CO_2 + H_2O$). The higher percentage of viable acrosome, intact spermatozoa with high membrane fluidity in comparison with the capacitating medium might be due to the higher pH of the medium. Also the presence of caffeine in fertilization medium could cause the found differences. However, the same concentration of caffeine had no additional effect in equine spermatozoa. Although the response in IVF medium in acquiring high M540-binding ability was extremely high, no PE exposure could be detected in viable sperm in both capacitating and fertilization media.

Strikingly, all studies into capacitation of porcine sperm so far have been performed in variants of Tyrode's medium, a medium which based on our preliminary observations seems to be not supportive of fertilization in this species. Careful testing is required to elucidate whether the lack of fertilization is due to unsuccessful activation of the sperm or due to an incompatibility of the oocyte with components in the Tyrode's medium. If our preliminary findings are confirmed, a revision of capacitation hallmarks might be indicated since PE exposure seems to be unnecessary to gain the ability to fertilize. Experiments monitoring sperm capacitation in established IVF media should lead to a revision of what are true capacitation hallmarks.

Conclusion

This study showed that bicarbonate induces early capacitation-related changes in the plasma membrane of equine spermatozoa, i.e. an increase in membrane fluidity in viable acrosome intact spermatozoa, within 15 minutes. However, PE exposure in the outer leaflet of the plasma membrane seems to be neither bicarbonate induced, nor under the control of the cAMP/sAC/PKA axis in equine spermatozoa, and is only seen in dead sperm. BSA and calcium modulated the bicarbonate effect and induced a subpopulation of live sperm with spontaneous acrosome reaction. This reaction seemed to be triggered by cAMP.

Parallel experiments with porcine sperm confirmed the absence of PE exposure under fertilizing conditions in vitro. Moreover, a revision of capacitation hallmarks might be indicated for species with successful IVF protocols. This might provide more reliable tools to monitor capacitation in equine spermatozoa and finally aid with the development of a medium that successfully supports equine IVF.

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Appendix

Experiment	Total motility	Progressive motility	N
1	61.04 ± 14.72	47.05 ± 17.39	6
2	68.71 ± 4.67	55.54 ± 6.65	6
3	70.60 ± 7.59	58.10 ± 6.99	6
4	79.47 ± 4.48	59.48 ± 2.04	2
7	76.95 ± 11.83	57.83 ± 13.90	5
8	60.73 ± 14.07	39.51 ± 9.5	3

Supplemental table 1; Total motility and progressive motility resulted from CASA measurements of samples processed for experimentation.

Time (min)	Bicarbonate (mM)	Macromolecule	viable acrosome intact spermatozoa (%)
15	0	PVA/PVP	70.09 ± 10.37
		BSA	67.91 ± 9.42
	15	PVA/PVP	67.02 ± 10.63
		BSA	64.75 ± 14.54
	30	PVA/PVP	69.59 ± 10.76
		BSA	62.49 ± 14.38
	60	PVA/PVP	69.60 ± 8.48
		BSA	65.90 ± 10.34
30	0	PVA/PVP	66.32 ± 9.23
		BSA	65.82 ± 9.14
	15	PVA/PVP	64.64 ± 11.23
		BSA	56.90 ± 19.64
	30	PVA/PVP	69.44 ± 10.75
		BSA	55.56 ± 19.77
	60	PVA/PVP	70.75 ± 8.32
		BSA	60.31 ± 12.58
60	0	PVA/PVP	66.26 ± 7.78
		BSA	60.51 ± 7.19
	15	PVA/PVP	63.08 ±- 11.02
		BSA	50.30 ± 15.28
	30	PVA/PVP	67.94 ± 10.76
		BSA	49.02 ± 15.80
	60	PVA/PVP	70.03 ± 7.36
		BSA	53.26 ± 10.07

Supplemental Table 2; Percentage of viable, acrosome intact spermatozoa per incubation condition and measuring time point (n=6 stallions).

Time (min)	Bicarbonate (mM)	Macromolecule	dead spermatozoa (%)
15	0	PVA/PVP	27.51 ± 10.91
		BSA	29.48 ± 9.55
	15	PVA/PVP	30.13 ± 10.42
		BSA	32.92 ± 12.84
	30	PVA/PVP	26.09 ± 11.65
		BSA	33.40 ± 10.97
	60	PVA/PVP	28.50 ± 9.17
		BSA	32.01 ± 10.56
30	0	PVA/PVP	31.06 ± 9.54
		BSA	31.59 ± 9.57
	15	PVA/PVP	32.23 ± 10.44
		BSA	38.27 ± 15.10
	30	PVA/PVP	27.75 ± 10.50
		BSA	38.36 ± 15.40
	60	PVA/PVP	27.39 ± 9.02
		BSA	34.87 ± 11.17
60	0	PVA/PVP	30.96 ± 8.54
		BSA	35.47 ± 8.72
	15	PVA/PVP	33.19 ± 10.83
		BSA	43.85 ± 12.19
	30	PVA/PVP	29.00 ± 10.59
		BSA	43.19 ± 13.05
	60	PVA/PVP	27.76 ± 8.56
		BSA	37.58 ± 9.55

Supplemental Table 3; Percentage of dead spermatozoa per incubation condition and measuring time point (n=6 stallions).

Time (min)	Medium	Macromolecule	calcium (mM)	dead spermatozoa (%)
15	Control	PVA/PVP	-	17.6 ± 8.4
	Cap			15.7 ± 6.8
	Control		2	18.7 ± 8.2
	Cap			17.3 ± 7.7
	Control	BSA	-	18.6 ± 8.0
	Cap			18.5 ± 7.7
	Control		2	19.5 ± 9.0
	Cap			19.9 ± 7.4
30	Control	PVA/PVP	-	19.2 ± 7.8
	Cap			17.7 ± 7.4
	Control		2	20.0 ± 8.3
	Cap			20.8 ± 6.3
	Control	BSA	-	21.0 ± 7.5
	Cap			20.6 ± 6.8
	Control		2	21.9 ± 6.6
	Cap			24.5 ± 9.0
60	Control	PVA/PVP	-	19.8 ± 7.2
	Cap			20.2 ± 7.8
	Control		2	20.2 ± 11.0
	Cap			21.8 ± 7.3
	Control	BSA	-	23.9 ± 9.0
	Cap			27.0 ± 10.7
	Control		2	27.5 ± 8.1
	Cap			32.0 ± 6.1

Supplemental Table 4; Percentage of dead spermatozoa per incubation condition and measuring time point (n=6 stallions).

Time (min)	Medium	Macromolecule	calcium (mM)	viable spermatozoa with PE exposure (%)
15	Control	PVA/PVP	-	1.18 ± 0.67
	Cap			0.82 ± 0.34
	Control		2	0.77 ± 0.67
	Cap			1.43 ± 0.48
	Control	BSA	-	0.96 ± 0.43
	Cap			0.84 ± 0.42
	Control		2	1.06 ± 0.38
	Cap			0.92 ± 0.81
30	Control	PVA/PVP	-	0.76 ± 0.11
	Cap			0.64 ± 0.44
	Control		2	0.48 ± 0.26
	Cap			0.94 ± 0.67
	Control	BSA	-	0.73 ± 0.34
	Cap			1.15 ± 1.05
	Control		2	0.98 ± 0.48
	Cap			1.12 ± 0.69
60	Control	PVA/PVP	-	0.76 ± 0.17
	Cap			0.56 ± 0.34
	Control		2	0.57 ± 0.39
	Cap			0.32 ± 0.38
	Control	BSA	-	0.87 ± 0.27
	Cap			0.84 ± 0.47
	Control		2	1.32 ± 0.52
	Cap			1.05 ± 0.74

Supplemental Table 5; Percentage of viable, acrosome intact spermatozoa with PE exposure in the outer leaflet of the membrane per incubation condition per measuring time point (n=6 stallions)

Courses

Writing for academic publication

Provided by BABEL

17/04/2018 – 29/05/2018

Course description

This was a course, organized especially for the honours program group to improve English academic writing.

Course contents:

- Improve writing and reading skills
- Learn to analyze structure of a academic publication
- Discuss corresponding language structures
- Discuss interpunction
- Improve formulation
- Expend vocabulary

Personal experience

The course was adapted to fit the needs of the HP students and in my opinion it succeed in this. I thought the peer-reviewing of our writing was really helpful. These sessions provided a better insight of my writing style, and helped me improve it. The English grammar and vocabulary sessions were really enjoyable and reminded me of my time in high school. Besides, it was also really helpful. Some communly used combinations of words have been discussed, and I was able to use this in my writing. I've learnt a lot during this course and I would definitely recommend it to others. It's really enjoyable, really helpful during writing, and really changes your view when reading published articles. This course should have been give somewhat later during the HP year, because now we were not able to take full advantage of the course during the results and discussion sessions. Most of us were not yet finished in the lab, so we did not have access to all our data. This made it quite hard to write discussion and results. So peer reviewing on these parts was not as beneficial as it was with the introduction an d material and methods.

Modern Methods in Data Analysis

Provided by MSc Epidemiology, University of Utrecht, Utrecht, the Netherlands

08/01/2018 – 26/01/2018

Course description

This course provides statistical methods to study the association between (multiple) determinants and the occurrence of an outcome event. The course starts with an introduction to likelihood theory, using simple examples and a minimum of mathematics. Next, the most important regression models used in medical research are introduced. Topics are: maximum-likelihood methods, logistic regression, model validation and regression diagnostics, Poisson regression, and analysis of `event-history´ data, including an extensive discussion of the Cox proportional hazards regression model. Also, the basic principles of longitudinal data analysis are taught.

Personal experience

This was a very well build course. I found this course very challenging, but also really useful. The only experience I had so far was part of the regular program of the bachelor in Veterinary Medicine. I learnt a lot about different statistic models. However, after the course I still found it really difficult to decide when to use which statistical method. My supervisor still had to help and advise me on statistics to process my own data. Although, I'm still glad that I followed this course. It was a nice experience, and I know for sure that this course will be very helpful in my future.

Attendance scientific meetings

- **Veterinary Science Day, Bunnik, The Netherlands, 16 November, 2017**
- **BRC meetings, weekly meeting with the fertility and reproduction department**
- **ISER Xii, Cambridge, United Kingdom, 22-28 July, 2018**
  Abstract accepted for Oral presentation
  Hoogendijk, R.[1,*], Gadella, B.M.[2,3], Stout, T.A.E.[1], Henning, H.H.W.[1] Inducing lipid reorganization and the acrosome reaction in stallion spermatozoa
  Departments of [1] Equine Sciences, [2] Biochemistry and Cell Biology, [3]Farm Animal Health; Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Acknowledgements

First, I would like to thank my daily supervisor Heiko Henning. I really enjoyed working with you. I really appreciated your way of working. I learnt a lot. I would also really like to thank you for all your time. You always made time whenever I needed help with experiments. You were really helpful during the writing process, and I could always count on you. Thank you for giving me the opportunity to work together with you and learn from you this year.
I would also like to thank Bart Gadella, my supervisor. Thank you for your critical insights on my results during the "HP meetings" and for your help during the writing process.

A special thanks to Arend Rijneveld. Thank you for the introductions in the sperm lab. It was really nice to have someone in the laboratory that was interested in my experiments, was willing to help me whenever I needed something, or just to have a nice chat about the weekend.

I would like to thank the team members of the clinic. They were always willing to help me with sperm collection from stallions in the clinic, despite their own busy schedules. Thank you!
Thanks to the Center for Cell Imaging (CCI) for allowing me to use the fluorescence microscope and confocal microscope. Also thanks for introducing me in confocal and fluorescence microscopy.

I'd like to give a special thanks to the MOC Onderzoek. Thank you for the opportunity to join this program. I'm very grateful for this experience. I've learned a lot this year, and definitely got a better insight in research and what comes with it.
Last but not least, thanks to my friends and family, who supported me during this year. A special thanks to my boyfriend, who drove me all the way to Belgium, so I was able to attend the, for me very special, meeting in Cambridge.