

# **Novel insights in capacitation of stallion spermatozoa: What is the role of the oviduct?**

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Breaking through barriers  
is a natural impulse



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# LIST OF ABBREVIATIONS

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AI	artificial insemination
AKAPs	A-kinase anchoring proteins
ALH	amplitude of lateral head displacement
ANOVA	analysis of variance
ANP A	atrial natriuretic peptide A
APM	apical plasma membrane
ART	assisted reproductive techniques
ATP	adenosine triphosphate
AU	arbitrary units
AV	artificial vagina
BCECF-AM	BCECF-acetoxymethyl
BP	band pass
BSA	bovine serum albumin
cAMP	cyclic 3'-5'-cyclic adenosine monophosphate
CASA	computer-assisted sperm analyzer
CATSPER	cation channels of sperm
CaVs	voltage-gated Ca <sup>2+</sup>
CD	completely cumulus-denuded oocytes
CNG	cyclic nucleic gated
COCs	cumulus-oocyte complexes
Con A	Canavalia ensiformis agglutinin
CTC	chlortetracycline
DABCO	1,4-Diazabicyclo[2.2.2]octane
DAG	diacylglycerol
DBA	Dolichos biflorus agglutinin
DIC	differential interference contrast
DMEM/F12	Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12
DMBT1	Deleted in malignant brain tumor 1
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
ET	embryo transfer
F	very condensed fragments of DNA
F-actin	filamentous actin
FBS	fetal bovine serum
FF	follicular fluid
FITC	fluorescein isothiocyanate-conjugated
Fluo-4 AM	Fluo-4 acetoxymethyl
Fura-2 AM	Fura-2 acetoxymethyl

HBS	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiP	hypotonic buffer solution
HPA	Helix pomatia agglutinin
ICSI	intracytoplasmic sperm injection
IP <sub>3</sub>	inositol 1,4,5-triphosphate
IU	International Units
IVF	<i>in vitro</i> fertilization
IVP	<i>in vitro</i> production of equine embryos
kDa	kilodalton
LCA	Lens culinaris agglutinin
LIN	linearity
LP	long pass
mBCD	methyl- $\beta$ -cyclodextrin
MII	metaphase of the second meiotic division
MTG	MitoTracker Green FM
OEC	oviduct epithelial cell
OPU	ovum pick-up
OSG	oviduct secretory glycoprotein or oviductin
PAI-1	plasminogen activator inhibitor-1
PB	polar body
PC	phosphatidylcholine
PD	partially cumulus-denuded oocytes
PE	phosphatidylethanolamine
PI	propidium iodide
PIP <sub>2</sub>	phosphatidylinositol-biphosphate
PKA	protein kinase A
PLC $\zeta$	phospholipase C zeta
PM	progressively motile sperm
PN	pronucleus
PNA	Arachis hypogaea [peanut] agglutinin
PS	phosphatidylserine
PSA	Pisum sativum [pea] agglutinin
PY	tail-associated protein tyrosine phosphorylation
RNE	redundant nuclear envelopes
ROS	reactive oxygen species
sAC	soluble adenylyl cyclase
SD	standard deviation
SH	sulphydryl
SM	sphingomyelin
SNA	Sambucus nigra agglutinin
SNARE	soluble NSF attachment protein receptor
STR	straightness



*List of Abbreviations*

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TALP	Tyrode's albumin pyruvate lactate
TIMP-1	tissue inhibitor metalloprotease 1
TM	total motile sperm
TRP	transient receptor potential
TUB	tubulin
UEA I	Ulex europaeus agglutinin I
UTJ	utero-tubal junction
VCL	curvilinear velocity
WGA	Triticum vulgare [wheat germ] agglutinin
ZP	zona pellucida



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# CHAPTER 1

## GENERAL INTRODUCTION

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**Adapted from:**

**Novel insights in capacitation of stallion spermatozoa: What is the role of the oviduct? A review.**

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*In preparation.*



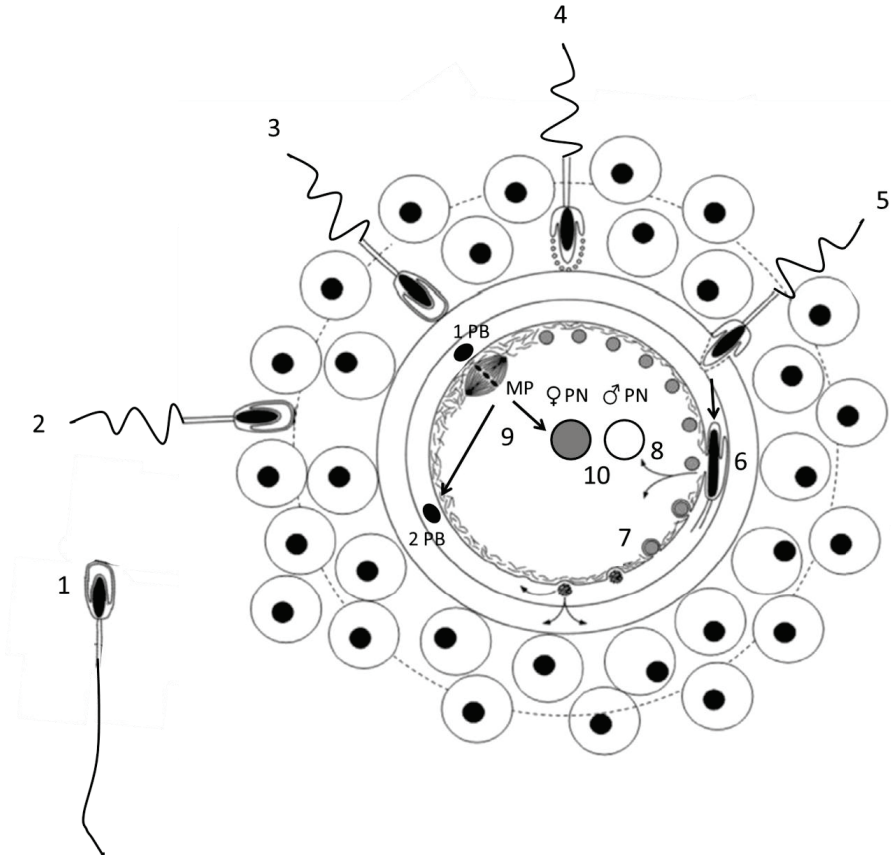
## 1. Assisted reproductive technologies in the horse

Up to the end of the nineteenth century, the vast majority of horses were bred by natural cover. The introduction of assisted reproductive techniques (ART) in equine reproduction started with the first reported pregnancy using artificial insemination (AI) [1]. Nowadays, the majority of mares are inseminated using fresh, cooled or frozen-thawed semen, enabling genetically valuable stallions to produce more offspring. The selection of genetically valuable mares can be accelerated by flushing the embryo from the uterus of a donor mare and subsequently transfer the embryo to a recipient mare which carries the foal to term. As such, multiple foals from the same donor mare can be produced in one season. The first successful embryo transfer (ET) in horses was performed in 1974 [2]. This technique is now a routine procedure in practice [3], e.g. >31000 *in vivo* derived equine embryos were worldwide transferred in 2012 [4]. With both techniques, early embryonic development occurs in the female reproductive tract, i.e. *in vivo*.

More recently, ART in equine reproduction were developed to breed foals from sub- or even infertile horses as well as from recently deceased mares or stallions. During an ovum pick-up (OPU) session, oocytes are retrieved from living donor mares by flushing oocytes from follicles using a transvaginal or transabdominal approach [5-7]. Oocytes can also be collected post mortem by scraping the follicles of ovaries obtained from slaughtered mares [6-8]. After oocyte maturation, the oocytes can be fertilized *in vitro* using intracytoplasmic sperm injection (ICSI) [9, 10] or *in vivo* by transferring the matured oocytes to the oviduct of an inseminated recipient mare (oocyte transfer) [11-13]. The first foal produced by the latter method was born in 1980 [11]. Later on, Carnevale and Ginther [12] reported success rates of 92% (11 / 12) when oocytes were retrieved from young donor mares. Since 1990, oocyte transfer is available for clinical applications to treat infertile mares [14, 15]. The first ICSI foal was born in 1996 by transferring newly injected oocytes to the oviduct of a synchronized recipient mare as an efficient *in vitro* culture system was lacking [16]. Shortly after, the first ICSI foals were born from *in vitro* cultured blastocysts which were transferred to the uterus of a synchronized recipient mare [5, 17, 18]. The first ICSI foal in the Benelux, Smicsi, was born in 2009 [8]. Equine ICSI is now considered as a valuable tool to produce healthy foals from reproductive sub- and infertile horses.

## 2. State of art: equine conventional *in vitro* fertilization

The birth of Louise Brown in 1978 announced the first successful conventional *in vitro* fertilization (IVF) [19] and at present, this technique is a well-established ART in human. In domestic animals, conventional IVF gained popularity as well. In 2012, 9930 calves were produced *in vitro*, i.e. 6.8% of the cattle population [4]. Also in pigs and various laboratory animals, dozens of offspring are born by conventional IVF each year [20, 21]. In 1991, the birth of two foals produced after IVF of *in vivo* matured oocytes was reported [22, 23]. Unfortunately, this procedure could not be repeated in other laboratories [24]. Conventional IVF implies fertilization after incubation of mature oocytes with capacitated sperm (Figure 1). Despite the birth of two IVF foals born after *in vitro* fertilization of *in vivo* matured oocytes with  $\text{Ca}^{2+}$  ionophore treated sperm [22], no repeatable protocol for IVF in equine with clinical application is established yet [24-29]. For example, the use of  $\text{Ca}^{2+}$  ionophore A23187 and heparin did not yield significantly higher fertilization rates [27, 30]. ZP (zona pellucida) proteins, caffeine and lysophospholipids supported sperm capacitation but fertilization rates remained very low as well [31]. An overview of the different equine IVF studies is given in Table 1 indicating that *in vitro* fertilization rates are very low and vary between 0-31%. In 2009, McPartlin *et al.* [32] showed that procaine treatment of stallion spermatozoa induced high fertilization rates, varying from 0 to 60%, by prompting hyperactivated motility [24, 26, 32]. However, the method of evaluation of fertilization can be questioned (only two pronuclei were observed). This will be further investigated in Chapter 6. The procedure is also difficult to repeat, so, five years later, this technique has not been confirmed yet. ZP drilling and partial removal of the ZP is also a way to improve equine fertilization rates [25, 30, 33], but polyspermy complicated the success of these protocols.



**Figure 1.** Schematic presentation of the subsequent events occurring during mammalian fertilization. (1) Inside the female genital tract, spermatozoa are activated during a process called capacitation. (2) Capacitated sperm becomes hypermotile, passes through the cumulus cell layer and (3) binds to the ZP. (4) Subsequently the acrosome reaction is triggered. The released hydrolytic enzymes lyse the extracellular matrix of the cumulus and / or the ZP (5) as such enabling the hyperactive spermatozoon to enter the perivitelline space and to bind to the oolemma, (6) fuse with and become incorporated into the oocyte. (7) As soon the oocyte is fertilized, the cortical reaction occurs, i.e. the content of the secretory granules just underlying the oolemma is extruded and alters the structure of the ZP and the oolemma, as such preventing polyspermy. Subsequently, the fertilizing spermatozoon activates the oocyte. (8) The sperm head will swell and (9) the oocyte, arrested at metaphase of the second meiotic division (MII) with its chromosomes arranged along the metaphase plate (MP), progresses through meiosis and extrudes the second polar body (2 PB). (10) In the end, the female and male pronuclei (PN) are formed as the final prelude to syngamy (Image adapted from Gadella and Luna [34]).

**Table 1:** Overview of equine conventional IVF results

Oocyte maturation	N oocytes	Sperm treatment	N fertilized oocytes (2 PN)	N cleaved oocytes	Reference
<i>In vivo</i>	113	Ca <sup>2+</sup> Ionophore A23187	16 (14%) <sup>a,b</sup>		[22]
<i>In vivo</i>	173	Ca <sup>2+</sup> Ionophore A23188	30 (17%) <sup>a</sup>	22 (13%) <sup>b</sup>	[23]
<i>In vitro</i>	57	Caffeine / Ca <sup>2+</sup> Ionophore A23187	2 (4%)		[25]
<i>In vitro</i>	232	Heparin	41 (18%)	0	[33]
<i>In vitro</i>	206	Heparin	18 (9%)	0	[26]
<i>In vitro</i>	203	Heparin	14 (7%)	5 (2%)	[24]
<i>In vitro</i>	349	Heparin / Ca <sup>2+</sup> Ionophore A23187	45 (13%)		[27]
<i>In vitro</i>	815	Ca <sup>2+</sup> Ionophore A23187	38 (5%)		[28]
<i>In vitro</i>	89	PVA / BSA / Br- AMP/Ionomycin	28 (31%)		[35]
<i>In vitro</i>	370	Progesterone	0 (0%)		[36]
<i>In vitro</i>	385	Heparin/Ca <sup>2+</sup> Ionophore A23187 / BSA	26 (7%)		[37]
<i>In vitro</i>	994	Ca <sup>2+</sup> Ionophore A23187	53 (5%)		[29]
<i>In vitro</i>	21	Caffeine	0 (0%)		[33]
<i>In vitro</i>	74	Procaine	47 (64%) <sup>c</sup>		[27]
<i>In vitro</i>	154	10% follicular fluid Procaine	(19%) <sup>c</sup> 69 (54%)		[34] [40]

N= number

<sup>a</sup> oocyte transfer<sup>b</sup> birth of 1 IVF foal [22] and 2 IVF foals [23] respectively from *in vivo* matured oocytes<sup>c</sup> sum of fertilized and cleaved oocytes

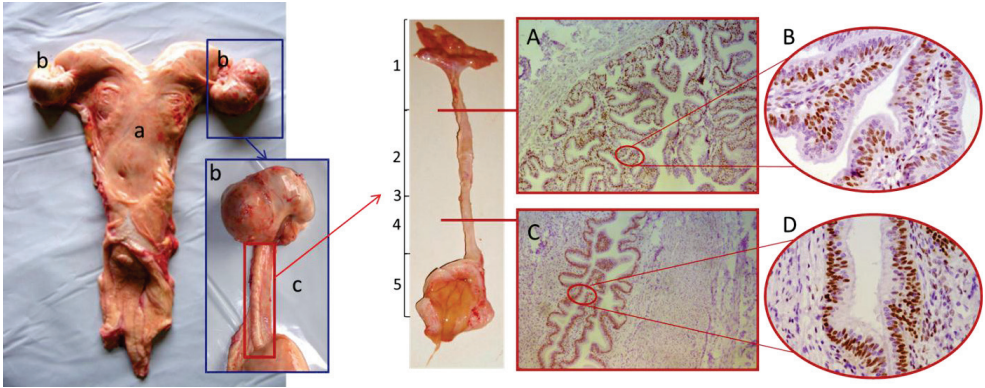
Theoretically, the deficiencies in equine IVF can be attributed to either an inefficient sperm capacitation or an inadequate oocyte maturation. However, the inability of the spermatozoa to penetrate the ZP is most likely due to a deficient activation of spermatozoa (capacitation). Indeed, Tremoleda *et al.* [36] showed that equine IVF conditions supported the binding between stallion sperm and the ZP but did not induce the acrosome reaction. Indeed, IVF using *in vivo* matured oocytes is not successful [22] while *in vitro* matured oocytes transferred to the oviduct of an inseminated mare showed similar pregnancy rates compared to



AI outcomes [28]. The latter indicates that *in vitro* matured oocytes are capable of being fertilized normally. Furthermore, irreversible ZP hardening, seen with premature release of cortical granules, or other oviduct-mediated changes affecting the ZP permeability of matured oocytes probably do not play a role in the failure of equine IVF [28, 41]. Generally, it is assumed that high rates of fertilization *in vivo* are caused by a capacitating supportive oviduct environment. Therefore, we need to gain more knowledge on how spermatozoa become activated in the oviduct environment and achieve the ability to fertilize the mature oocyte. In this thesis, we have focussed on the events which the sperm cells in the oviduct must undergo in order to be able to fertilize the oocyte. These events include (1) sperm-oviduct binding (Chapter 3), (2) physiological changes of the sperm related to capacitation (Chapter 4 and 5) and (3) fertilization (Chapter 6).

### 3. The oviduct: morphology and ultrastructural features

Each oviduct consists of an expansive infundibulum covering the ovary's ovulation fossa, a highly tortuous ampulla which is about 6 mm in diameter and a less tortuous isthmus which is half the diameter of the ampulla (Figure 2). The isthmus terminates in a small uterine ostium at a papilla in the endometrium in the tip of each uterine horn. The circular muscle sphincter at the utero-tubal junction (UTJ) serves as a valve, preventing reflux of uterine contents to the oviduct. The uterine ostium of the tube is 2–3 mm in diameter while the abdominal ostium of the infundibulum is about 6 mm in diameter. When effacing the loops and removing the suspending mesosalpinx, the actual length of the equine oviducts can be determined, i.e. 20 to 30 cm long. Half of the oviduct consists of the ampulla. Irregular fimbriae are present along the margin of the funnel-shaped infundibulum. As some fimbriae are attached to the cranial pole of the ovary, the infundibulum also covers the ventral located ovulation fossa. The mucous membrane of the fimbriae is highly folded, especially in the ampulla where secondary and tertiary ridges branch from the longitudinal folds. The lining of the simple columnar epithelium is intermittently ciliated (pseudo stratified cilia epithelium). Ciliogenesis and ciliary motion toward the uterus are depending on the sexual cycle stage. A thin, well vascularized lamina propria supports the epithelium. Inner, circularly disposed smooth muscle fibers are covered by outer, longitudinally arranged fibers that continue into the mesosalpinx [42].



**Figure 2:** Morphology and ultrastructural features of the mare’s oviduct: (a) uterus, (b) ovaries and (c) oviduct. The oviduct consist of (1) infundibulum, (2) ampulla, (2) ampullary-isthmic junction, (4) isthmus and (5) utero-tubal junction (UTJ). (A) and (B) represent ultrastructural images of the ampulla and (C) and (D) of the isthmus (Images kindly provided by Dr. Hilde Nelis; RBU, Ugent).

## 4. Journey of the sperm through the mare’s genital tract

Freshly ejaculated spermatozoa have to undergo a maturation process in the female reproductive tract called “capacitation” in order to prepare the spermatozoa to fertilize the oocyte. In the end, only a small fraction of the total inseminated sperm population reaches the site of fertilization and undergoes capacitation. Based on *in vitro* studies, it is hypothesized that only these sperm cells with superior viability, morphology and motility reach the oviduct [43, 44]. Moreover, the capacitated state of this sperm population is very short-lived, e.g. 1-4 h *in vitro* in men [45], due to the reactive oxygen species (ROS) generated by mammalian spermatozoa. Although the sperm cells are very sensitive to oxidative stress, low levels of ROS are essential to promote capacitation by redox regulation (see 1.5). Inseminated sperm cells not participating in fertilization are only shortly protected against oxidative stress as they have only a restricted amount of cytoplasm containing the limited stock of anti-oxidants. The over-capacitation of spermatozoa eventually results in a state of senescence and the activation of the intrinsic apoptotic cascade. As a consequence, once spermatozoa started capacitation they become very instable which reduces their life span severely [46, 47].

Sperm capacitation is a strictly ordered process of sequential events in all mammals, including the horse [48]: (1) spermatozoa are ejaculated into the uterine body and transported to the UTJ; (2) a reservoir of non-capacitated spermatozoa is established at the UTJ and the caudal isthmus; (3) spermatozoa within the reservoir become capacitated near ovulation; (4) the capacitated spermatozoa acquire hyperactivated motility and are released from the sperm reservoir, (5) the released spermatozoa meet the mature oocyte at the ampullary-isthmic junction and bind to the ZP or to the intercellular matrix of the cumulus-oocyte complex [49, 50], (6) the acrosome reaction is triggered allowing the sperm to penetrate the cumulus and ZP and enter the perivitelline space, after which (7) the fertilizing spermatozoon can bind and fuse with the oolemma. All these steps are initiated after the spermatozoa have made contact with the epithelial cells and the pre-ovulatory stage-mediated secretions of the oviduct.

### **1. Migration through the uterine lumen**

After ejaculation or insemination, stallion spermatozoa are transported from the mare's uterine body towards the oviduct mainly by uterine contractions (passive sperm transport) and less by active sperm motility [51]. The first spermatozoa are observed in the oviduct within 2 h post-insemination while most spermatozoa reach the oviduct 4 h post-insemination [52, 53]. Only a small number of spermatozoa are able to enter the oviduct [54]. There are several mechanisms to clear the uterus and eliminate the redundant spermatozoa. Firstly, myometrial contractions will mechanically remove the sperm through the cervix [55, 56]. Secondly, 0.5 h post-insemination, an influx of polymorphonuclear neutrophils (PMNs) into the uterine lumen has been observed playing an important role in sperm phagocytosis [57-59]. This inflammation response is harmful for all the spermatozoa passing the uterine lumen. However, it has recently been demonstrated that only dead spermatozoa are very susceptible to this elimination cascade whereas viable spermatozoa are protected from binding to PMNs and phagocytosis. Seminal plasma factors play an important role in this mechanism. For example, lactoferrin enhances the cell-to-cell interaction between PMNs and spermatozoa (living and dead) in the uterus [60] while CRISP-3 causes a strong reduction in the binding between living spermatozoa and PMNs [61]. As such, the transport of living spermatozoa to the oviduct is allowed while PMNs phagocytize the dead sperm population in the uterus.

During sperm migration towards the oviduct, the onset of capacitation in living sperm needs to be inhibited. To prevent a premature capacitation response, spermatozoa contact decapacitation factors after ejaculation. Moreover, mammalian seminal plasma contains several extracellular vesicles such as prostasomes. Prostasomes are secreted by prostate epithelium and probably act as a decapacitation factor as they have a lipid content consisting of mainly saturated fatty acids and high concentrations of cholesterol and sphingomyelin [62-64]. The high cholesterol content might inhibit the plasma membrane changes and acrosome reaction by stabilizing the sperm plasma membrane [65-67], as such preventing premature sperm capacitation. On the other hand, some studies have suggested that prostasomes actually promote capacitation-related events like the acrosome reaction [68, 69] and the  $\text{Ca}^{2+}$ -induced onset of hyperactivated motility, a requirement for ZP penetration [70].

## **2. Passing through the utero-tubal junction (UTJ)**

In the horse, the oviduct reservoir selects morphologically normal spermatozoa with superior progressive motility [54, 71, 72]. After migrating through the uterus, stallion spermatozoa need to pass the UTJ, a closed muscular structure which opens during the pre-ovulatory period in order to allow spermatozoa to enter the oviduct. The key factors evoking the relaxation of the UTJ have not been elucidated yet but oestrogens might be a candidate factor. It is hypothesized that during oestrus the UTJ might open under the influence of oestrogens while it remains closed during the progesterone phase. The transport of the developing embryo from the ampullary-isthmic junction to the uterus 6-6.5 days after ovulation, i.e. during the progesterone phase, can provide additional insights in the regulation of the UTJ [73]. The late morula or early blastocyst passes the UTJ retrogradely to the uterus [74] under the influence of prostaglandin E2 produced by the equine conceptus [75]. Unfertilized eggs (UFOs) or parthenotes on the other hand are retained in the oviduct because of their inability to produce this signal. A similar mechanism of gamete / embryo-maternal communication or interaction can be hypothesized when considering the migration of sperm through the UTJ.

### 3. The oviduct as a microenvironment for capacitation / fertilization

Interactions between gametes and the female reproductive tract include final stage maturation and transport of both male and female gametes, fertilization, early cleavage-stage embryonic development and transport of the embryo to the uterus. These events are regulated by the oviduct and its secretions which provide a proper environment to support all the critical events of early stage reproduction. In many species, conventional IVF is successful since events like sperm capacitation, fertilization and blastocyst development can occur *in vitro* in the absence of the epithelia of the female reproductive tract. In horses, however, cell-to-cell contact between gametes and these epithelia seems important as IVF is not successful so far. Theoretically, incubation of equine gametes with oviduct cells and secretions should result in an improved gamete selection and preparation to undergo fertilization and embryonic development [17].

The interaction between spermatozoa and oviduct environment has been well studied in many mammals. Considerably less data are available in the horse due to the lack of equine reproductive tissues being available to study this topic. Additionally, *in vivo* studies are quite problematic since invasive surgery or laparoscopy is required to reach the oviduct. Therefore, a representative *in vitro* model is necessary to study the early events in equine sperm capacitation and fertilization, and to mimic the equine *in vivo* circumstances near ovulation as close as possible. To this end, three oviduct cell models which have been used so far predominantly in bovine research will be discussed, i.e. oviduct monolayers, explants and apical plasma membranes (APM).

#### 3.1. Choice of equine oviduct model: monolayers, apical plasma membranes or explants?

Considering both types of *in vitro* oviduct cell models, the explant model has several advantages to study sperm-oviduct binding interactions when compared to monolayers: (1) it has been demonstrated in cattle [76, 77] and horses [78, 79] that proliferating oviduct cells grown in monolayers dedifferentiate resulting in a reduced cell height, loss of cilia and loss of secretory granules and bulbous protrusions. Therefore, they reflect less accurately the *in vivo* situation [76, 77, 80]. (2) Explants of bovine [76, 81-83] and equine [84] oviduct epithelial

cells show constant vigorously beating cilia, an important marker of cell viability. Nelis *et al.* [84] showed that equine oviduct cell explants still contained healthy beating cilia and numerous microvilli after 20 days in culture as confirmed by transmission electron microscopy. Moreover, the epithelial cells bordering the explants maintained a highly differentiated morphology at the ultrastructural level as well, including numerous mitochondria and rough endoplasmic reticulum, which is highly similar to the oviduct epithelium *ex vivo*. (3) The *in vitro* secretion of IGF2 from bovine oviduct explants was also significantly higher compared to monolayers indicating a more optimal cell function [85]. (4) Moreover, explants can be used already within 6 to 12 hours after harvest whereas monolayers require several days of culture before use [83]. (5) Bull sperm heads bind preferentially to the cilia or in deeper regions of ciliated epithelial cells and not to the secretory epithelial cells [86, 87]. In addition, Baillie *et al.* [88] reported that human spermatozoa bind preferably to explants compared to monolayers. This was also confirmed by Sostaric *et al.* who demonstrated that the binding capacity of bull spermatozoa to oviduct explants was much higher compared to oviduct monolayers [89]. Although these arguments all favor the use of “*in vivo*-like” oviduct explants, it is difficult to reliably quantify the sperm-oviduct binding due to the invaginated and irregular surface of the oviduct explants. Therefore, sperm-binding studies are regularly performed using equine oviduct monolayers with a flattened surface [78, 79]. As the ultrastructural properties of the oviduct during *in vitro* culture are conserved, we preferred to use the oviduct explant model instead of a monolayer system in which the oviduct cells dedifferentiate *in vitro* (Chapter 3, 4 and 5). Moreover, to standardize the quantification of the sperm-binding more accurately, parallel experiments were performed using oviduct explants and nitrocellulose-coated oviduct apical plasma membranes (Chapter 3).

### 3.2. Sperm oviduct binding: formation of a sperm reservoir

#### **GENERAL ASPECTS OF EQUINE SPERM-OVIDUCT BINDING**

After entering the oviduct, spermatozoa bind to the oviduct epithelium and form a sperm reservoir at the UTJ and caudal isthmus during the peri-ovulatory period [71]. Particularly in the mare, spermatozoa need to survive rather long considering the prolonged estrous period. They bind to the oviduct epithelium by means of species-specific carbohydrate

moieties (horse: [78, 90]; cow: [91, 92]; pig: [44]). The interaction between oviduct epithelial cells and spermatozoa serves different aims: (1) providing a suitable environment for sperm storage and survival until ovulation, (2) selecting a superior viable, morphological and motile sperm population, (3) preparing sperm to capacitation and achieving the capability to fertilize a mature oocyte and finally (4) preventing polyspermic fertilization [93].

In cattle and horses, only viable spermatozoa with intact acrosome and plasma membranes bind to the oviduct epithelium [89, 94-97]. Stallion spermatozoa bind to oviduct epithelial explants *in vitro* via the apical region of the sperm head [96]. In oviduct monolayers, sperm binding properties are not affected by cycle stage [98] while in equine oviduct explants, a higher sperm-oviduct binding capacity is observed during the pre- and post-ovulatory period when compared to the luteal stage [97]. The latter study also showed that the motility of spermatozoa bound to follicular stage oviduct explants is higher when compared to other cycle stage explants. Moreover, enhanced sperm-oviduct explant binding was demonstrated using isthmic oviduct explants compared to ampullar oviduct explants [97]. In contrast to these *in vitro* findings, no cycle dependence and no regional differences in sperm-oviduct binding was observed *in vivo* after flushing the oviduct of inseminated mares [52, 99]. However, probably not all the bound spermatozoa were collected after flushing. Moreover, stallion spermatozoa in co-culture with oviduct monolayers maintained superior motility in contrast to spermatozoa in conditions without oviduct cells [100-102]. Direct membrane contact between stallion spermatozoa and oviduct APM *in vitro* is also required to maintain low intracellular calcium [ $\text{Ca}^{2+}$ ] concentrations [103, 104], which causes delayed capacitation and prolongs the viability of the bound spermatozoa. Probably a similar scenario in the isthmic sperm reservoir allows *in vivo* a continuous competent sperm subpopulation to be functionally active at the time of fertilization.

Near ovulation, stallion sperm bound to oviduct epithelium undergoes certain modifications related to capacitation. Co-culture of spermatozoa with oviduct monolayer cells induced capacitation of stallion spermatozoa, whereas capacitation did not occur in sperm suspensions cultured in the absence of oviduct cells. This sperm population had an increased affinity for the ZP as well [105]. At this stage, sperm has to be released from the oviduct epithelium. It has been demonstrated that the intracellular  $\text{Ca}^{2+}$  concentration is many times higher in released stallion spermatozoa compared to bound spermatozoa [103, 104]. Also in bovine, an increased intracellular  $\text{Ca}^{2+}$  concentration accompanies the oviduct epithelium

release of bull spermatozoa after induction of capacitation with heparin [106]. Additionally, after previous contact with capacitation triggers like  $\text{Ca}^{2+}$  ionophore and heparin, significantly fewer spermatozoa are able to bind to oviduct monolayers. Likewise in the horse, contact with oestradiol and heparin induced an increased sperm release from oviduct monolayers [107]. Altogether, these observations strongly suggest that sperm release is initiated by capacitation-related events.

### **BIOCHEMICAL KEY FACTORS OF SPERM-OVIDUCT BINDING**

In many species, sperm binding to somatic cells like sertoli cells or oviduct epithelium and to a glycoprotein layer like the ZP, is regulated by species-specific lectin interactions [108-111]. In the oviduct, carbohydrates and / or glycoproteins expressed on the surface of the oviduct plasma membrane recognize the proteins present on the sperm plasma membrane of non-capacitated spermatozoa [92, 93, 112].

This mechanism was first demonstrated in the hamster in which sialic acid, a component of the glycoprotein fetuin, competitively inhibited sperm-oviduct binding. This sequence appears in terminal positions on the oligosaccharides attached to the protein core of fetuin. Colloid gold-labelled fetuin interacted with the acrosomal region of the sperm head and also bound to sperm plasma membrane proteins after Western blot extraction [113]. In pigs, it has been demonstrated that the formation of a sperm reservoir was regulated by high sperm affinity for oviduct epithelium with oligomannose N glycan expression [114, 115].

In cattle, fucoidan and fucose competitively inhibited sperm-oviduct binding [92, 110]. A more effective inhibition of sperm-oviduct binding was demonstrated by the trisaccharide Lewis A ( $\text{Le}^a$ ;  $\alpha$ 1-4 fucose linked to N-acetylglucosamine) in contrast to any other linkage [116]. Additionally, fucose-binding lectins (*Lotus tetragonolobus* and *Ulex europaeus*) detected clearly the presence of fucose on the surface of the bovine oviduct epithelium [110]. Subsequently, pretreatment of oviduct epithelium with fucosidase reduced significantly sperm binding [110]. On bull sperm, the fucose-binding receptor was detected using fluorescent-labelled fucose and  $\text{Le}^a$ . These molecules labelled live sperm across the acrosomal region [116-118] and were identified as PDC 109, a major heparin-binding protein present in seminal plasma [118]. During ejaculation, this protein has been associated with the bull sperm plasma membrane [119]. The lectin-based sperm-oviduct binding appeared  $\text{Ca}^{2+}$ -dependent. When  $\text{Ca}^{2+}$  was removed from the medium, bull sperm was not able to bind to the oviduct



epithelium [116]. Though, this effect was reversible and could be restored by supplementing  $\text{Ca}^{2+}$ . *In vitro*, bull sperm has similar affinities for both the oviduct epithelium from the isthmus and the ampulla [110, 120] as the lectin affinity for fucose residues exists in both parts of the oviduct [110]. *In vivo*, however, the sperm reservoir is limited to the isthmus which can be explained by the fact that the isthmus is the first segment of the oviduct where the sperm initially contact their carbohydrate ligands in the narrow oviduct lumen filled with mucus.

Using an equine oviduct monolayer model, asialofetuin blocked the *in vitro* sperm-oviduct binding more effectively than fetuin. When sialic acid was removed from fetuin, the principal carbohydrate, D-galactose, was exposed at the end of the oligosaccharide chains [121]. Moreover, a competitive inhibitory effect of D-galactose was shown on *in vitro* sperm-oviduct binding using the oviduct monolayer model [90, 121] while galactose-binding proteins were detected on the surface of stallion spermatozoa by Sabeur and Ball [122]. A similar galactosyl receptor was found in the human testis and spermatozoa. This receptor appeared to be a  $\text{Ca}^{2+}$ -dependent lectin playing a role in the cell-cell interaction during spermiogenesis and sperm-zona binding [123]. It has been demonstrated that galactosyl residues were highly expressed in the isthmus part of the equine oviduct [124], suggesting their involvement as ligands for sperm adhesion. These residues were slightly less expressed in the ampulla while cycle-related differences were observed with a maximum galactosyl expression during oestrus [124]. These observations were supported by the study of Thomas *et al.* [97] in which a slight improved sperm-oviduct explant binding was demonstrated at the isthmus epithelium compared to the ampullar epithelium. Moreover, a similar sperm-oviduct binding density was observed during oestrus and the post-ovulatory stage. However, when considering the sperm motility, it has been demonstrated that the oestrous oviduct epithelium supported motility better than the oviduct epithelium from the post-ovulatory and diestrous stage [97]. On the other hand, horse seminal plasma protein-7 (HSP-7), one of the major seminal plasma proteins, may be involved in the sperm reservoir formation. This protein is the equine homologue of spermadhesin AWN in pigs [125] and is associated with the sperm plasma membrane during ejaculation. These HSP-7 proteins are interacting with the carbohydrates expressed on the oviduct epithelium. Additionally, SP-1 and SP-2 (previously HSP-1 and HSP-2), which are members of the Fibronectin-2 proteins, have also been identified in equine seminal plasma. They are also homologues of bovine seminal plasma

proteins which are involved in establishing the bovine sperm oviduct reservoir [126]. Still, the role of these proteins in equine sperm-oviduct binding needs to be elucidated further. In Chapter 3, the role of various carbohydrates, glycosaminoglycans and capacitation triggers in equine sperm-oviduct binding has been verified using the oviduct explant and apical membrane model instead of the monolayer model.

### 3.3. Sperm-oviduct release

At the late pre-ovulatory period, spermatozoa bound at the isthmic side are flooded by oviduct secretions released mainly from the ampulla site. A select group of bound spermatozoa must be released and migrate upstream to the fertilization site at the ampullary-isthmic junction. Theoretically, following mechanisms of sperm release can be considered: (1) sperm binding sites on the oviduct might decrease, (2) the sperm plasma membrane may undergo capacitation-related changes, (3) competitive binding molecules may be secreted or released, (4) spermatozoa may acquire the hyperactivated motility state, (5) the spermatozoa may get in contact with disulphide-reductants, or (6) sperm release may be caused by a combination of these mechanisms. Current evidence suggests that a combination of capacitation-related events, induction of plasma membrane changes and acquiring hyperactivated motility, are regulating the sperm release from the oviduct epithelium since:

- (1) In cattle and horses, no decrease in sperm binding sites on oviduct epithelium was observed during the hormonal transition at the time of ovulation [97, 120, 127].
  
- (2) Bull spermatozoa are *in vitro* effectively capacitated by heparin [128]. After having contact with heparin, they show less affinity for the oviduct epithelium [91]. Analogously, a reduced affinity of heparin-capacitated bull spermatozoa was observed for fucosylated BSA [117]. These observations suggest that changes in the sperm head plasma membrane, which is part of the capacitation process, are responsible for the decreased oviduct epithelium affinity. This reduced oviduct affinity is due to a loss or modification of PDC-109, the major fucose / Le<sup>a</sup>-binding protein [118].

- (3) Moreover, heparin is also a strong inhibitor of fucose binding to the sperm plasma membranes of non-capacitated bull spermatozoa, even at a very low concentration. Most likely, this is due to competitive binding of heparin to fucose-binding ligands on the sperm plasma membrane of non-capacitated spermatozoa. The binding competition between fucose and heparin is independent of sperm capacitation [92, 116].
  
- (4) In hamster, it has been reported that capacitated and hyperactivated spermatozoa lose their ability to bind to oviduct epithelium [129]. DeMott and Suarez [130] observed in mice that only hyperactivated spermatozoa were released from oviduct epithelium. Likewise, Pacey *et al.* [131] showed in human that hyperactivated motility was essential to release the sperm from *in vitro* cultured oviduct epithelium. Also in bull spermatozoa, heparin-induced capacitation induces the hyperactivated motility during sperm-oviduct release, as illustrated by the increase in flagellar-beat frequency in combination with a high linear motility [132]. Furthermore, these capacitated spermatozoa showed enhanced ZP binding and fertilization competence [133]. It is evident that, in cooperation with plasma membrane changes, hyperactivated motility participates in the sperm-oviduct release by generating an increased force to move the sperm away from the oviduct surface.
  
- (5) Disulphide-reductants are also present in the peri-ovulatory oviduct fluid and act in bovine as a release signal for the spermatozoa from the oviduct reservoir [134] as they reduce the S-S covalent bridges (disulphide; thiol interaction), between the sperm and oviduct-surface, to SH (sulphydryls). *In vitro*, bovine sperm could be reversibly released from oviduct monolayers by disulphide-reductants like penicillamine and reduced glutathione [135, 136]. Recovery of adhesion was associated with reoxidation of the sperm-surface protein SH.

### 3.4. Role of oviduct fluid in sperm capacitation

Each event in the mammalian oviduct needs to contribute to a cycle-dependent optimization of the microenvironment. In horses, only very few data are available on the oviduct paracrine factors which might modulate gamete preparation for fertilization. The successful clinical application of oocyte transfer (*in vivo* or *in vitro* matured) to the oviduct of an inseminated recipient mare emphasizes the importance of oviduct factors on gamete interaction [28]. Ovarian steroids i.e. oestrogens and progesterone regulate the composition of the oviduct fluid which is constituted by selective serum transudation and active biosynthesis and secretion from the oviduct secretory cells [137, 138]. Secretory activity of the oviduct cells is region-dependent: the ampulla exhibits the highest secretory activity while the infundibulum shows intermediate and the isthmus even a minimal secretory activity (sheep: [139]; pig: [140]; horse: [141]). The volume of oviduct fluid is also cycle-dependent with the largest volume measured during the follicular phase under estrogen influence (cattle: [142, 143]; pig: [143]; mare: [144]). As such, a dilution effect is probably present in the oviduct fluid with the concentration of molecules lower in oestrus when compared to dioestrus (see Table 2; [144, 145]).

The majority of the follicular fluid passes through the fimbriae into the peritoneal cavity after ovulation, though a very small amount of fluid enters the infundibulum of the oviduct [146]. This follicular fluid probably also influences the oviduct micro-environment to a certain extent as components of follicular fluid might contain important factors to activate or capacitate the oviduct entered sperm population, as was already demonstrated in human [147-149], in cattle [92], in hamster [150] and in rabbit [151, 152]. As such, progesterone has been designated as a key factor for capacitation of human spermatozoa [153-156], while in cattle glycosaminoglycans were identified as the main capacitation activators [92]. In equine, the identity of capacitation factors in follicular fluid remains to be elucidated although progesterone might play a role as well [157-159].

Up to now, only very few oviduct secreted factors have been studied. The most important factors are listed below:

- (1) Oestrogen-dependent oviduct secretory glycoprotein (OSG) or oviductin is a unique oviduct protein conserved in many mammalian species. This protein enhances the sperm-oocyte binding and penetration through the ZP, and also plays a role in early embryonic development (cattle: [160]; sheep: [161]; pig: [162]). In the horse, however, Mugnier *et al.* [29] demonstrated that OSG is a pseudogene in the equine genome which implies that this protein is not expressed. Nevertheless, this finding can still be due to a not well annotated fault in the equine genome sequencing.
  
- (2) A second major protein synthesized and released by the oviduct is identified as tissue inhibitor metalloprotease 1 (TIMP-1) which is known as a specific inhibitor of matrix metalloproteinases like collagenases, stromelysins and gelatinases (pig: [163]). Several cell functions also have been attributed to TIMP-1 including cell growth [164], embryonic development [165] and maintenance and remodeling of extracellular matrix [166]. TIMP-1 regulates tissue remodeling and steroidogenesis in the oviduct while it exhibits growth activity in the ovary [167].
  
- (3) Plasminogen activator inhibitor-1 (PAI-1) is also commonly secreted by the oviduct. This serine protease is the primary inhibitor of urokinase plasminogen activator and tissue-type plasminogen activator. Both plasminogen activators initiate proteolytic cascades by converting plasminogen to plasmin. PAI-1 is involved in a number of activities such as remodeling extracellular matrix, fibrinolysis, cell migration, and tumor metastasis [168, 169]. Similar to TIMP-1, little is known about the specific actions of PAI-1 in the oviduct but it has probably similar functions.
  
- (4) Other factors identified in the oviduct of various mammals, include complement C3b, immunoglobulin A, prepro-collagen, clusterin [170], cytokines and growth factors [171, 172].

- (5) Exclusively in the horse, osteopontin, atrial natriuretic peptide A (ANP A) and deleted in malignant brain tumor 1 (DMBT1) are identified as oviduct secretory proteins. When stallion sperm was capacitated with  $\text{Ca}^{2+}$  ionophore, co-incubation of mature equine oocytes with either equine or porcine oviduct epithelial explants or monolayers increased equine IVF rates (0 versus 9%). Although there was no significant effect of osteopontin and ANP A on fertilization, osteopontin slightly increased the IVF rates [29]. Moreover, Ambruosi *et al.* [40] reported increased monospermic equine fertilization rates when mature oocytes were pre-incubated with DMBT1 and subsequently fertilized by procaine capacitated sperm.
- (6) Unidentified oviduct secreted proteins with affinity for the sperm plasma membrane or affecting the final maturation of the equine oocyte [173]. Little is known about their function in the equine oviduct [174].
- (7) Besides proteins, many other factors like electrolytes ( $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$ ,...), lipids including steroids and carbohydrates contribute to the composition of the oviduct fluid to create optimal fertilization conditions. The current knowledge on the composition of equine oviduct fluid compared to blood serum is summarized in Table 2. Concentrations of electrolytes in the oviduct fluid of mares tend to be similar of those in serum, except for  $\text{Mg}^{2+}$ . This concentration is 2-5 times higher in the oviduct of the mare compared to serum concentrations [144].

**Table 2:** Composition of the equine oviduct fluid compared to blood serum

	<b>Oviduct fluid</b>	<b>Blood serum</b>
<b>Electrolytes (mM)</b>	[144]	[175, 176]
Na <sup>+</sup>	130 <sup>b</sup>	132 – 142
Cl <sup>-</sup>	ND	96 – 107
K <sup>+</sup>	7.9 <sup>b</sup>	3.0 – 5.9
Total Ca	2.3 <sup>b</sup>	2.4 – 3.3
Ca <sup>2+</sup>	ND	1.4 – 1.7
Mg <sup>2+</sup>	4.6 <sup>b</sup>	0.8 – 1.2
P	0.4 <sup>b</sup>	0.8 – 1.8
HCO <sub>3</sub> <sup>-</sup>	ND	20 – 28
<b>Energy substrates (mM)</b>	[144]	[175, 176]
Glucose	2.84-5.92	3.9 – 5.6
Lactate	ND	0.7 – 1.2
Pyruvate	ND	ND
<b>Amino acids (μM / ml)</b>	[145]	[145]
Alanine	0.140 <sup>a,b</sup>	0.055*
Arginine	0.031	0.010
Aspartic acid	0.022	0.004
Cystine	0.003	<0.001
Glutamic acid	0.057	0.025
Glycine	0.263 <sup>a,b</sup>	0.137 <sup>a</sup>
Histidine	0.020 <sup>c</sup>	0.020
Isoleucine	0.025	0.017
Leucine	0.053 <sup>b</sup>	0.029
Lysine	0.053 <sup>b</sup>	0.057 <sup>a</sup>
Methionine	0.014 <sup>c</sup>	0.002
Phenylalanine	0.026	0.016
Tryptophan	ND	ND
Proline	0.048 <sup>c</sup>	0.047 <sup>b</sup>
Serine	0.051 <sup>c</sup>	0.137 <sup>a</sup>
Threonine	0.038	0.026
Tyrosine	0.041	0.018
Valine	0.041	0.042
<b>Growth factors</b>	[177]	
Platelet derived growth factor (PDGF)	present	present

<sup>a</sup>: most prevalent amino acids; cycle-dependent significant difference (<sup>b</sup>: oestrus < dioestrus; <sup>c</sup>: oestrus > dioestrus) probably due to dilution; the concentration amino acids in oviduct fluid was twice the amino acid concentration in blood serum; ND: not determined.

## 5. Sperm capacitation-related events on cellular level

### 1. Capacitation triggers

*In vivo*, capacitation events are induced when sperm is exposed to the oviduct environment near ovulation while capacitation events are mimicked *in vitro* by a density gradient centrifugation (e.g. Percoll®) to separate sperm from seminal plasma, followed by the incubation in capacitating medium containing  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and albumin. In mammalian species, these three capacitation factors are known to induce the physiological sperm changes required for acquiring fertilization potential. However, species-specific exceptions are known. In cattle, for example, heparin-like molecules like glycosaminoglycans are one of the central capacitation triggers [128]. Unfortunately, in the horse, the exact capacitation triggers are still unknown. So far, two different media are generally used in stallion capacitation studies. Non-capacitating medium lacks any capacitation trigger and is used as control medium while capacitating medium theoretically contains triggers to induce capacitation / fertilization. Because in the horse, however, the exact triggers for full capacitation - resulting in a repeatable, working equine IVF system - are still unknown, the currently used equine capacitating media are based on capacitation triggers known in other species and as such, does not support full capacitation and needs further optimization [178]. In Table 3, an overview of the molecules frequently used as capacitation triggers are given. As the capacitation process of stallion sperm cells is still poorly understood, we screened various biological capacitation triggers in an oviduct explant model which induced tail-associated protein tyrosine phosphorylation and hyperactivated motility (Chapter 4 and 5). Finally, we assessed the ability of tail-associated protein tyrosine phosphorylated, hyperactivated sperm cells to fertilize the equine oocyte (Chapter 6).

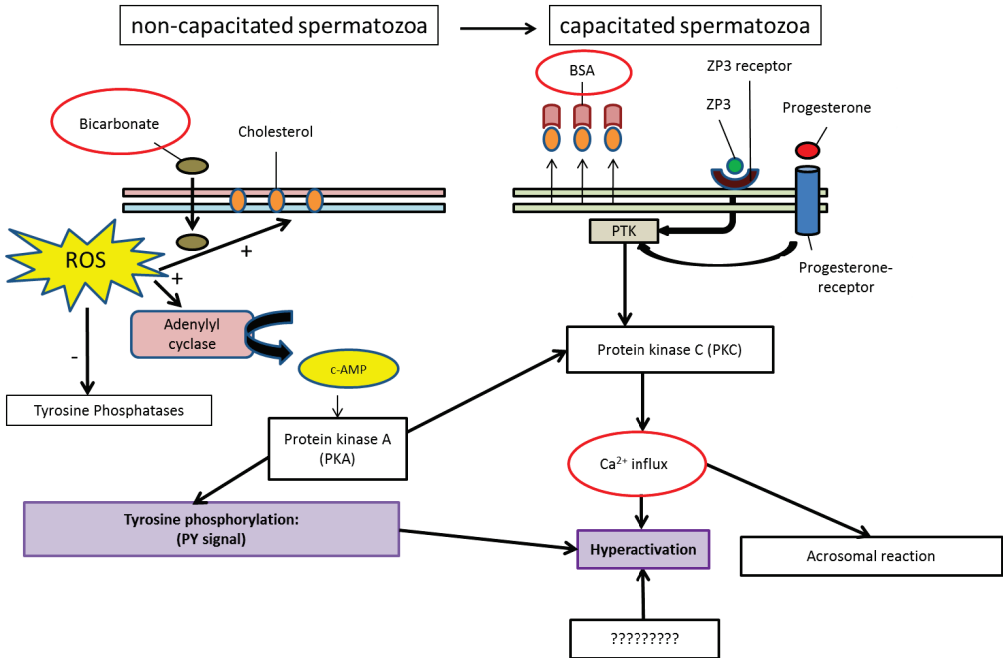


**Table 3:** Overview of capacitation triggers and their *in vitro* capacitation effect in different mammalian species

Capacitating trigger	Capacitation effect	Species	Reference
Ca <sup>2+</sup>	Membrane fluidity	Mice	[179, 180]
	Protein tyrosine phosphorylation	Men	[181]
	Hyperactivation	Pig	[182]
	Acrosome reaction	Cattle	[183, 184]
		Horse	[178]
HCO <sub>3</sub> <sup>-</sup>	Membrane fluidity	Mice	[179, 180]
	Protein tyrosine phosphorylation	Hamster	[185]
	Hyperactivation	Men	[181]
	Acrosome reaction	Pig	[182, 183]
		Cattle	[159]
	Horse	[178]	
Albumin	Cholesterol extraction	Mice	[179, 180]
		Pig	[182, 184]
		Horse	[178]
Methyl-β-cyclodextrin	Cholesterol extraction	Mice	[186]
		Pig	[187]
		Horse	[188]
Heparin	Membrane fluidity	Cattle	[106, 128, 183]
	Protein tyrosine phosphorylation		
	Hyperactivation		
	Acrosome reaction		
Progesterone	Acrosome reaction	Horse	[157, 158]
	Hypermotility	Men	[155]
Ca <sup>2+</sup> ionophore A23187	Acrosome reaction	Mice	[189]
		Men	[190, 191]
		Pig	[192]
		Cattle	[193]
		Horse	[194]
Lysophosphatidylcholine	Acrosome reaction	Horse	[31]
c-AMP and caffeine	Protein tyrosine phosphorylation	Cattle	[183]
		Horse	[195]
	Hypermotility	Pig	[196]
ROS	Protein tyrosine phosphorylation	Cattle	[183]
		Horse	[197]
Alkaline medium pH	Protein tyrosine phosphorylation	Horse	[198]
	Hypermotility	Cattle	[199]
Procaine	Hyperactivation	Guinea pig	[200]
		Horse	[32]

## 2. Capacitation events

Since the first description of capacitation in 1951 [201, 202], the capacitation process is still not fully understood. It is known that capacitation involves a series of changes including (1) the removal of seminal plasma and surface-adhered decapacitation factors from the sperm plasma membrane, (2) reorganization of the sperm plasma membrane and (3) activation of intracellular capacitation pathways (Figure 3).



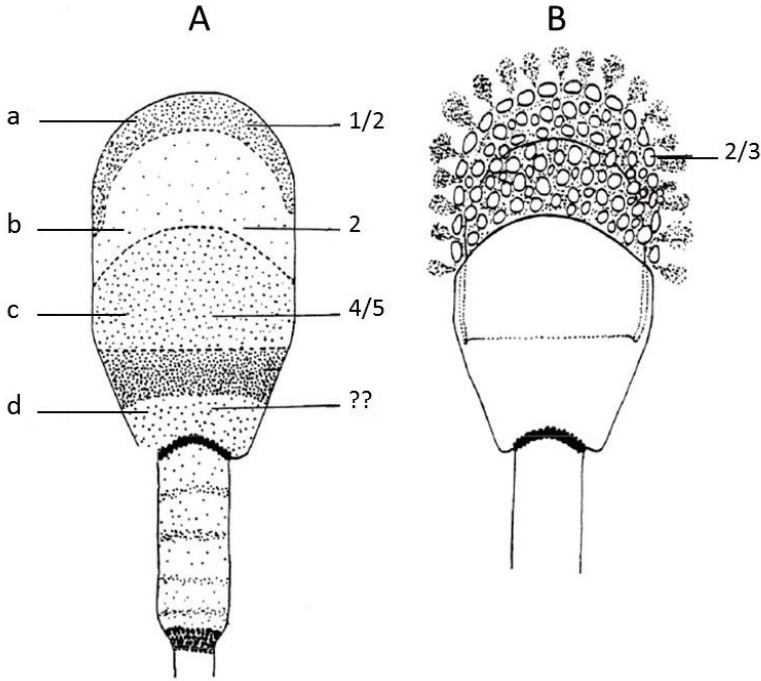
**Figure 3:** Pathways involved in stallion sperm capacitation (ROS: reactive oxygen species; PTK: protein tyrosine kinase; ZP3: ZP protein 3; ZP3-receptor: ZP protein 3-receptor; +: activation; -: inhibition).

After seminal plasma removal, rapid membrane changes (<10 minutes) are induced by an increasing intracellular  $\text{HCO}_3^-$  concentration and the activation of second messenger systems, including a soluble adenylyl cyclase (sAC) and a rise in intracellular  $\text{Ca}^{2+}$  [159, 182, 203]. The activation of sAC and the concomitant production of cAMP results in the depletion of cholesterol from the sperm plasma membrane by a cholesterol acceptor like albumin (>1 hour), which is followed by a slower series of functional membrane changes whereby lipid ordered microdomains are aggregated at the apical ridge of the sperm head (>1 hour) [204]. These microdomains contain functional ZP binding protein complexes [205] and the soluble

NSF attachment protein receptor (SNARE) proteins, which play an important role in the induction of the acrosome reaction [206, 207]. Simultaneously, the production of cAMP enables the activation of protein kinase A (PKA) which is essential to phosphorylate the tyrosine residues on sperm proteins [159, 208, 209]. In various species, this cAMP-dependent protein tyrosine phosphorylation, especially in the sperm tail, has been related to the acquisition of hyperactivated sperm motility and is considered as a marker for some essential elements of the capacitation process [179, 180, 210-213].

### *2.1. Plasma membrane changes*

Spermatozoa have a highly polarized morphology and their heterogenic surface is differentiated into at least four surface membrane domains: the apical ridge, the pre-equatorial, the equatorial and the post-equatorial surface area (Figure 4). The functional regions do not contain junctional barriers to maintain these regions [214]. When sperm is capacitating and some decapacitating factors are removed from the extracellular sperm coat, the lipid and protein ordering on the sperm plasma membrane will change dramatically [48, 215, 216]. Each of the sperm head surface regions play a specific role in fertilization: (1) the apical ridge facilitates the zona pellucida binding [217], (2) the apical ridge together with the pre-equatorial surface area is involved in the acrosome reaction while (3) the equatorial surface area initiates the binding to the oolemma and the subsequent fertilization fusion. These events occur in the region of the sperm head where the sperm plasma membrane including the lipid rafts (microdomains) covers the acrosome which plays an essential role in the dynamics of sperm capacitation [203].



**Figure 4:** Sperm surface involved in gamete interactions that lead to fertilization. Panel A: the surface of the sperm head, with (a) the apical ridge, (b) the pre-equatorial, (c) equatorial and (d) post-equatorial area. Panel B: a surface view of the sperm head after the acrosome reaction. The numbers indicate the processes leading to fertilization: 1. zona binding, 2. the acrosome reaction, 3. sperm-zona penetration, 4. sperm-oolemma binding, 5. fertilization fusion and activation of the oocyte (Image adapted from Gadella [218]).

**COLLAPSE OF PLASMA MEMBRANE ASYMMETRY**

Phospholipid scrambling is the first lipid architectural change of the sperm plasma membrane during the capacitation process. Consequently, endogenous aminophospholipids are exposed at the surface of the sperm plasma membrane. More precisely, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are pre-dominantly present in the inner leaflet of the membrane bilayer in non-capacitated spermatozoa, while glycolipids, phosphatidylcholine (PC) and sphingomyelin (SM) are present in the outer leaflet of the plasma membrane [219]. After  $\text{HCO}_3^-$ -induced *in vitro* capacitation, the sperm plasma membrane lipid asymmetry was clearly disrupted in stallion sperm [220]. A marked increase of PC and SM levels in the inner leaflet was observed while the normal inward movement of PE and PS was considerably

slowed down. A non-specific bidirectional phospholipid scramblase is playing a central role in these events which coincide with an increased membrane fluidity and cholesterol efflux [221].

### **CHOLESTEROL EXTRACTION**

The redistribution of the surface phospholipid molecules in the sperm head is an essential step in the capacitation process to prepare the sperm to achieve the ability to fertilize. The increased  $\text{HCO}_3^- / \text{sAC} / \text{cAMP}$ -mediated membrane fluidity causes the redistribution of cholesterol from the equatorial area to the apical part of the sperm plasma membrane while seminolipids on the other hand show a retrograde movement [222, 223]. Both molecules are considered as plasma membrane stabilizers preventing membrane fusions prior to capacitation. Finally, the  $\text{HCO}_3^-$ -mediated repacking of the sperm surface lipids is essential to allow the efflux of cholesterol. This cholesterol extraction is a major event which additionally increases the plasma membrane fluidity by the aggregation of lipid rafts, another essential step in the membrane reorganization.

The extraction of cholesterol from the sperm plasma membrane occurs *in vitro* by (1) an active cholesterol transporter that provides free cholesterol to the hydrophobic pocket of albumin [221] and (2) the oxidation of membrane sterols [224]. Brouwers *et al.* [225] showed that the production of reactive oxygen species (ROS) is an essential step in oxysterol formation. The production of ROS at low levels during sperm capacitation was first demonstrated in 1993 indicating that sperm capacitation should be considered as an oxidative process which depends on the active generation of ROS [226]. The latter probably also has a regulating effect on protein tyrosine phosphorylation, another important capacitation marker, by increasing the cAMP production [47, 227] and suppressing the tyrosine phosphatase activity [228]. Furthermore, it was demonstrated that peroxynitrite played a central role in ROS- induced sperm capacitation [229, 230]. As oxysterols are more hydrophilic, they move freely through the plasma membrane and facilitate the binding of oxysterols to sterol acceptor molecules like albumin. Subsequently, cholesterol is extracted from the plasma membrane followed by an enhanced membrane fluidity which eventually results in sperm capacitation. Indeed, the presence of bovine serum albumin (BSA) in capacitating medium is essential considering its unique ability to scavenge hydrophilic oxidation products [231] and to facilitate the cholesterol extraction [159, 178]. An alternative macromolecule which is not of

animal origin (important in terms of risk for disease transmission), is methyl- $\beta$ -cyclodextrin (mBCD). In contrast to albumin, which extracts 20% cholesterol from the non-raft sperm plasma membrane fraction, mBCD extracts 50% cholesterol from the complete sperm plasma membrane including the lipid rafts [187]. However, mBCD seems to have a sperm deteriorative effect, even in low concentrations. Recently, a deteriorative of mBCD effect on mouse oocytes was observed too [232].

Remarkably, it has been shown that standard capacitating conditions, including  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and BSA (individual or in combination), absolutely not facilitated the cholesterol removal from the sperm plasma membrane in stallion sperm. Though,  $\text{HCO}_3^-$  induced an increase in ROS that was abolished by the addition of  $\text{Ca}^{2+}$  or BSA [233].

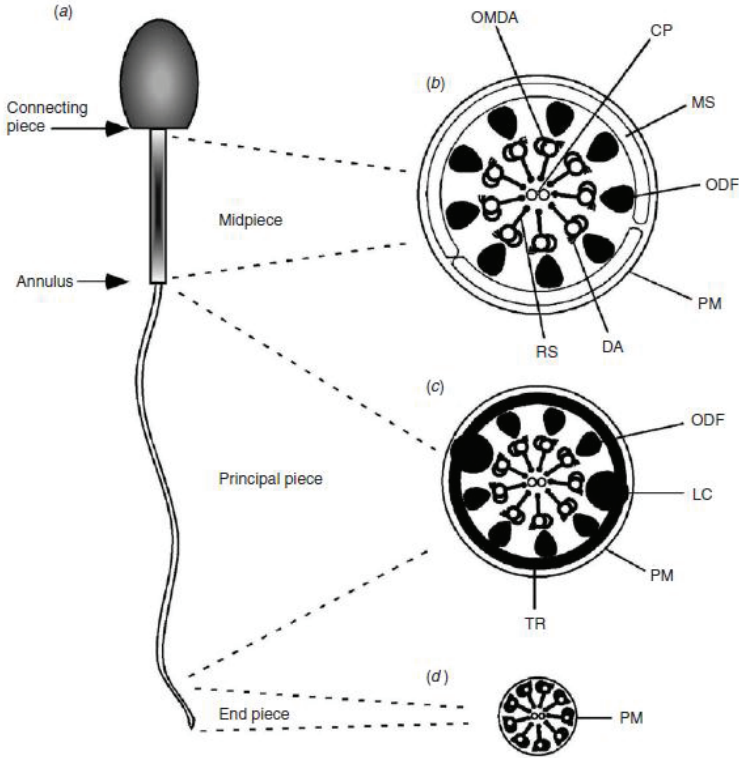
#### **AGGREGATION OF LIPID RAFTS**

Following the cholesterol depletion and the subsequent increased membrane fluidity, the lateral segregated molecules are redistributed by aggregation of lipid ordered microdomains at the apical ridge area of the sperm head, as demonstrated in pigs [231]. Subsequently, the proteins and lipids which are part of these microdomains also show a capacitation-dependent membrane distribution containing higher proportions of cholesterol, sphingomyelin, gangliosides, phospholipids with saturated long-chain acyl chains and lipid-modified proteins such as GPI anchored proteins [204, 234]. Moreover, caveolin-1 and flotillin-1 were identified as lipid raft-specific markers [204]. Caveolin is a cholesterol interacting protein involved in clathrin-independent endocytosis [235, 236]. Although the function of flotillin is not completely understood yet, it has a prohibitin homology (PHB) domain that might interact with lipid rafts by constituting a primordial lipid recognition motif [237]. Beside caveolin-1 and flotillin-1, the microdomains also contain functional ZP binding protein complexes. In pigs, it was shown that isoforms of AQN-3 (spermadhesin), P47 (porcine homologue of SED-1), fertilin  $\beta$  and peroxiredoxin 5 were indisputably identified as key proteins regulating the primary binding between capacitated spermatozoa and the ZP [205].

## 2.2. *Hyperactivated motility*

### **REGULATION OF SPERM MOTILITY IN GENERAL**

If a sperm cell wants to generate progressive motility,  $\text{Ca}^{2+}$  needs to interact with the phosphorylated dynein molecules along the microtubules of the axoneme (Figure 5). The axoneme of the sperm tail consists of nine microtubule doublets surrounding a central pair of single microtubules which are interconnected with radial spokes [238]. This central pair of microtubules regulate the shape and size of the flagellar bending as signals are transmitted to the outer microtubule doublets via these core structures (Figure 5) [239]. The outer microtubule doublets are interconnected by inner and outer dynein arms containing molecular dynein motor activities [240-242]. Symmetrical flagellar or progressive motion requires the activation of dynein ATPases which is initiated after protein phosphorylation on dynein arms and causes the sliding of adjacent outer axonemal doublet microtubules [243]. This sliding force is subsequently translated into a bend in the sperm tail when the doublets slide along one another [244, 245]. The normal flagellar waveform requires an asynchronous phosphorylation and dephosphorylation of the dynein arms along the complete axoneme length [239]. Dephosphorylation of dyneins is evoked by the calmodulin-dependent protein phosphatase calcineurin which completely opposes the effect on axoneme movement of phosphorylation of target proteins that supports sperm motility [246, 247] (Figure 5).



**Figure 5:** Schematic ultrastructural representation of a mammalian spermatozoon. (a) Mammalian sperm are divided structurally in 4 areas: the connecting piece, the mid-piece, the principal piece and the end-piece. The end of the mid-piece and the start of the principal piece are demarcated by the annulus. (b) Schematic cross-section through a representative segment of the mid-piece showing the plasma membrane (PM) and mitochondrial sheath (MS) surrounding the nine outer dense fibers (ODFs). Within the ODFs are the components of the axoneme; the nine outer microtubule doublets of the axoneme (OMDA) with associated dynein arms (DA) and radial spokes (RS) and the central pair of microtubule doublets (CP). In an actual sperm, several projections are present on the CP, which are not shown in this figure. (c) Schematic cross-section through a representative segment of the principal piece showing the PM surrounding 7 ODFs. The ODFs 3 and 8 have been replaced by the longitudinal columns of the fibrous sheath (LC). The 2 LC are connected by transverse ribs (TR). The axonemal components are unchanged. (d) Schematic cross-section through a representative segment of the end-piece. The ODFs and FS tapered at the termination of the principal piece and are no longer present in the end-piece, thus leaving only the PM to surround the axoneme (Image from Turner [248]).



In mammals, sperm motility is mostly initiated and maintained by  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ -driven cAMP-dependent phosphorylation of flagellar proteins [244, 249-253]. Like  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  also directly regulates sAC, which results in generating cAMP and activating PKA [248, 254, 255]. An important downstream target of cAMP in the sperm flagel is serine / threonine kinase PKA [256]. The phosphorylation of serine / threonine activates PKA which results in the downstream phosphorylation of tyrosine kinases whose targets are primarily located in the sperm tail (human: [257]; mouse: [258]). So far, only a few target proteins of tyrosine phosphorylation in the sperm tail have been identified. For example, phosphorylation of one specific protein on the axonemal dynein seems essential to initiate sperm motility [245]. On the other hand, serine / threonine phosphatases are necessary to provide a balance for the cAMP-driven serine / threonine kinases in the sperm tail. The resulting net phosphorylation represents the sperm motility status very well [245, 247]. If serine / threonine phosphatase activity is dominant, spermatozoa are mainly immotile while serine / threonine kinase activity highly correlates with increased motility (men and rhesus monkeys: [259]; bull: [260]).

#### **$\text{Ca}^{2+}$ AS THE MAJOR TRIGGER FOR HYPERACTIVATED SPERM MOTILITY**

Sperm cells must acquire hyperactivated motility (1) to leave the oviduct reservoir and release the oviduct binding [130, 261], (2) to provide a powerful force to migrate through the viscous lumen of the oviduct [262, 263] and (3) to penetrate the cumulus matrix and ZP of the mature oocyte in order to fuse with the oolemma [264-266]. In many species, hyperactivated motility is characterized by a highly asymmetrical and high-amplitude flagellar beating pattern giving rise to a whip-like motion of the sperm tail which evokes circular, figure eight or zigzag swimming trajectories [48, 267]. The onset and maintenance of hyperactivated motility is associated with an influx of  $\text{Ca}^{2+}$  to the cytosol of the sperm tail [268, 269]. *In vitro*,  $\text{Ca}^{2+}$  ionophores such as A23187 or ionomycin can induce hyperactivation in mouse spermatozoa [250, 263]. Other pharmacological agents such as caffeine [270], procaine [271], thimerosal [270, 272] and thapsigargin [270, 273] also initiate asymmetrical beating by an intracellular  $\text{Ca}^{2+}$  rise. Ho *et al.* [253] showed in demembrated bull sperm that symmetrical, progressive sperm movement was maintained with an intracellular  $\text{Ca}^{2+}$  level of ~50 nM. When hyperactivated motility was initiated, the intracellular  $\text{Ca}^{2+}$  concentration increased until 400 nM.

In mammals such as human, cattle and mouse, CATSPER channels present on the principal piece of the sperm tail must be activated to induce hyperactivated motility. There are 4 CATSPER genes coding for proteins which are structurally similar to subunits of conventional voltage-gated cation channels. When mice were knocked-out for a single CATSPER gene, they were infertile as spermatozoa were not able anymore to achieve hyperactivated motility [274, 275]. The general trigger of these CATSPER channels is alkaline depolarization evoked by a change in the ionic oviduct environment and resulting in an elevated pH of the oviduct fluid [276, 277]. Contact between the spermatozoa and the alkaline oviduct environment increases the intracellular pH and activates the CATSPER channels. A clear increase in the oviduct pH was observed in Rhesus monkeys near ovulation (7.1-7.3 to 7.5-7.8) [278]. In mouse, a  $\text{Na}^+$ -dependent  $\text{Cl}^- / \text{HCO}_3^-$  exchange controls the intracellular pH of the sperm [279], while in human, an outflow of  $\text{H}^+$  is initiated by activating a voltage-gated proton channel [280]. To maximize the subsequent  $\text{Ca}^{2+}$  entry through the CATSPER channels, both a pH-sensitive efflux of  $\text{K}^+$  by KSPER [281] and the activation of  $\text{Cl}^-$  channels by closing  $\text{Na}^+$  channels might support the sperm plasma membrane hyperpolarization (mouse: [282]). This hyperpolarization also plays a central role in the acrosome reaction and chemotaxis as well. In human sperm, it has been demonstrated that CATSPER channels are alternatively activated by progesterone and, to a lesser extent, prostaglandins. Interestingly, both factors potentiated CATSPER activation by another CATSPER binding site [155, 283]. Other  $\text{Ca}^{2+}$  channels identified in the mammalian sperm tail, include: (1) transient receptor potential, which may affect sperm motility and resequestration of  $\text{Ca}^{2+}$  into sperm stores [284], (2) cyclic-nucleotide-gated [285] and (3) voltage-gated [286-290]  $\text{Ca}^{2+}$  channels. However, it is still unclear if these channels are involved in the physiological activation of hyperactivated motility.

Beside the extracellular  $\text{Ca}^{2+}$  influx through CATSPER channels, intracellular  $\text{Ca}^{2+}$  stores, being located at the base of the sperm tail and called redundant nuclear envelopes (RNE), also provide  $\text{Ca}^{2+}$  to the sperm cytoplasm [270, 273]. Inositol 1,4,5-triphosphate ( $\text{IP}_3$ )-gated channels on the membrane of the RNE stores trigger the release of  $\text{Ca}^{2+}$  to the sperm cytoplasm while calreticulin, a  $\text{Ca}^{2+}$  binding protein, sequesters  $\text{Ca}^{2+}$  in the RNE (bull: [270, 273]; man: [291]). In mouse, it has been shown that ryanodine receptors on the RNE membrane also play a role in the intracellular  $\text{Ca}^{2+}$  release [292]. So, both  $\text{Ca}^{2+}$  sources, external  $\text{Ca}^{2+}$  influx via CATSPER channels and  $\text{Ca}^{2+}$  release from intracellular RNE stores,

contribute to initiate and maintain high  $\text{Ca}^{2+}$  levels during sperm hyperactivation [270, 273]. More specifically, the intracellular  $\text{Ca}^{2+}$  elevation due to CATSPER activation amplifies and propagates forward by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the RNE [293-295]. Recently, it has been shown in mouse spermatozoa that the  $\text{Ca}^{2+}$  release from the RNE produces a reversed hyperactivation pattern [296]. Furthermore, the acrosome reaction is also supported by the cytoplasmic  $\text{Ca}^{2+}$  increase [297-299]. Activation of CATSPER and  $\text{IP}_3$ -gated channels, which both initiate the intracellular  $\text{Ca}^{2+}$  rise, showed clearly an effect on the induction of the acrosome reaction [266, 298, 300, 301].

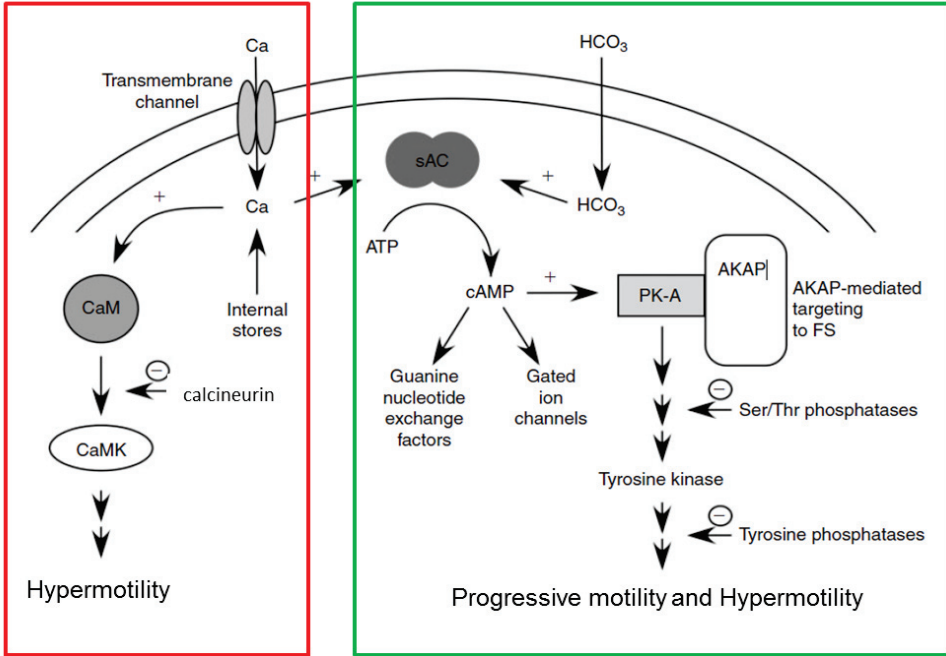
To control the intracellular  $\text{Ca}^{2+}$  levels, spermatozoa contain several  $\text{Ca}^{2+}$  clearance mechanisms [302-304]. Although a constant  $\text{Ca}^{2+}$  leakage from the extracellular environment ( $\text{Ca}^{2+}$  levels in mM) through the sperm plasma membrane into the cytosol was demonstrated, spermatozoa were able to maintain the intracellular  $\text{Ca}^{2+}$  concentration in nM levels (hamster: [268]; bull: [253]; mouse: [305]). Important  $\text{Ca}^{2+}$  clearance mechanisms are plasma membrane  $\text{H}^+ / \text{Ca}^{2+}$  ATPases and the  $\text{Na}^+ / \text{Ca}^{2+}$  exchanger to remove  $\text{Ca}^{2+}$  out of the sperm cell or into the RNE (mouse: [302]). Considering the close connection between RNE and the mitochondrial sheet at the base of the flagellar midpiece (bull: [270, 306]), it is clear that mitochondria can also act as a  $\text{Ca}^{2+}$  buffer and clear the cytosol from  $\text{Ca}^{2+}$  [307].

### **$\text{HCO}_3^-$ AND $\text{Ca}^{2+}$ -DRIVEN PATHWAYS INDUCE HYPERMOTILITY**

So far, it is not completely known yet how the sperm cells modify the beat of their tails at the molecular level. It is clear that the  $\text{Ca}^{2+}$  rise and the subsequent onset of hyperactivated motility changes the sliding of the microtubules [308]. In sperm of primates, pigs and rodents, it has been demonstrated that hyperactivated motility is associated with a highly increased cAMP-dependent tyrosine phosphorylation of the flagellar proteins [212, 309-315]. The generator of tyrosine phosphorylation events PKA is connected with the fibrous sheath of the sperm tail by A-kinase anchoring proteins (AKAPs). These proteins play very likely an important role in hyperactivated sperm motility (Hamster: [311]). Moreover, it has been suggested that AKAPs are tethering PKA to specific subcellular regions in close proximity of motility-related targets in the axonema [316-318]. It has been demonstrated that AKAP-3 and AKAP-4 play a central role in the onset of tyrosine kinases which cause extensive tyrosine phosphorylation of proteins in the sperm tail (hamster: [311]; human: [319]). This altered protein tyrosine phosphorylation status of the tail proteins is required to

obtain the hyperactivated sperm motility (hamster: [311]; human: [257]). Additionally, a testis-specific  $\text{Ca}^{2+}$ -binding protein, CA-BYR, has been identified in the tyrosine phosphorylated proteins of the sperm tail during capacitation. It is known that CA-BYR interacts with the AKAPs on the sperm fibrous sheet though their role in sperm motility is still unclear [320, 321]. Nevertheless, changes in AKAP-mediated protein targeting surely play an essential role to induce hyperactivated sperm motility.

$\text{Ca}^{2+}$  is also involved in sperm motility regulating pathways independent of PKA. Calmodulin, located in the principal piece of the sperm tail [322], is an essential  $\text{Ca}^{2+}$  binding protein in the  $\text{Ca}^{2+}$ -dependent modulation of mammalian sperm motility [306, 323-325]. It has been suggested that this pathway progresses parallel with cAMP / PKA activity although both are acting independently [322, 326, 327]. Binding of  $\text{Ca}^{2+}$  to calmodulin activates  $\text{Ca}^{2+}$  / calmodulin-dependent kinases [328] which phosphorylate a specific axonemal protein, resulting in hyperactivated motility [269]. Calmodulin kinases were identified in the flagella of bull [325] and men [328] sperm showing a relationship with hyperactivated motility. On the other hand, phosphatase activities were observed as well to reverse this effect by regulating dynein ATPase activities and thus these are directly involved in the axoneme function [329, 330] (Figure 6). Additionally, it has been shown that calmodulin kinases have also an effect on the acrosome reaction [329, 330].



**Figure 6:** Schematic representation of signaling pathways involved in the regulation of mammalian sperm (hyper)motility. Progressive motility is regulated by low level activity of the sAC / cAMP / PKA pathway (green box) while hyperactivated motility is induced if the latter pathway acts on a much higher level in combination with the activation of the calmodulin kinase (CaMK) pathway (red box) (Image adapted from Turner [248]).

**IS HYPERACTIVATION LINKED TO PROTEIN TYROSINE PHOSPHORYLATION IN STALLION SPERM CAPACITATION?**

As discussed above, a clear relationship is suggested between hyperactivation and protein tyrosine phosphorylation in various mammalian species. On the other hand, it has been shown that hyperactivation and increased protein tyrosine phosphorylation can occur independently (mouse: [331]; bovine: [271]).

In horse, type 10 sAC was observed to induce protein tyrosine phosphorylation in stallion sperm without the induction of hyperactivated motility [332]. Moreover, an increase in PKA activity and protein tyrosine phosphorylation without inducing hyperactivation was clearly observed after *in vitro* incubation of stallion spermatozoa with membrane soluble cAMP analogues and phosphodiesterase inhibitors (caffeine) [195, 333]. Also ROS [197] and

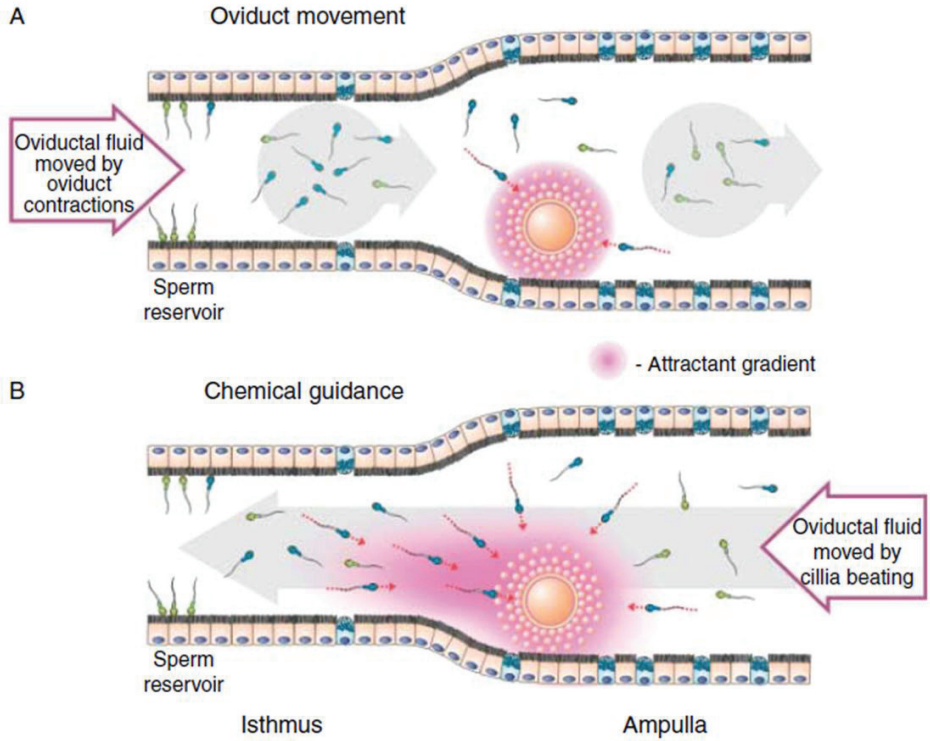
modified-Whittens capacitating medium with increased alkalinity (pH=7.8-8.0) [198, 334, 335] only induce protein tyrosine phosphorylation. If stallion sperm is incubated in capacitating conditions at a physiological pH of 7.4, a  $\text{Ca}^{2+}$ -mediated inhibitory effect on protein tyrosine phosphorylation has been demonstrated due to the formation of a  $\text{Ca}^{2+}$  / calmodulin complex which supports sperm phosphatase activity [198]. Under elevated pH capacitating conditions (pH=7.8-8.0), however,  $\text{Ca}^{2+}$  / Calmodulin-dependent kinases become dominant and play a downstream role in PKA-dependent protein tyrosine phosphorylation of stallion sperm. Subsequently, focal adhesion kinases act as activators of protein tyrosine phosphorylation in stallion spermatozoa downstream of PKA [334].

Both the  $\text{Ca}^{2+}$  / calmodulin-kinase and cAMP / PKA pathways must be activated to acquire functional (hyper)motility in stallion sperm [336]. Hyperactivation triggers for stallion sperm which do not support protein tyrosine phosphorylation in the sperm tail, are known as well. For example, incubating stallion sperm in 10% follicular fluid resulted in a decrease of three motility parameters (straight line velocity, straightness and linearity) which is indicative for acquiring hyperactivated motility [39]. Procaine was also reported to induce effective hyperactivated motility in stallion spermatozoa without protein tyrosine phosphorylation [32, 337]. So hyperactivation can be induced in non-capacitated spermatozoa by increasing intracellular  $\text{Ca}^{2+}$  [32, 337, 338], even when external  $\text{Ca}^{2+}$  is lacking [337]. Surprisingly, the CATSPER channels did not participate in procaine-induced hyperactivation in stallion sperm [337]. In contrast to other mammals, ionomycin, progesterone and prostaglandin  $\text{E}_1$  did not show any association with an intracellular  $\text{Ca}^{2+}$  rise and subsequent hyperactivated motility in stallion sperm [337]. These controversial observations can be explained as a consequence of a timing difference between hyperactivated motility and tail-associated protein tyrosine phosphorylation.

Interestingly, a premature induction of protein tyrosine phosphorylation is observed after cryopreservation, commonly referred to as 'cryocapacitation' [339, 340]. The increased osmolarity associated with cryopreservation may induce the ROS-dependent increase of protein tyrosine phosphorylation in stallion sperm [341, 342]. Subsequent to cryopreservation, sperm cells have an increased intracellular  $\text{Ca}^{2+}$  concentration, an increased generation of ROS, and a reduced antioxidant capacity. This premature capacitation of sperm after cryopreservation may be responsible for the reduced longevity of sperm typically noted after freezing and thawing.

### **CHEMOTAXIS AND HYPERACTIVATED SPERM MOTILITY**

Sperm hyperactivation is initiated in the lower oviduct, far from the fertilization site. The flagellar beat pattern determines the swimming path that the sperm has to follow to reach the oocyte. Besides, the movement of the capacitated spermatozoa to the mature oocyte is also facilitated by smooth muscular contractions of the oviduct [343]. However, spermatozoa are additionally guided towards the oocyte by a chemical gradient, indicated as chemotaxis (Figure 7). The molecular triggers for chemotaxis in mammalian sperm are not completely understood yet. In various marine invertebrates, amino acids, peptides, lipids and sulfated steroids were identified as chemotactic key factors [344, 345] while in mammalian sperm, odorant-like factors seem potential candidate molecules to induce sperm chemotaxis. Receptors for these molecules were found at the base and the mid-piece of mature dog [346], mouse [347] and rat [348] spermatozoa. In human, it was demonstrated that sperm was chemotactically attracted by follicular fluid [349-351] or cumulus cell secretions [352]. A progesterone gradient around the cumulus-oocyte complex was considered as the active component [353, 354] as progesterone is the ligand that binds to the chemoreceptor on the CATSPER channels and subsequently initiates hyperactivated motility by inducing  $\text{Ca}^{2+}$  oscillations and increased bend amplitudes of the sperm tail [155, 156].



**Figure 7.** Schematic representation of sperm transport and sperm retention at the fertilization site mediated by chemical guidance and oviduct movement. (A) The oviduct movement mechanically propels oviduct fluid droplets containing free-swimming capacitated spermatozoa towards the ovary. (B) The egg complex continuously secretes an attractant forming a gradient in the cumulus surroundings which may be expanded toward the isthmus by the cilia beating. This attractant gradient may chemically guide capacitated spermatozoa towards the egg. The attractant gradient disrupted during the oviduct contractions may be restored by the cilia beating during the quiescence period between contractions. These two mechanisms, the chemical guidance and the oviduct movement, would alternate as long as a viable egg complex is available in the oviduct (blue spermatozoa: capacitated; green spermatozoa: non-capacitated) (Image from Guidobaldi *et al.* [355]).



### 2.3. Acrosome reaction

The  $\text{Ca}^{2+}$ -dependent release of the acrosomal content is an essential step in mammalian fertilization as it facilitates the penetration through the acellular glycoprotein barrier of the oocyte. The sperm cell which passes through the ZP, will subsequently fuse with the oolemma of the mature oocyte [48] (Figure 1).

The acrosome reaction physiologically occurs in the female genital tract at the site of fertilization. It is a multipoint membrane fusion event of the sperm plasma membrane and the outer acrosomal membrane [206, 357], which results in the generation of mixed vesicles containing plasma membrane and outer acrosomal membrane material. The remaining unfused acrosomal membranes, i.e. the equatorial area of the outer acrosome and the sperm plasma membrane connected to the inner acrosomal membrane covering the apical part of the nucleus, take over the surface function of the sperm plasma membrane [358, 359]. This newly designed sperm membrane contains a hairpin structure which binds to the oolemma resulting in gamete fusion and oocyte activation [48]. In boar sperm, it was demonstrated that SNARE interactions play a fundamental role in the interaction between the sperm plasma membrane and the outer plasma membrane. During capacitation these two membranes become docked by the formation of a *trans* ternary SNARE protein complex. Key factors involved in this process are syntaxin 1B and VAMP 3 from the plasma membrane and SNAP 23 from the outer acrosomal membrane [206]. However, an additional  $\text{Ca}^{2+}$  entry (*in vitro* by use of  $\text{Ca}^{2+}$  ionophores; *in vivo* after ZP binding) is required to facilitate the conversion to *cis* SNARE complexes which will result in acrosomal exocytosis. This event allows spermatozoa to penetrate the ZP [360-362]. SNARE complexes showed many interactions with different protein-like complexins [206, 363, 364], dynamins [365], synaptogamins [366], multi-PDZ domain protein MUPP1, calmodulin and calmodulin kinase II $\alpha$  [367, 368], .... The specific role of these interactions is unknown but most likely they are involved in the stabilization of the *trans* SNARE complexes or in the  $\text{Ca}^{2+}$ - mediated conversion to *cis* SNARE complexes.

In mice, one of the main molecules triggering the acrosome reaction in capacitated spermatozoa is ZP glycoprotein 3 (ZP3) present on mature oocytes [369]. More recently, it was observed that just the contact with the intercellular matrix of the cumulus cell complex induced the acrosome reaction and mouse spermatozoa were able to pass through the ZP [49, 50]. In many other mammals, including the horse, it is shown that capacitated, acrosome-

intact spermatozoa initiate the ZP binding [48]. Nevertheless, stallion spermatozoa showed a low incidence of acrosome reaction after 1 h *in vitro* binding to the ZP [105, 370, 371]. Beside the ZP glycoproteins, other factors might be responsible for inducing the acrosome reaction in the horse. Cheng *et al.* [158] demonstrated that the acrosome reaction in stallion spermatozoa can be induced by progesterone, present in follicular fluid or cumulus cell secretions [39, 157, 372, 373], and is mediated by plasma membrane non-genomic progesterone receptors (Figure 3). Interestingly, the progesterone-induced acrosome reaction did not act in a PKA but in a PKC and PTK-dependent manner [159] which is very similar to ZP-mediated induction of the acrosome reaction. Indeed, Breitbart and Naor [210] showed that ZP3 activates a sperm PTK coupled to phospholipase C (PLC) which in turn stimulates PKC by generating diacylglycerol (DAG) from phosphatidylinositol-biphosphate (PIP<sub>2</sub>). How exactly the generation of DAG results in the onset of the acrosome reaction is not known yet. In contrast, if stallion sperm *in vitro* is incubated in HCO<sub>3</sub><sup>-</sup>-enriched conditions, the acrosome reaction is mainly supported by the PKA pathway instead of in a PTK and PKC-dependent manner [159], indicating that progesterone and HCO<sub>3</sub><sup>-</sup> induce the acrosome reaction in a different way. There are various physiological inducers of the acrosome reaction, like ZP3 and follicular fluid, but *in vitro* it can also be evoked by non-physiological inducers, like Ca<sup>2+</sup> ionophore in combination with HCO<sub>3</sub><sup>-</sup> [220]. *In vivo*, a biological effective acrosome reaction will depend on both the presence and activity of physiological inducers as the ability of spermatozoa to respond to these inducers. Recently, McPartlin *et al.* [333] showed that the cAMP-driven activation of guanine-nucleotide exchange factors (RAPGEF3 / RAPGEF4) induced a sperm membrane depolarization in capacitated stallion spermatozoa. Depolarization-dependent Ca<sup>2+</sup> influx subsequently initiated acrosomal exocytosis. However, the activation of these factors did not play any role in the activation of PKA and protein tyrosine phosphorylation (Figure 5).

## 6. Conclusion

So far, conventional IVF in the horse still does not work. Since more than 20 years, however, co-incubation of mature oocytes with capacitated sperm is the standard method to produce *in vitro* embryos in several species such as human, cattle, pigs and many laboratory animals (rats, mice,...). The development of a standardized equine conventional IVF system is important as this technique allows the production of foals from sub- and infertile horses with probably a greater efficiency when compared to ICSI. *In vitro*, stallion sperm is not able to penetrate the ZP of the mature oocyte. Theoretically, both sperm and oocytes can be responsible for this failure of fertilization. However, stallion sperm incubated in capacitating conditions is able to bind to the ZP while the acrosome reaction cannot be induced [36]. Moreover, *in vivo*-matured oocytes are not able to be fertilized *in vitro* while *in vitro*-matured oocytes can be fertilized *in vivo* after transfer to the oviduct of an inseminated mare [22, 28]. These findings strongly indicate that insufficient capacitation of stallion spermatozoa under *in vitro* conditions probably is the major obstacle why equine IVF does not work. We hypothesize that one or more oviduct-derived factor(s) is / are essential to allow an adequately capacitation-triggered stallion sperm cell to penetrate the oocyte. It is very likely that a sperm cell cannot fertilize the oocyte without adding this oviduct factor to equine IVF media. Since *in vivo* sperm capacitation takes place in the oviduct in the peri-ovulatory period, capacitation events in stallion sperm were studied in this thesis by means of an *in vivo*-like oviduct explant and an oviduct apical plasma membrane model.

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## CHAPTER 2

# AIMS AND OUTLINES OF THE THESIS

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How do stallion spermatozoa interact with the oviduct environment in the peri-ovulatory period and obtain their fertilizing capability? Understanding this fundamental question will provide essential knowledge to effectively induce *in vitro* capacitation of stallion spermatozoa and equine fertilization. As fertilization occurs *in vivo* in the oviduct, we hypothesize that oviduct factors are essential to induce capacitation of the sperm cells *in vitro*. If we are able to identify the capacitation triggers present in the oviduct and add these factors to the current equine IVF media, we will establish a repeatable conventional equine IVF system. To this end, we used an oviduct explant and apical plasma membrane (APM) model to study the sperm-oviduct interactions, induction of sperm capacitation, release of oviduct epithelium-bound spermatozoa and fertilization in the horse.

The specific scientific aims of this thesis were:

1. To examine the involvement of various carbohydrates, glycosaminoglycans, S-S reductants and capacitation triggers ( $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and albumin) in sperm-oviduct binding using two different sperm-oviduct binding inhibition assays (oviduct explant and oviduct APM assay) (CHAPTER 3).
2. To identify whether or not binding of stallion sperm to oviduct explants in the late pre-ovulatory period induces tail-associated protein tyrosine phosphorylation and if so, which regulating mechanism causes this effect (CHAPTER 4).
3. To evaluate the role of various maternal reproductive tract fluids and cells on the release of viable, tail-associated protein tyrosine phosphorylated stallion spermatozoa, probably involved in the initiation of hyperactivated sperm motility (CHAPTER 5).
4. To assess if viable, hyperactivated and tail-associated protein tyrosine phosphorylated spermatozoa are able to *in vitro* fertilize mature equine oocytes after inducing both capacitation characteristics in 2 different ways: (1) using *in vivo*-like capacitating conditions based on the results of chapter 5, and (2) after incubation in air followed by procaine exposure. We question previously published equine IVF results based on *in vitro* sperm capacitation by procaine. Additionally, we assess the direct effect of procaine on equine oocytes (CHAPTER 6).



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**CHAPTER 3**  
**AFFINITY OF STALLION SPERM FOR**  
**OVIDUCT BINDING IS INHIBITED BY**  
**THE COMBINATION OF BICARBONATE**  
**AND ALBUMIN, BUT BINDING**  
**RELEASE IS NOT TRIGGERED BY**  
**CALCIUM, CARBOHYDRATES OR S-S**  
**REDUCTANTS**

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## ABSTRACT

In many species, sperm binding to oviduct epithelium is believed to be an essential step in generating a highly fertile capacitated sperm population primed for fertilization. In several mammalian species, this interaction is based on carbohydrate-lectin recognition. D-galactose has previously been characterized as a key-molecule that facilitates sperm-oviduct binding in the horse. We used oviduct explant and oviduct apical plasma membrane (APM) assays to investigate the effects of various carbohydrates, glycosaminoglycans, lectins, S-S reductants and the capacitating factors albumin,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  on sperm-oviduct binding in the horse. Carbohydrate-specific lectin staining indicated that *N*-acetylgalactosamine, *N*-acetylneuraminic acid (sialic acid), and D-mannose or D-glucose were the most abundant carbohydrates on equine oviduct epithelia whereas D-galactose moieties were not detected. However, in a competitive binding assay, sperm-oviduct binding density was not influenced by any tested carbohydrates, glycosaminoglycans, lectins or penicillamine, nor did the glycosaminoglycans induce sperm tail-associated protein tyrosine phosphorylation. Furthermore, *N*-glycosidase F (PNGase) pretreatment of oviduct explants and APM did not alter sperm-oviduct binding density. By contrast, a combination of the sperm capacitating factors albumin and  $\text{HCO}_3^-$  severely reduced (>10 fold) stallion sperm affinity for oviduct binding by inducing rapid head-to-head agglutination, both of which events were independent of  $\text{Ca}^{2+}$  and an elevated pH (7.9). Conversely, neither albumin,  $\text{HCO}_3^-$  or any other capacitating factor could induce release of oviduct-bound sperm. In conclusion, a combination of albumin and  $\text{HCO}_3^-$  markedly reduced sperm affinity for binding to oviduct epithelium presumably in part due to the head-to-head sperm agglutination induced.

## INTRODUCTION

Sperm storage after mating is a female reproductive tract phenomenon that has been observed in various animals with the presumed aim to preserving sperm fertilizing capacity in species in which mating and ovulation are poorly synchronized [1]. In the mare [2, 3] and many other mammals (rabbit: [4]; pig: [5]; sheep: [6]; mouse: [7]; cattle: [8]; hamster: [9]), an oviductal sperm reservoir is established at the uterotubal junction and the caudal part of the oviductal isthmus. This sperm reservoir contains spermatozoa bound to the epithelial surface by their apical head region. Most of the bound sperm are found in pockets formed by mucosal folds. The precise identity of molecules involved in sperm-oviduct interaction is not clear, but there are indications that the interaction in several mammals is mediated by carbohydrate ligands in a species-specific manner [10]. In general, spermatozoa contain lectin-like receptors on their plasma membrane with affinity for carbohydrate moieties found on the surface of oviduct epithelial cells. Interactions between the two can therefore be studied using competitive carbohydrate-lectin binding assays. It has also been hypothesized that, when ovulation is imminent, oviduct bound spermatozoa become capacitated and subsequently release. The releasing factor needs to have either a stronger affinity for the carbohydrate-ligand receptors on the sperm plasma membrane, with sperm release as a result of a competitive interaction as shown in cattle [11-13], or enzymatic activity capable of disturbing the lectin recognition site or substrate.

Bull spermatozoa actively bind via the apical part of the sperm head surface to the oviduct epithelium [14]. Only non-capacitated bovine spermatozoa are able to bind to oviductal epithelium *in vitro* [15, 16]. Importantly, the bound spermatozoa appear to remain in a rather quiescent state in the early pre-ovulatory stage oviduct. This has been demonstrated by incubation of rabbit, bull, boar and stallion spermatozoa with the apical plasma membranes of pre-ovulatory oviductal epithelial cells; binding to oviductal epithelium prolonged sperm longevity [17, 18] by maintaining low cytoplasmic  $\text{Ca}^{2+}$  levels [19]. Oviduct binding of non-capacitated bull sperm can be inhibited by fucose and fucoidan (a sulphated polysaccharide that predominantly consists of fucose) [11], while pretreatment of bovine oviduct explants with fucosidase significantly reduces sperm binding [20]. Not only the presence of fucose, but its position within the complex carbohydrate moieties is important for functional sperm oviduct binding. For instance, only trisaccharide Lewis A ( $\alpha$ -1-Fuc[1, 4]- $\beta$ -D-Gal[1, 3]-d-

GlcNAc) significantly reduced binding of spermatozoa to oviduct epithelia whereas other fucose-containing oligosaccharides failed to influence sperm-oviduct binding [11, 21]. A competitive carbohydrate binding inhibition assay, using fetuin and sialic acid individually, was successfully used to block hamster sperm-oviduct binding [22]. In pigs, the major inhibitory effect could be mimicked by biantennary structures containing a mannose core with 6-sialylated lactosamines at one or more termini. In pigs, as in cattle, binding to carbohydrate moieties was very specific; different isomers of the specific motif did not bind sperm [23].

At the late pre-ovulatory stage, it is thought that oviduct bound sperm undergo final maturation or capacitation and are released from oviduct epithelial cells by undergoing plasma membrane changes and achieving hyperactivated motility at various rates [24]. In many mammals, this release coincides with raised levels of capacitation factors [25]. In cattle, sulfated glycosaminoglycans induce a reduction in fucose binding by spermatozoa, which is regulated by direct competition. This suggests that glycosaminoglycans released into the bovine oviduct near the time of ovulation display a stronger affinity for sperm plasma membrane receptors than fucose moieties expressed on the oviduct membrane [11]. In addition, disulphide-reductants like penicillamine inhibit sperm-oviduct binding and facilitate sperm release from the oviduct epithelium. It was suggested that reversible adhesion of bull spermatozoa to the oviduct epithelium is modulated by redox control of sperm surface protein sulfhydryls [26].

Much less is known about sperm-oviduct epithelium binding in the horse. Inhibition of stallion sperm binding to oviduct epithelial monolayers has been reported after adding fetuin, asialofetuin or D-galactose to the culture medium (D-galactose gave the most prominent effects) [27]. Moreover, since galactose-binding proteins have been observed on the rostral and post-acrosomal regions of the sperm head of non-capacitated stallion spermatozoa [28], the authors concluded that D-galactose was the key molecule facilitating the binding of non-capacitated spermatozoa to oviduct epithelium in the horse [27, 29]. Nevertheless, equine oviduct epithelium expresses very few D-galactose moieties [30, 31], and it is more likely that other, as yet undefined, factors are involved in regulating binding of non-capacitated stallion sperm to the oviduct epithelium.

For release of sperm from the epithelium lining the equine oviduct, high concentrations of sulfated glycosaminoglycans in oviductal fluid (originating from the ovulatory follicle or secreted from the oviduct epithelium) may be important [32]. Glycosaminoglycans and disulphide-reductants have been reported to play a role in bovine sperm-oviduct binding and sperm release from the epithelium [12]. We therefore decided to investigate the involvement of various carbohydrates in the regulation of equine sperm-oviduct binding and release, as well as that of known capacitation factors such as albumin,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  ions.

## MATERIALS AND METHODS

### Chemicals and reagents

Dulbecco's phosphate buffered saline (DPBS), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline (HBS), fetal bovine serum (FBS) (Batch: 07G8814F) and Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 (DMEM/F12) were purchased from Gibco® Life Technologies (Merelbeke, Belgium). Various carbohydrates and glycosaminoglycans (D-galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, fetuin, asialofetuin, D-fucose, fucoidan, D-mannose, mannan, *N*-acetylneuraminic (sialic) acid, chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate), D-penicillamine, fatty acid-free bovine serum albumin (A9418; cell culture tested), ethylenediaminetetraacetic acid (EDTA) and all chemicals not otherwise listed were obtained from Sigma-Aldrich (Bornem, Belgium). Various lectins (non-fluorescein conjugated and fluorescein conjugated): PNA (*Arachis hypogaea* [peanut] agglutinin), DBA (*Dolichos biflorus* agglutinin), HPA (*Helix pomatia* agglutinin), WGA (*Triticum vulgare* [wheat germ] agglutinin), UEA I (*Ulex europaeus* agglutinin), SNA (*Sambucus nigra* agglutinin), Con A (*Canavalia ensiformis* agglutinin), LCA (*Lens culinaris* agglutinin), PSA (*Pisum sativum* [pea] agglutinin) were purchased from Labconsult SPRL (Vector Labs, Brussels, Belgium). Hoechst 33342 and Alexa Fluor 488-conjugated goat anti-mouse antibody were obtained from Molecular Probes (Ghent, Belgium). Monoclonal 4G10®Platinum, anti-phosphotyrosine mouse antibodies were obtained from Millipore (Overijse, Belgium). Protease inhibitors (Comlate Mini, EDTA-free) were purchased from Roche (Mannheim, Germany).

## Animals

Oviducts were collected at a local slaughterhouse (Euro Meat Group, Moeskroen, Belgium) from healthy Warmblood mares aged between 5 and 22 years and without any visible reproductive tract pathology. Only oviducts from mares with growing follicles and without a corpus luteum on the ovaries in combination with estrous oedema in the uterine wall, indicating that the mare was in estrus, were used for this study.

## Preparation of oviduct explants and isolation of oviduct apical plasma membranes

Five oviducts per experiment were prepared for oviduct explant culture, as previously described by Nelis *et al.* [33]. Briefly, oviducts from mares in early estrus were dissected free of extraneous connective tissue, clamped at both ends and transported on ice in sterile 0.9% saline containing 50 µg / ml gentamycin. Upon arrival at the lab, the oviducts were washed in PBS and the epithelial cells were harvested by scraping the ampullary-isthmic mucosa of the longitudinally incised oviduct. The harvested cellular material was transferred to a tube containing HEPES-buffered Tyrode's albumin pyruvate lactate (HEPES buffered TALP) (10 µg / ml gentamycin sulfate, 10 mM HEPES, and 3 mg / ml BSA; based on [34]) and left to settle for 10 min, after which the cell pellet was resuspended in 3 ml of fresh HEPES-buffered TALP washing medium. The process of sedimentation was repeated twice. The time-span from slaughter of mares to seeding of the cells was approximately 3 to 4 h. Next, the harvested cellular material was washed and cultured overnight in Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) at 38.5 °C in a humidified atmosphere of 5% CO<sub>2</sub>-in-air.

To isolate oviduct apical plasma membrane (APM) [17], oviducts from early estrous mares were collected as for oviduct explant preparation. All further processing of the oviducts in the laboratory took place on ice (0-4°C). Upon arrival in the lab, oviducts were dissected free from surrounding tissue, opened longitudinally and rinsed with ice-cold saline. Subsequently, oviduct epithelia from the ampullary-isthmic mucosa was gently harvested by scraping and then suspended in hypotonic buffer solution (HiP; 50 mM mannitol, 2 mM Tris-HCl, pH=7.1). This suspension was homogenized by Turrax blending (IKA T-18 Ultra Turrax Digital Homogenizer; 115 VAC; Metrohm Belgium n.v., Antwerp, Belgium) at maximum speed for 2 x 3 min, and further sonicated for 5 x 5 sec. To this homogenized suspension, 10

mM solid  $MgCl_2$  (hexahydrate; Bornem, Belgium) was added, to cross-link non-apical cell plasma membranes. After incubation on a rotation plate for 30 min, the suspension was centrifuged at 3000g for 15 min to remove large cellular debris and agglutinated non-apical plasma membranes. The pellet was discarded from the supernatant and the supernatant was subsequently centrifuged at 27 000g for 30 min. After these centrifugation steps, the resulting pellet was resuspended in Mannitol buffer (MB; 100mM mannitol, 20mM Tris-Hepes buffer, pH=7.4) and homogenized by pipetting for 5 min. In order to improve the degree of purity, unwanted debris was washed from the APM fraction using a 15 min 6000g centrifugation step. The resulting pellet was discarded and the supernatant was centrifuged at 23 000g for 30 min to pellet the washed APM vesicles. Pelleted APM vesicles were then suspended in HBS supplemented with protease inhibitors and stored at  $-80^{\circ}C$  until further use. Purity of the membrane isolates was assessed by measuring apical plasma membrane  $\gamma$ -glutamyl transpeptidase activity [17]. Fractions of oviduct APM samples were collected, and protein content was determined using a modification of the method described by Lowry *et al.* [35], using BSA as the standard.

### **Semen collection and preparation**

Semen was collected using an artificial vagina (Colorado State University AV) from three adult stallions of proven fertility. The raw ejaculate was filtered through gauze to remove the gel fraction and any debris, before visual evaluation of sperm motility by light microscopy (200x) on a heated stage at  $37.0^{\circ}C$ . Semen with adequate sperm motility was immediately transported to the laboratory for further processing. The nuclei of spermatozoa in fresh semen with a concentration of 100 to  $300 \times 10^6$  spermatozoa / ml were labelled by pre-incubating one ml semen with  $3.2 \mu M$  Hoechst 33342 for 10 min. Subsequently, the suspension of Hoechst stained spermatozoa was washed using a 45 / 90% Percoll® gradient [36, 37]. Next, the sperm pellet was diluted in Whitten's medium (100 mM NaCl, 4.7 mM KCl, 1.2 mM  $MgCl_2$ , 5.5 mM glucose, 22 mM HEPES, 2.4 mM sodium lactate, 1.0 mM pyruvate and 0.05% polyvinylpyrrolidone ; pH=7.4 and 280-300 mOsm / kg; adapted from McPartlin *et al.* [38]; further referred as non-capacitating medium) to the desired concentration (10, 20 or  $50 \times 10^6$  spermatozoa / ml). At least 3 replicates of each experiment were performed using an ejaculate from each of the three stallions. The study was approved by the Ethical Committee of Ghent University's Faculty of Veterinary Medicine (EC2013/175

and EC2013/176).

### **Sperm-oviduct explant and sperm-oviduct APM assay**

Oviduct explants were cultured in DMEM/F12 medium with 10% FBS, equilibrated with 5% CO<sub>2</sub> in a humidified atmosphere at 38.5 °C, as described by Nelis *et al.* [33]. After overnight incubation, oviduct explant material contained vesicular epithelial cell clumps; vesicles with a diameter of < 200 µm were selected, washed and transferred in groups of 5 to 45 µl incubation droplets. Sperm binding to oviduct explants was performed in non-capacitating medium at 38.5 °C in air. To provide sperm capacitation supporting conditions, Whitten's medium was modified to capacitating medium by replacing the sodium lactate with 2.4 mM calcium lactate and adding 25 mM NaHCO<sub>3</sub> and 7 mg / ml bovine serum albumin (BSA; pH=7.4 and 280-300 mOsm / kg; osmolality was adjusted by stepwise addition of NaCl); this medium was pre-equilibrated for at least 2 h in a humidified atmosphere containing 5% CO<sub>2</sub> at 38.5°C (adapted from McPartlin *et al.* [38]). Elevated pH conditions were achieved by incubating capacitating medium in air until a pH of 7.9 was attained. In general, a final concentration of 2 million (carbohydrate-glycosaminoglycan-penicillamine pre-incubation) or 5 million (lectin pre-incubation) Hoechst stained spermatozoa / ml was produced by adding 5 µl Percoll® washed and diluted sperm (20 or 50 x 10<sup>6</sup> / ml spermatozoa) to the 45 µl medium droplets containing the oviduct explants [39]. The 50 µl medium droplets (38.5 °C in air) were covered under mineral oil to prevent evaporation. Similar experiments were repeated under capacitating conditions (38.5 °C in 5% CO<sub>2</sub>-in-air). Sperm-oviduct binding was additionally assessed in various non-capacitating conditions with individual or combined addition of BSA, calcium lactate, NaHCO<sub>3</sub> or EDTA (pH=7.4; media were pre-equilibrated for 2 h at 38.5 °C in 5% CO<sub>2</sub>-in-air to avoid a pH change in the medium). Sperm-oviduct binding was also tested in capacitating medium at pH 7.9. Identical conditions were used to test sperm release from oviduct explants, for which sperm-oviduct explants were previously established in non-capacitating conditions, subsequently washed twice and transferred to the sperm release conditions. Ultimately, the effect of sperm concentration (1, 2, 5, 10, 25, 50, 75, 100 x 10<sup>6</sup> spermatozoa / ml) on binding capacity of pre-incubated sperm to oviduct explants was assessed in both non-capacitating and capacitating conditions. Each replicate was performed using different ejaculates.

To establish a sperm-oviduct APM binding assay, a dot blot technique was adapted from Tsai *et al.* [40]. In brief, dot blotting was performed using the Easy-Titer™ ELIFA dot blot system (Pierce, Rockford IL, USA). Nitrocellulose membranes (Sigma-Aldrich, Bornem, Belgium) were rinsed in Milli-Q and oviduct APM samples containing 20 µg protein were subsequently pipetted (10 µl of 2 µg / µl APM protein) into separate wells. Membranes and adhering proteins were dried using a vacuum system (flow rate 100 µl / 1.5 min / well). After blotting, non-specific binding was blocked using 50 ml 10% BSA in HBS for 1 h at room temperature. Oviduct APM coated nitrocellulose membrane was subsequently washed twice with non-capacitating medium and the spots were then individually cut. Per tested condition, three APM coated blots were further co-incubated with carbohydrate and glycosaminoglycan pre-incubated, Hoechst-stained spermatozoa (1 x 10<sup>6</sup> spermatozoa / ml) under non-capacitating conditions (38.5°C) in a 2 ml Eppendorf tube (Sigma-Aldrich, Bornem, Belgium). Similar experiments were repeated under capacitating conditions (38.5 °C in 5% CO<sub>2</sub>-in-air). After 2 h co-incubation, APM coated blots were washed 3 times. Quantitative analysis of dot blot labeling was performed by scanning the blots with a GS-700 densitometer. Absorbance results achieved by the sperm-oviduct APM assay were corrected for the control absorbance (nitrocellulose membrane without coated oviduct APM).

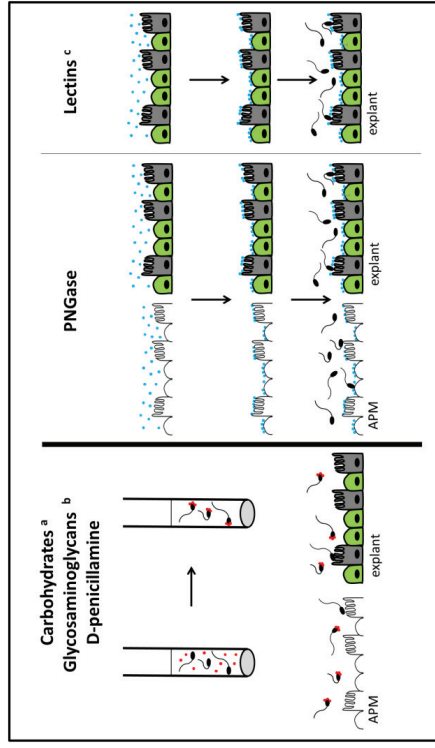
### **Effect of carbohydrate, glycosaminoglycan, penicillamine, lectin, Ca<sup>2+</sup>, HCO<sub>3</sub><sup>-</sup> and albumin pre-incubation on sperm-oviduct binding**

Before adding sperm to either oviduct explants or oviduct APM, Hoechst-stained spermatozoa were pre-incubated with various carbohydrates (50 mM; D(+)-galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, D(+)-fucose, D(+)-mannose, *N*-acetylneuraminic (sialic) acid: 5 mg / ml; asialofetuin, fetuin, fucoidan, mannan: 10 µg / ml; chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate), or penicillamine (0.125, 0.25, 0.5, 1, 5 and 10 mM) (Figure 1). Hoechst-stained sperm were pre-incubated at a concentration of 10 or 20 x 10<sup>6</sup> spermatozoa / ml in 500 µl non-capacitating and capacitating conditions at 38.5°C to saturate sperm plasma membrane receptors so that they could have a competitive inhibitory effect on sperm-oviduct binding. After 20 min, sperm suspensions were washed by centrifugation (600g; 5 min) with 500 µl non-capacitating or capacitating medium and 5 µl of the washed sperm solution was added to either oviduct explants (2 x 10<sup>6</sup> spermatozoa / ml) or oviduct APM (1 x 10<sup>6</sup> spermatozoa / ml) (control condition). The

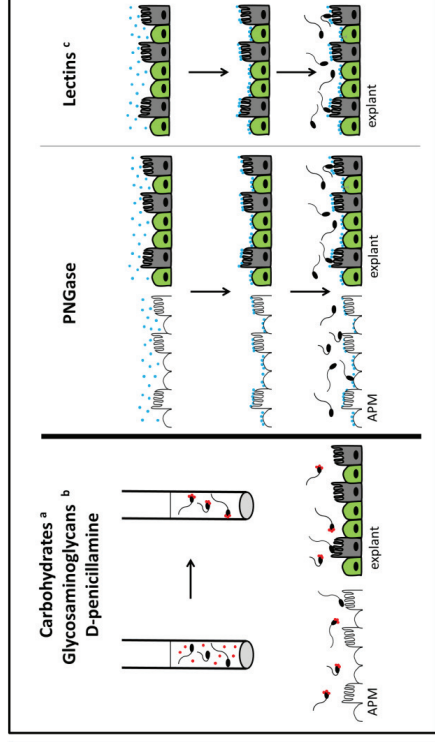


competitive inhibitory effect of lectins on sperm-oviduct binding was assessed by pretreating oviduct explants with individual, or mixtures of, various lectins (50  $\mu\text{g}$  / ml: PNA, DBA, HPA, WGA, UEA I, SNA, Con A, LCA, PSA or in case of the lectin mix; 20  $\mu\text{g}$  / ml of each lectin; supplementary table) in 50  $\mu\text{l}$  droplets of non-capacitating medium under mineral oil at 38.5°C (Figure 1). After 2 h, oviduct explants were washed in non-capacitating medium and 5  $\mu\text{l}$  Hoechst-stained sperm was added to the 45  $\mu\text{l}$  oviduct-explant containing non-capacitating droplets ( $5 \times 10^6$  spermatozoa / ml). These incubations were repeated using capacitating conditions in which Hoechst-stained spermatozoa were pre-incubated in either full capacitation medium, individual or combinations of 7 mg / ml BSA, 2.4 mM calcium lactate or 25 mM  $\text{NaHCO}_3$  in non-capacitating medium (pH=7.4; 2 h pre-equilibrated in 5%  $\text{CO}_2$  in air to avoid a pH change in the medium). To test the role of  $\text{Ca}^{2+}$  in sperm-oviduct binding, 2 mM (non-capacitating medium) or 4 mM (capacitating medium) EDTA [41, 42] was added (pH=7.4; 2 h pre-equilibrated in 5%  $\text{CO}_2$  in air to avoid a pH change in the medium). In addition, the effect of elevated pH 7.9 in capacitating medium was assessed. Similar incubation conditions were used to test the ability of the capacitating factors to trigger sperm-oviduct release after establishment of sperm-oviduct complexes in non-capacitating medium.

## Non-capacitating medium



## Capacitating medium



<sup>a</sup> D(+)-galactose, N-acetylgalactosamine, N-acetylglucosamine, D(+)-fucose, D(+)-mannose, N-acetylneuraminic (sialic) acid, asialofetuin, fetuin, fucoidan, and mannan

<sup>b</sup> chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, and keratan sulfate

<sup>c</sup> PNA, DBA, HPA, WGA, UEA I, SNA, Con A, LCA, PSA

**Figure 1.** Schematic overview of the experimental design. The role of various molecules in sperm-oviduct binding was evaluated by a competitive sperm-oviduct binding assay using both the oviduct explant and apical plasma membrane (APM) model. The effect of carbohydrates, glycosaminoglycans and D-penicillamine was tested by pre-incubating the sperm cells with the different molecules respectively and subsequently adding this sperm suspension to either the APM or the explant model. The effect of different lectins and PNGase on the other hand was evaluated by first pre-incubating the APM and oviduct explants; or the oviduct explants only with PNGase or the different lectins, respectively, before adding the sperm cells to the pre-incubated oviduct models.

Supplementary table: Various used lectins

Lectin	Lectin source	Sugar specificity	Inhibitory sugar
PNA	Arachis hypogea	Gal $\beta$ 1,3GalNAc	D-Gal
DBA	Dolichos biflorus	$\alpha$ GalNAc	GalNAc
HPA	Helix pomatia	$\alpha$ GalNAc	GalNAc
WGA	Triticum vulgaris	$\beta$ GlcNAc $\gg$ NeuNAc	GlcNAc
UEA I	Ulex europaeus	A-L-Fuc	L-Fuc
SNA	Sambucus nigra	Neu5Ac $\alpha$ 6Gal/GalNAc	NeuNac
Con A	Canavalia ensiformis	A -D-Man $\gg$ $\alpha$ -D-Glc	D-Man and D-Glc
LCA	Lens culinaris	$\alpha$ -D-Man, $\alpha$ -D-Glc	D-Man and D-Glc
PSA	Pisum sativum	$\alpha$ -D-Man, $\alpha$ -D-Glc	D-Man and D-Glc

Gal, Galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuNac, N-acetyl neuraminic (sialic) acid; Fuc, Fucose; Man, Mannose; Glc, Glucose

### Individual and combined effect of $\text{Ca}^{2+}$ , $\text{HCO}_3^-$ or albumin on head-to-head agglutination of stallion spermatozoa

Head-to-head sperm agglutination was assessed after 0.5 and 2 h in non-capacitating medium containing 7 mg / ml BSA, 2.4 mM calcium lactate or 25 mM  $\text{NaHCO}_3$  individually or in combinations. To test the role of  $\text{Ca}^{2+}$  in sperm-oviduct binding, 2 mM (non-capacitating medium) or 4 mM (capacitating medium) EDTA [41, 42] was added (pH=7.4; 2 h pre-equilibrated in 5%  $\text{CO}_2$  in air to avoid a pH change in the medium). The effect of elevated pH (7.9) in capacitating medium was also assessed. Sperm was incubated at a concentration of  $10 \times 10^6$  spermatozoa / ml in 500  $\mu\text{l}$  of each medium sample at 38.5°C. After 0.5 and 2 h incubation, sperm suspensions were evaluated for sperm agglutination by placing a 10  $\mu\text{l}$  aliquot onto a pre-warmed glass slide covered with a warm glass cover slip. For each medium sample, 200 randomly selected motile spermatozoa were examined and, subsequently, the percentage of head-to-head agglutinated sperm was calculated.

### **Effect of N-glycosidase F (PNGase) treatment on sperm-oviduct binding**

To elucidate the involvement of N-linked carbohydrates in sperm-oviduct interaction, oviduct explants and oviduct APM were treated for 24 hours with and without (control) the *N*-Glycosidase F enzyme [43, 44]. To this end, 10 oviduct-explants or 20 µg oviduct APM protein were incubated in PBS to which 3 µl *N*-Glycosidase F was then added. Enzyme activity was evaluated by Con A-FITC staining of oviduct explants before and after *N*-Glycosidase F treatment [30]. The enzyme-treated oviduct explants and oviduct APM were further processed for sperm binding assays as previously described. Incubations were performed under non-capacitating and capacitating conditions (Figure 1).

### **Carbohydrate expression on oviduct explant epithelial cells**

To examine which carbohydrates could play a key role in sperm-oviduct binding, the expression of carbohydrate moieties on the oviduct epithelial explants was assessed using fluorescein (FITC) conjugated lectins (PNA, DBA, HPA, WGA, UEA 1, SNA, Con A, LCA, PSA). Specificity of the lectins was previously demonstrated by Desantis *et al.* [30, 31]. Briefly, after fixing in 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, oviduct explants were washed twice in 500 µl blocking buffer (PBS containing 1% BSA) and stained with 50 µg / ml lectin-FITC in DPBS [30, 31] for 15 min at room temperature. After two washes with DPBS, the stained oviduct explants were mounted as described above using 1.4-diazabicyclo[2.2.2] octane (DABCO) as antifade. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at 400x magnification. Quantification of carbohydrate expression was performed by scoring the fluorescence intensity (+++, ++, + and -). Imaging of the lectin-stained oviduct explants was performed using standard settings (camera exposure: 204.1 ms; camera capture format: 2560 x 1920).

### **Tail-associated protein tyrosine phosphorylation after exposure to various sulfated glycosaminoglycans**

After 6 h incubation, the same glycosaminoglycan conditions were used as during the pre-incubation steps to assess competitive inhibition of sperm–oviduct binding. A portion of the treated Hoechst-stained sperm suspensions ( $10 \times 10^6$  spermatozoa / ml) were further processed to detect protein tyrosine phosphorylation, as previously described [39]. In brief, spermatozoa were washed twice in 1 ml DPBS (600g; 5 min) and fixed in 500 $\mu$ l 4% paraformaldehyde in PBS at room temperature for 15 min. The fixative was removed by three washing steps using 1 ml DPBS (600g; 5 min). The washed spermatozoa were subsequently incubated in 500  $\mu$ l 0.1% Triton X-100 in DPBS for 10 min at room temperature to ensure complete membrane permeabilization. The immobilized and permeabilized spermatozoa were then washed twice (600g; 5 min) in 1 ml DPBS and further incubated in 500  $\mu$ l blocking buffer (DPBS containing 1% BSA) for 10 min at room temperature. Next, spermatozoa were incubated in 200  $\mu$ l buffer containing 0.1% BSA and supplemented with a mouse monoclonal 4G10@Platinum IgG<sub>2b</sub> protein anti-phosphotyrosine antibody (diluted 1:500) at 4°C. After overnight incubation, unbound antibody was removed by washing the spermatozoa twice using 1 ml of DPBS containing 0.1% BSA (600g; 5 min). The resulting spermatozoa were then stained with a monoclonal goat anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Molecular Probes, Ghent, Belgium) for 1h at room temperature. After immunolabelling, the non-bound antibody conjugates were removed by washing three times using 1 ml PBS containing 0.1% BSA (600g; 5 min), and once using PBS. The immunolabelled spermatozoa were mounted on glass slides as described above and sealed with nail polish. The proportion of the total sperm population with green fluorescent tails (and Hoechst 33342 fluorescent heads) was determined by randomly assessing 200 spermatozoa. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at 400x magnification.

### **Quantification of sperm-oviduct binding and tail-associated protein tyrosine phosphorylation**

Density of sperm binding to oviduct APM after 2 h co-incubation was determined after two washing steps, in either non-capacitating or capacitating medium, by quantitative analysis of dot blot labeling. This analysis was performed by scanning the blots with a GS-700 densitometer equipped with a UV lamp (Bio-Rad Laboratories, Hercules, CA, USA) using Quantity One acquisition software (version 4.3, Biorad). Densitometric quantitation was carried out using the Gel-Pro Analyzer software (version 3.0; MediaCybernetics, Silver Spring, MD, USA).

Density of sperm binding to the oviduct explants after 2 h co-incubation was similarly determined in five microscopic fields (400x magnification), after two washing steps. The percentage of spermatozoa with tail-associated protein tyrosine phosphorylation (PY+ / Hoechst+) was determined by evaluating 200 randomly selected spermatozoa (Hoechst+). Both parameters were evaluated by means of fluorescence microscopy using a Leica DMR microscope equipped with excitation filters BP 340/380 nm, BP 450/490 nm, BP 560/40 nm and a 100 W mercury lamp. Alexa Fluor 488-conjugated goat anti-mouse antibody and Hoechst 33342 were sequentially excited using 499 nm and 345 nm wavelengths. Emission spectra of the dyes were then filtered at 519 nm and 478 nm. These emission spectra were detected by blue (LP 425 nm), green (LP 515 nm), and red (BP 645/75 nm) filters, corresponding to the emission peaks of the dyes. Images were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium). In addition, the surface area of the oviduct explants in each microscopic field (at 400x magnification) was measured using this program. The two fluorophores were checked for signal overlap, and no leakage of signals was detected.

Head-to-head sperm agglutination after 0.5 and 2 h in various medium conditions was determined by evaluating 200 randomly chosen, motile spermatozoa and observed at 400x magnification using the DIC and warm-stage (38.5 °C) equipped Leica DM 5500 B fluorescence microscope described above.

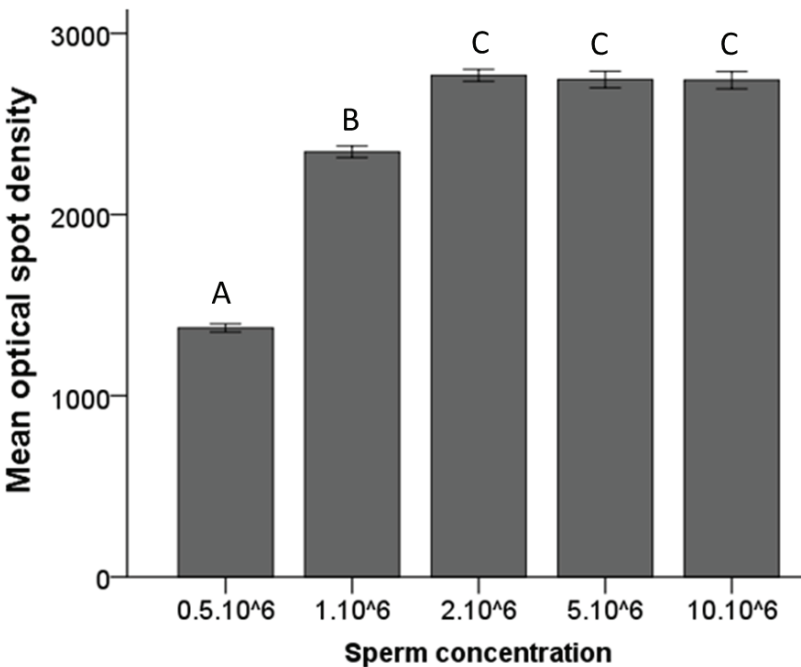
### Statistical analysis

Competitive carbohydrate / glycosaminoglycans / lectin / penicillamine / N-glycosidase F inhibition of sperm-oviduct binding, the effect of various glycosaminoglycans on protein tyrosine phosphorylation and the effect of various capacitating factors (individual and combined effects of  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  or albumin) on sperm-oviduct binding, release and head-to-head agglutination were assessed by analysis of variance (ANOVA). Significant differences in the number of oviduct-bound spermatozoa, or optical spot density, and the percentage of tyrosine phosphorylated (PY+) and head-to-head agglutinated spermatozoa over time were determined using repeated measures ANOVA with Greenhouse-Geisser and Bonferroni correction, as implemented in the general linear model. Scheffé post-hoc tests were performed for pair-wise comparisons. Statistical analysis and graph plotting was performed using SPSS version 20 for Windows (SPSS IBM, Brussels, Belgium). Differences were considered significant if  $P < 0.05$ .

## RESULTS

### Standardization of sperm-oviduct explant and sperm-oviduct APM assays

We previously showed that the saturation concentration for sperm binding to oviduct explants in non-capacitating conditions was reached at  $5 \times 10^6$  spermatozoa / ml [39]. To test the ability of carbohydrates to competitively inhibit sperm-oviduct explant binding, a sperm concentration of  $2 \times 10^6$  spermatozoa / ml was used. A similar experiment was performed for the sperm-oviduct APM assay, and sperm binding to oviduct APM was saturated at  $2 \times 10^6$  spermatozoa / ml. Therefore, a below saturation concentration of  $1 \times 10^6$  spermatozoa / ml was used to test competitive carbohydrate inhibition of sperm-oviduct binding (Figure 2). To test competitive lectin inhibition of sperm-oviduct explant binding, a concentration above the saturation level was used ( $5 \times 10^6$  spermatozoa / ml).



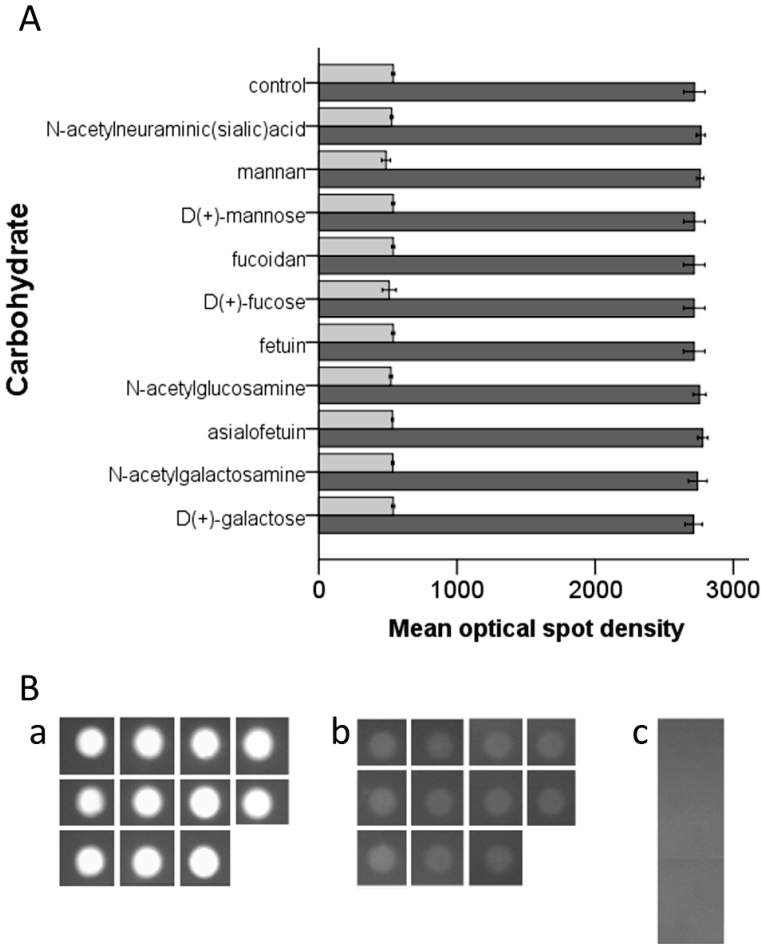
**Figure 2.** Effect of sperm concentration on binding density of stallion sperm to equine oviduct APM under non-capacitating conditions. Saturation of sperm-oviduct explant binding occurred at  $2 \times 10^6$  spermatozoa / ml. Data represent mean ( $\pm$  SD) number of spermatozoa bound to APM ( $n=30$  per group) in three replicates. Values that differ significantly are indicated by different capitals. One-way ANOVA, followed by Bonferroni post hoc tests for pairwise comparison.



To standardize the sperm-oviduct APM assay, the purity of the apical plasma membrane fractions was checked using well-established enzyme assays (see Smith and Nothnick [17] for full validation).  $\gamma$ -Glutamyl transpeptidase was used as a marker for the apical plasma membrane, and proved to be enriched  $21 \pm 4$  times in plasma membrane preparations ( $n = 3$ ).

### **Tested carbohydrates did not competitively reduce sperm-oviduct APM binding**

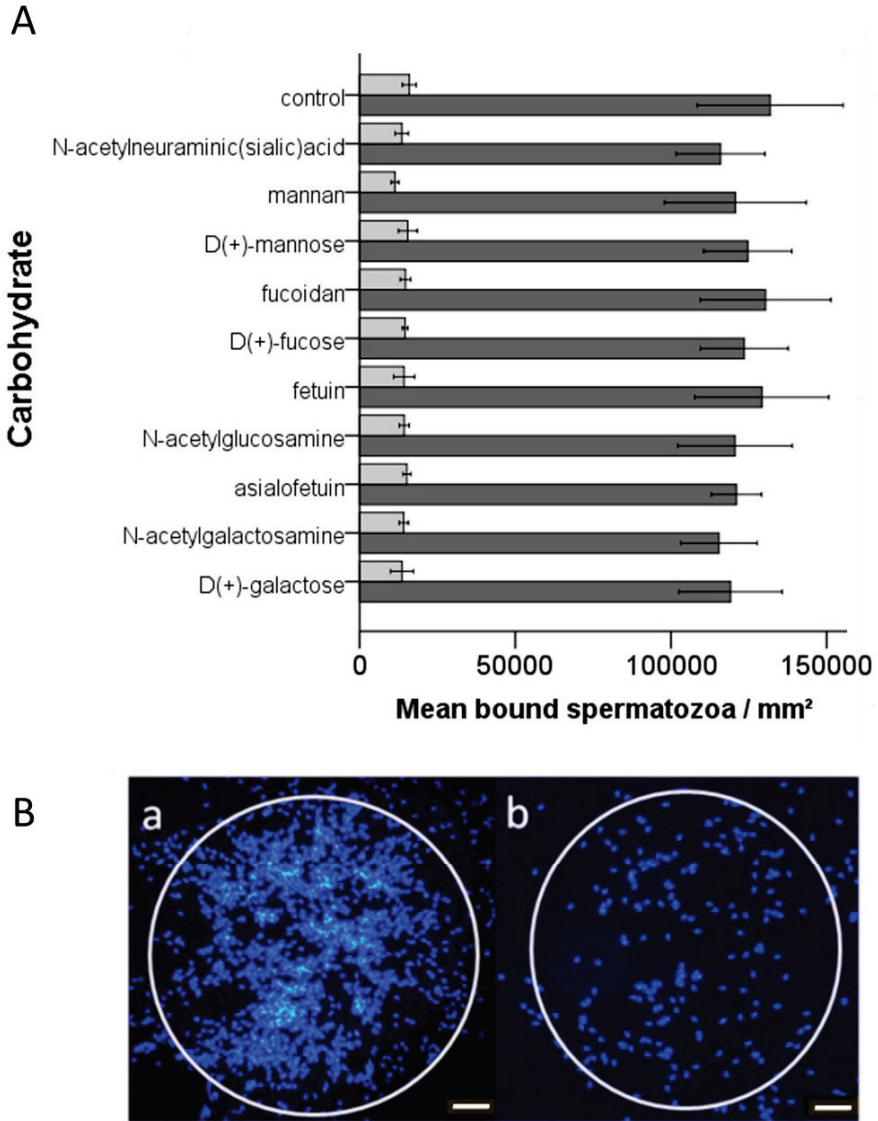
As demonstrated by Suarez [10, 25], species-specific carbohydrate moieties expressed on the epithelium facilitated binding of the head of the spermatozoa that ultimately populate the sperm reservoir. In order to assess the importance of carbohydrates in sperm-oviduct binding in equids, we used our oviduct derived APM to test competitive carbohydrate inhibition of sperm binding. Sperm binding to nitrocellulose coated with oviductal APM was assessed after pre-incubating spermatozoa with carbohydrates (D-galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, asialofetuin, fetuin, D-fucose, fucoidan, *D*-mannose, mannan, *N*-acetylneuraminic (sialic) acid). None of the tested carbohydrates reduced the sperm-oviduct APM binding density in either non-capacitating ( $P > 0.95$  for all comparisons) or capacitating conditions ( $P > 0.22$  for all comparisons). Similar to our previous study [39], sperm-oviduct binding density was significantly higher in non-capacitating than capacitating conditions ( $P < 0.001$  for all comparisons; Figures 3A and 3B).



**Figure 3.** (A) Effect of different carbohydrates (D-galactose, N-acetylgalactosamine, N-acetylglucosamine, asialofetuin, fetuin, D-fucose, fucoidan, D-mannose, mannan and N-acetylneuraminic (sialic acid) on equine sperm-oviduct APM binding density under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions. Data represent mean ( $\pm$  SD) optical spot density ( $n=10$  per group) over three replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparisons. (B) Fluorescent spots represent Hoechst stained spermatozoa bound to oviduct APM coated on nitrocellulose after pre-incubation with the test carbohydrate followed by 2 h co-incubation in (a) non-capacitating and (b) capacitating medium. Each spot represents one tested carbohydrate (row 1 to 3 from left to right: D-galactose, N-acetylgalactosamine, asialofetuin, N-acetylglucosamine, fetuin, D-fucose, fucoidan, D-mannose, mannan, N-acetylneuraminic (sialic acid) and controls (row 3). (c) Nitrocellulose paper without oviduct APM was included as an additional control and the optical density of the paper subtracted from the optical spot density of the other spots (a,b,c: original magnification).

### **Tested carbohydrates did not reduce sperm-oviduct explant binding**

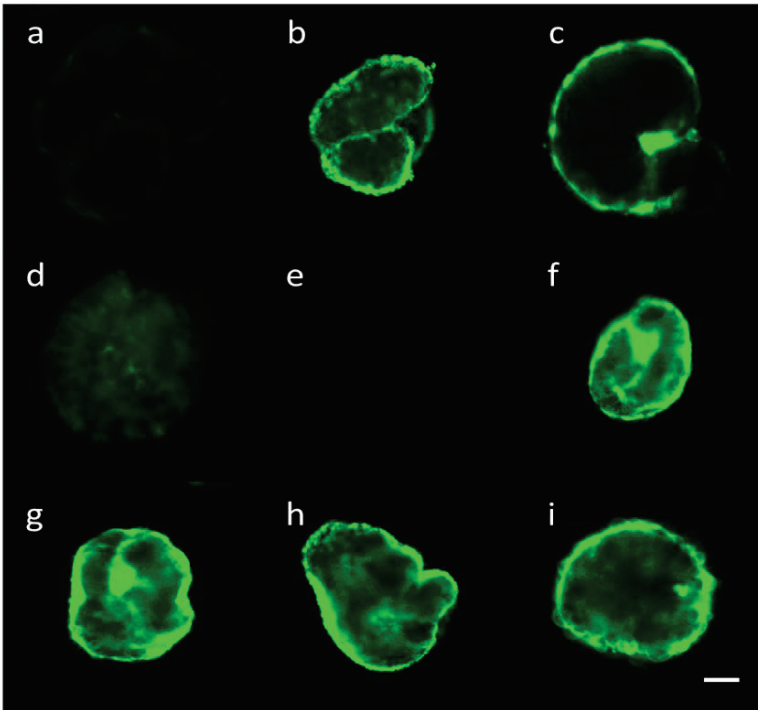
To validate the results obtained for the oviduct-derived APM model (described above), the same experiments were carried out using oviduct explants. In accordance with the oviduct APM experiments, none of the tested carbohydrates reduced the density of sperm binding to oviduct explants in either non-capacitating ( $P>0.96$  for all comparisons) or capacitating conditions ( $P>0.59$  for all comparisons). As for APM, sperm-oviduct binding density was significantly higher in non-capacitating than capacitating conditions ( $P<0.001$  for all comparisons; Figures 4A and 4B).



**Figure 4.** (A) Effect of different carbohydrates (D-galactose, N-acetylgalactosamine, asialofetuin, N-acetylglucosamine, fetuin, D-fucose, fucoidan, D-mannose, mannan, and N-acetylneuraminic (sialic) acid) on equine sperm-oviduct explant binding density under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over five replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison. (B) Fluorescence micrographs represent Hoechst stained spermatozoa bound to oviduct explants after 2 h co-incubation in (a) non-capacitating and (b) capacitating medium (b, c: original magnification, 400x, scale bar = 25  $\mu$ m).

### N-acetylgalactosamine and D-mannose / D-glucose moieties are expressed on equine oviduct explant epithelium

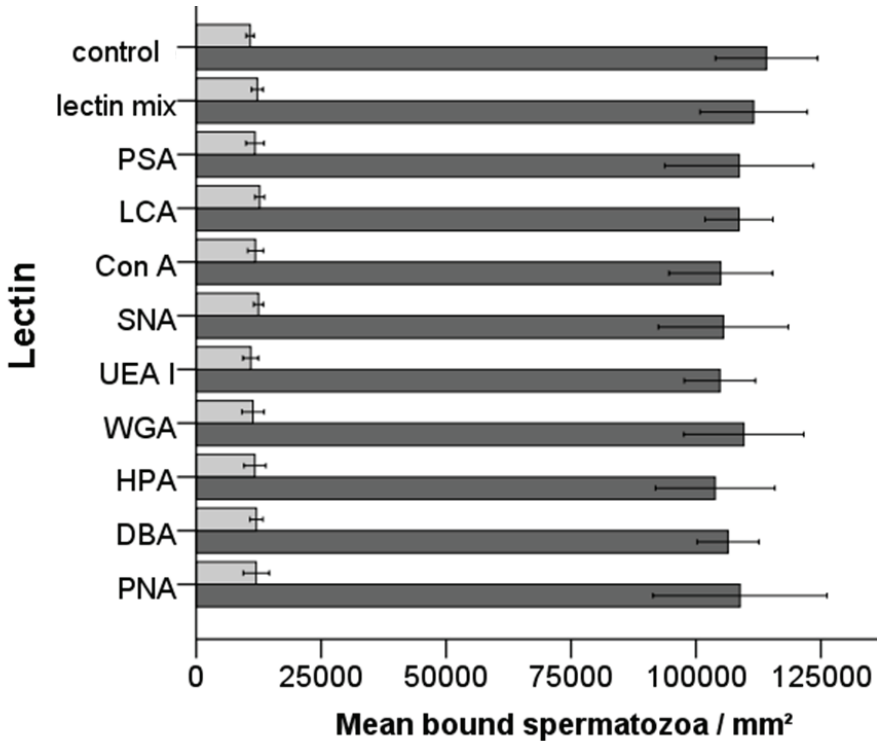
Lectin histochemistry of oviductal epithelium was used to demonstrate that *N*-acetylgalactosamine (DBA and HPA +++), *N*-acetylneuraminic (sialic) acid (SNA +++), and D-mannose / D-glucose (Con A, LCA and PSA +++), were the pre-dominant carbohydrate moieties expressed on equine oviduct epithelium. WGA-FITC (+), a lectin that binds to *N*-acetylglucosamine moieties and to a lesser extent *N*-acetyl neuraminic (sialic) acid, displayed a weaker expression pattern probably because of expression of the latter carbohydrate. D-galactose (PNA) and L-fucose moieties were not detected on equine oviduct epithelium (UEA I) (Figure 5).



**Figure 5:** Fluorescence micrographs of carbohydrate expression patterns on equine oviduct explant epithelia. Carbohydrate expression was assessed using various lectin-FITC conjugates; (a) PNA +/-, (b) DBA +++, (c) HPA +++, (d) WGA +, (e) UEA I -, (f) SNA +++, (g) Con A +++, (h) LCA +++, and (i) PSA +++. (a, b, c, d, e, f, g, h, i: original magnification, 400x, scale bar = 25  $\mu$ m).

**Tested lectins did not reduce sperm-oviduct explant binding**

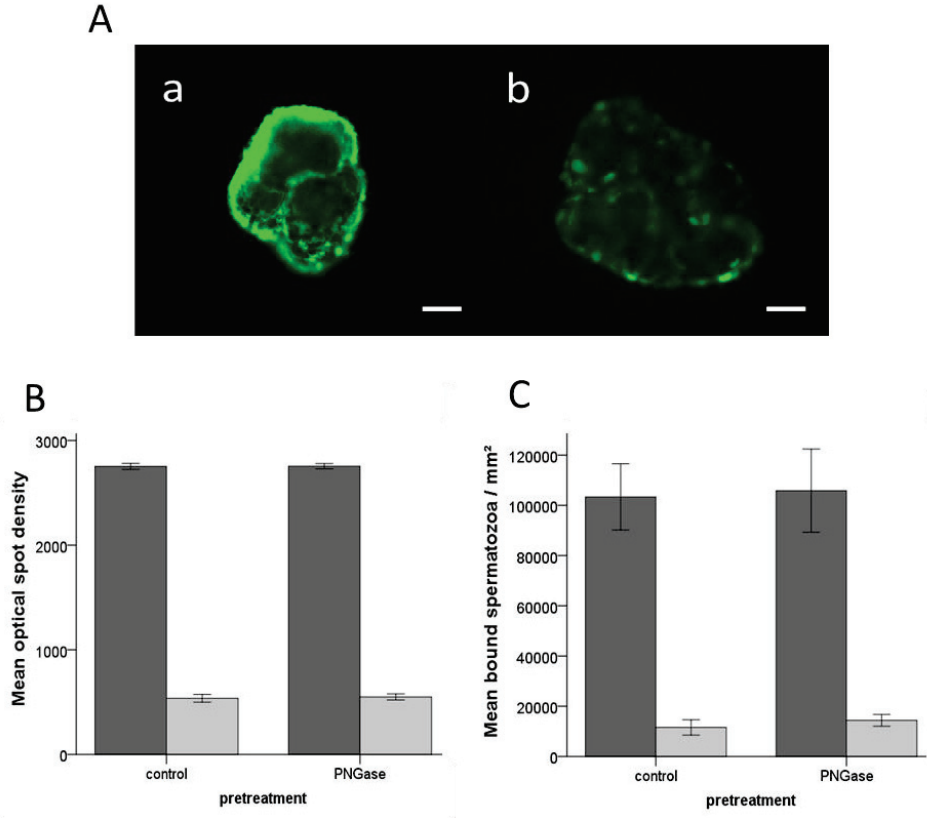
Various lectins were also tested in the sperm-oviduct assays to alternatively examine the role of oviduct carbohydrate moieties in sperm-oviduct binding. Sperm binding to oviduct explants was examined after pre-incubating oviduct explants with individual or a mix of lectins (PNA, DBA, HPA, WGA, UEA I, SNA, Con A, LCA and PSA), and compared to control explants not exposed to lectins. Similar to the carbohydrate studies, none of the lectins reduced the sperm-binding density to oviduct explants in either non-capacitating ( $P>0.99$  for all comparisons) or capacitating conditions ( $P>0.96$  for all comparisons; Figure 6); again, sperm-oviduct binding density was significantly higher in non-capacitating compared to capacitating conditions ( $P<0.001$  for all comparisons).



**Figure 6:** Effect of an individual or the mix of different lectins (PNA, DBA, HPA, WGA, UEA I, Con A, LCA, PSA, SNA) on sperm-oviduct explant binding density under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over three replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.

### **N-linked carbohydrates do not regulate sperm-oviduct binding**

*N*-linked glycosylation is prevalent in proteins destined for extracellular locations including proteins expressed on the extracellular side of the plasma membrane, secreted proteins and proteins present in body fluids [45, 46]. The possible involvement of *N*-glycosylated moieties in sperm oviduct binding was tested by pretreating either oviduct APM or oviduct explants for 24 hours with the enzyme *N*-glycosidase F (PNGase), which removes *N*-linked glycosylated moieties from glycoproteins. Indeed, the enzyme treatment effectively removed Con A binding sites, given that Con A-FITC staining was markedly reduced after *N*-glycosidase F treatment (Figure 7A). However, the *N*-glycosidase F pre-treatment of oviduct epithelium did not significantly affect sperm-oviduct binding densities in either the oviduct APM (Figure 7B) or oviduct explant systems (Figure 7C), and in either non-capacitating (APM assay:  $P > 0.75$  for all comparisons; oviduct explant assay:  $P > 0.09$  for all comparisons) or capacitating conditions (APM assay:  $P > 0.95$  for all comparisons; oviduct explant assay:  $P > 0.17$  for all comparisons).



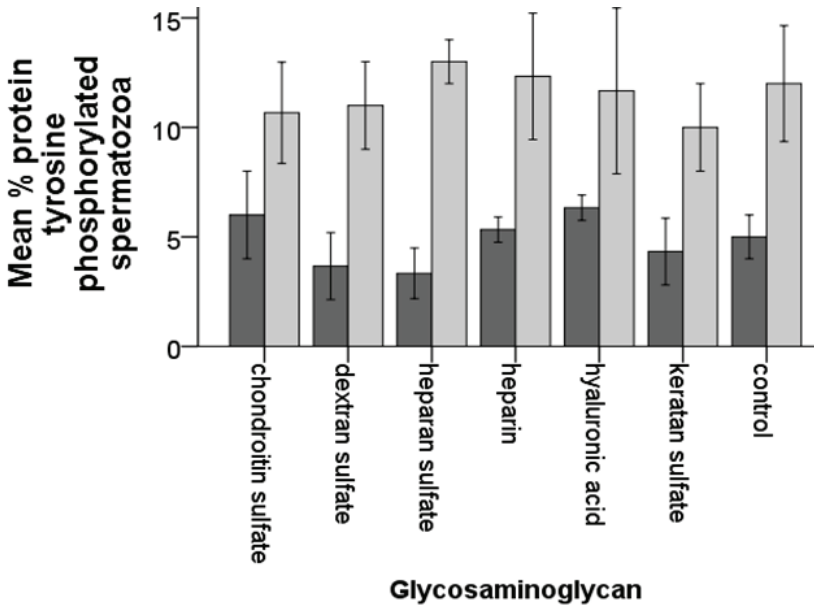
**Figure 7:** (A) Fluorescence micrographs of Con A-FITC stained equine oviduct explants (a) before and (b) after PNGase treatment (a, b: original magnification, 400x, Bar = 25  $\mu$ m). The effect of PNGase pretreatment on (B) sperm-oviduct APM and (C) sperm-oviduct explant binding density under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions was also assessed. Data represent mean optical spot densities (n=3 spots per group) and mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants (n=10 per group) over three replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.

***In vitro* capacitation induces protein tyrosine phosphorylation in equine sperm tails whereas glycosaminoglycans have no effect**

In cattle, heparin-like sulfated glycosaminoglycans, normally present in follicular and oviductal fluid, have been proposed to be potential *in vivo* capacitation agents [36, 47, 48]. Heparin also initiated sperm release from the bovine oviduct epithelium [13], associated with



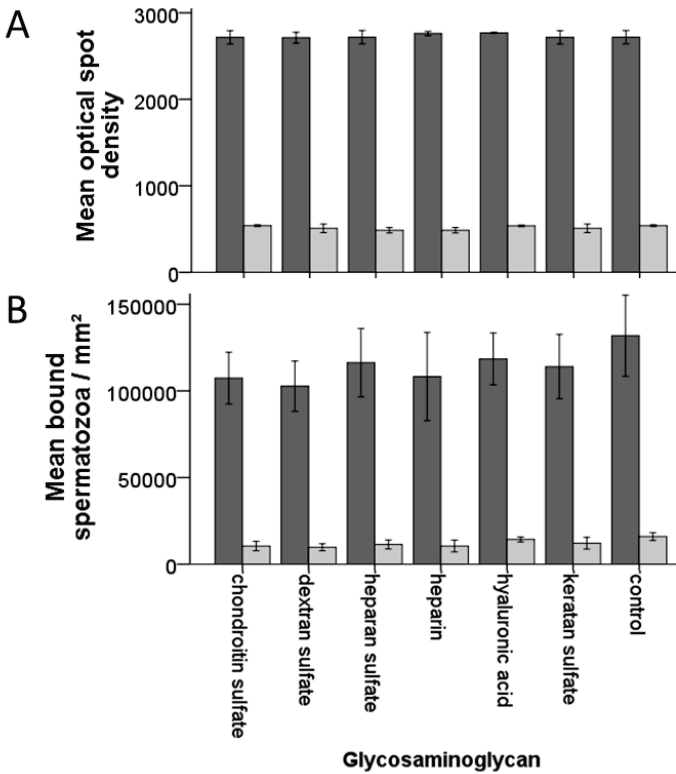
protein tyrosine phosphorylation [49]. Interestingly, in the mare high concentrations of sulfated glycosaminoglycans have been reported in follicular and oviductal fluid [32]. Therefore, the *in vitro* effect of added (sulfated) glycosaminoglycans on protein tyrosine phosphorylation (as a marker for sperm capacitation) was monitored. None of the tested glycosaminoglycans had any stimulatory or inhibitory effect on tail-associated protein tyrosine phosphorylation of spermatozoa in suspension after 6 h incubation in non-capacitating conditions ( $8 \pm 4\%$ ;  $P > 0.09$  for all comparison; Figure 8). Moreover, the increase in tyrosine phosphorylated sperm ( $P < 0.001$  for all comparisons) induced by capacitating conditions ( $15 \pm 7\%$ ; Figure 8) was not affected by addition of glycosaminoglycans ( $P > 0.79$  for all comparisons). These results indicate that glycosaminoglycans do not induce equine sperm capacitation.



**Figure 8:** Effect of different glycosaminoglycans on protein tyrosine phosphorylation in the tail of stallion sperm incubated in non-capacitating and capacitating media for 6 h. No effect of added glycosaminoglycans on protein tyrosine phosphorylation was observed in either condition. Data represent mean ( $\pm$  SD) percentages of protein tyrosine phosphorylated spermatozoa in non-capacitating (light grey bars) and capacitating media (dark grey bars) ( $n=200$  spermatozoa in each group; three replicates). Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.

### **Glycosaminoglycans do not competitively inhibit sperm-oviduct explant binding**

In cattle, after contact with various capacitation inducers (e.g. glycosaminoglycans and  $\text{HCO}_3^-$ ) the affinity of non-capacitated spermatozoa for carbohydrate moieties on the oviduct epithelium decreases dramatically, while the affinity for zona pellucida glycoproteins rises [11]. Initially, competition for oviduct carbohydrate binding receptors plays a role in sperm release from oviduct epithelium, while capacitation induces membrane changes that further modify the affinity of sperm receptors. We assessed the ability of spermatozoa to bind to oviduct APM or oviduct explants during a 2 h co-incubation after pretreatment with various sulfated glycosaminoglycans (chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate). None of the tested glycosaminoglycans decreased the sperm-oviduct binding density in either non-capacitating (APM assay:  $P>0.93$  for all comparisons; Figure 9A. Oviduct explant assay:  $P>0.20$  for all comparisons; Figure 9B) or capacitating conditions (APM assay:  $P>0.49$  for all comparisons; Figure 9A. Oviduct explant assay:  $P>0.09$  for all comparisons; Figure 9B). Similar to other experiments, only capacitating conditions decreased the density of sperm-oviduct epithelium binding (APM and oviduct explant assays:  $P<0.001$  for all comparisons).

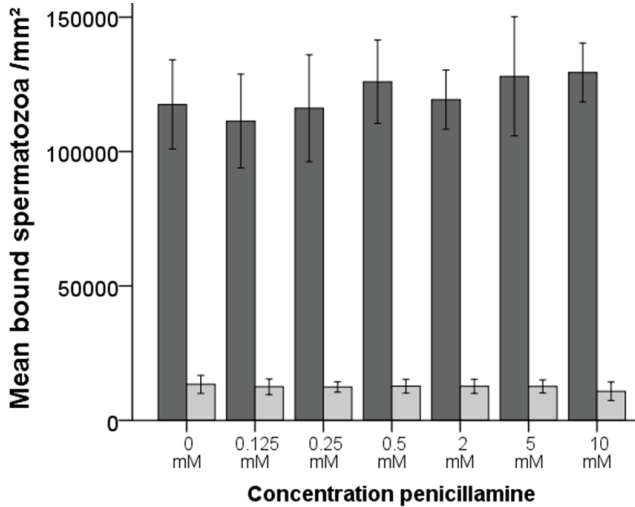


**Figure 9:** The effect of different glycosaminoglycans (chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate) on (A) sperm-oviduct APM and (B) sperm-oviduct explant binding density under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions was assessed. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over five replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.

### Penicillamine does not reduce sperm-oviduct explant binding density

In cattle, disulphide-reductants like penicillamine markedly reduce the affinity of spermatozoa for carbohydrate moieties on the oviduct epithelium [26]. Moreover, release of bull spermatozoa from oviduct epithelium following exposure to penicillamine was provoked by redox control of sperm surface protein sulfhydryls ( $S-S \leftrightarrow 2SH$ ). We assessed the ability of spermatozoa to bind to oviduct explants after pretreatment with various concentrations of penicillamine (0, 0.125, 0.25, 0.5, 1, 5 and 10 mM), none of which reduced oviduct membrane binding density in either non-capacitating ( $P>0.93$  for all comparisons) or

capacitating ( $P>0.81$  for all comparisons) conditions. Similar to other experiments, only capacitating conditions decrease the density of sperm-oviduct epithelium binding ( $P<0.001$  for all comparisons; Figure 10).

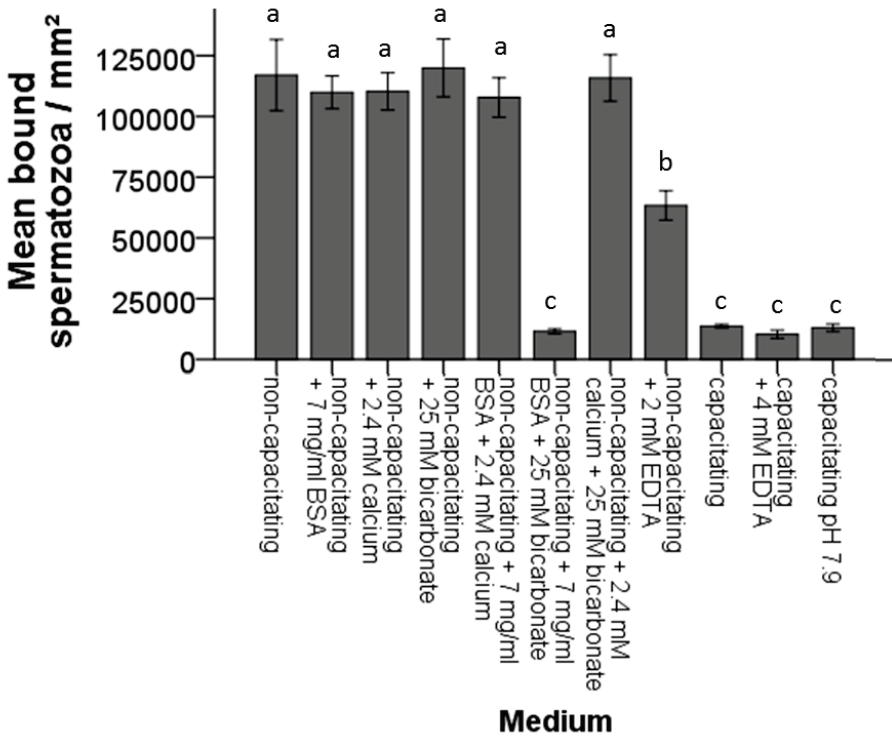


**Figure 10:** The effect of different concentrations of penicillamine (0, 0.125, 0.25, 0.5, 1, 5 and 10 mM) on initial density of equine sperm binding to oviduct explants under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions. No effect of penicillamine on sperm-oviduct binding was evident in either condition. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over five replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.

### **Sperm-oviduct binding is not regulated by $\text{Ca}^{2+}$ , but a combination of albumin and $\text{HCO}_3^-$ reduces sperm-oviduct explant binding density**

As shown in previous experiments, capacitating conditions supported sperm-oviduct binding at a significantly lower level than non-capacitating conditions. In cattle,  $\text{Ca}^{2+}$  - dependent lectin binding has been shown to initiate sperm-oviduct binding whereas bicarbonate decreased sperm-oviduct affinity [11]. We therefore tested the effect of preincubation for 20 min with each of the three major factors required for capacitation *in vitro* (albumin and / or  $\text{Ca}^{2+}$  and / or  $\text{HCO}_3^-$ ), individually or in combinations, on the ability of spermatozoa to bind to oviduct explants during a subsequent 2 h co-incubation. The results indicated that binding of stallion spermatozoa to oviduct explant epithelium was independent of  $\text{Ca}^{2+}$  and alkalinity (pH 7.9). However, sperm pre-incubated in  $\text{HCO}_3^-$  and albumin

enriched media showed a significantly decreased binding affinity for the oviduct in all groups ( $P < 0.001$ ; Figure 11) while individual exposure to either  $\text{HCO}_3^-$  or albumin had no effect. Sperm-oviduct binding density was also significantly lower when 2 mM EDTA was added to non-capacitating medium ( $P < 0.001$ ; Figure 11). Despite the latter finding, we conclude that equine sperm-oviduct binding is  $\text{Ca}^{2+}$ -independent because levels of sperm-oviduct binding were still very high in  $\text{Ca}^{2+}$  free conditions. Moreover, removal of  $\text{Ca}^{2+}$  by 4 mM EDTA in capacitating conditions did not significantly lower sperm-oviduct binding compared to the same conditions without EDTA ( $P = 0.98$ ; Figure 11). These results suggest that sperm binding to oviduct epithelium is not initiated by a  $\text{Ca}^{2+}$ -dependent lectin interaction, whereas combined exposure to  $\text{HCO}_3^-$  and albumin significantly reduced the affinity of spermatozoa for oviduct binding.

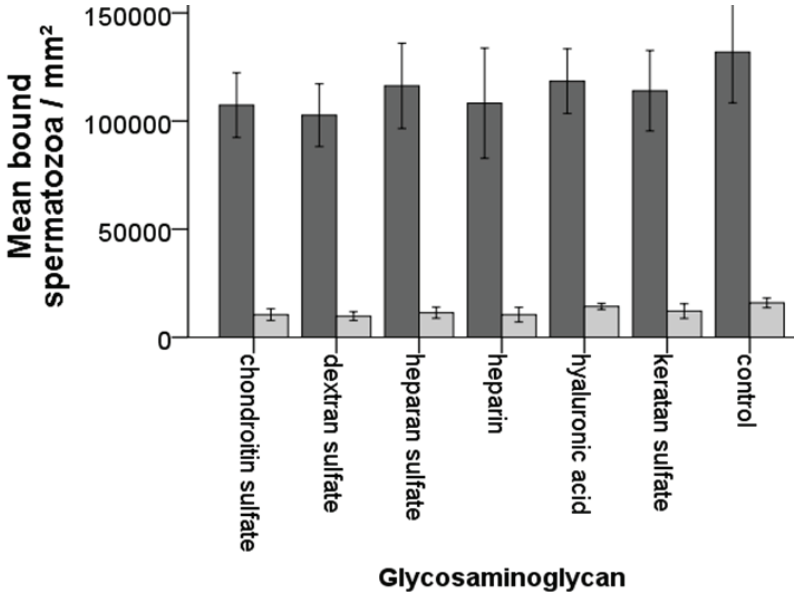


**Figure 11.** Individual and combined effects of albumin,  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and EDTA on initial density of equine sperm-oviduct. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) per  $\text{mm}^2$  over three replicates.  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and albumin did not affect sperm-oviduct binding density individually, whereas the combination of these 3 capacitating factors supported sperm-oviduct binding at a significantly lower density. Additionally, sperm-oviduct binding transpired not to be  $\text{Ca}^{2+}$  dependent. Values that differ significantly are indicated by different small letters. One-way ANOVA was followed by Bonferroni post hoc tests for pairwise comparison.

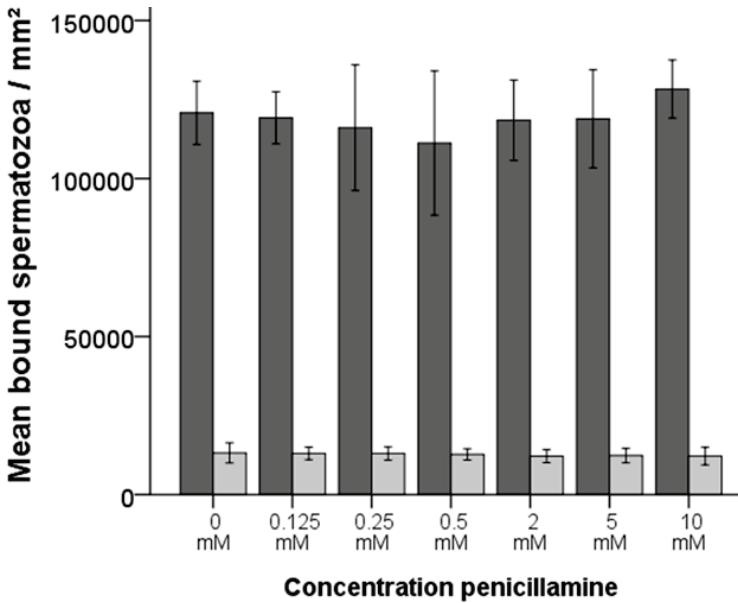
**None of glycosaminoglycans, penicillamine,  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$ , albumin or alkalinity (pH 7.9) induce release of oviduct bound spermatozoa.**

It has previously been shown that heparin [13] and penicillamine [26] initiate sperm release from the bovine oviduct epithelium. Washed sperm-oviduct explants, established in non-capacitating conditions, were incubated for 2 h with glycosaminoglycans, penicillamine and / or the three major capacitation agents (BSA,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ ) added individually or in combinations. Interestingly, while the density of sperm-oviduct binding clearly decreased in the combined presence of  $\text{HCO}_3^-$  and albumin, sperm release from oviduct explants could not be induced using glycosaminoglycans (Figure 12), penicillamine (Figure 13), albumin,  $\text{HCO}_3^-$

alone or  $\text{Ca}^{2+}$  (Figure 14). Moreover, neither capacitating conditions at pH 7.4 or at an elevated pH 7.9 were able to influence the density of spermatozoa binding to oviduct explant epithelium (Figure 14).

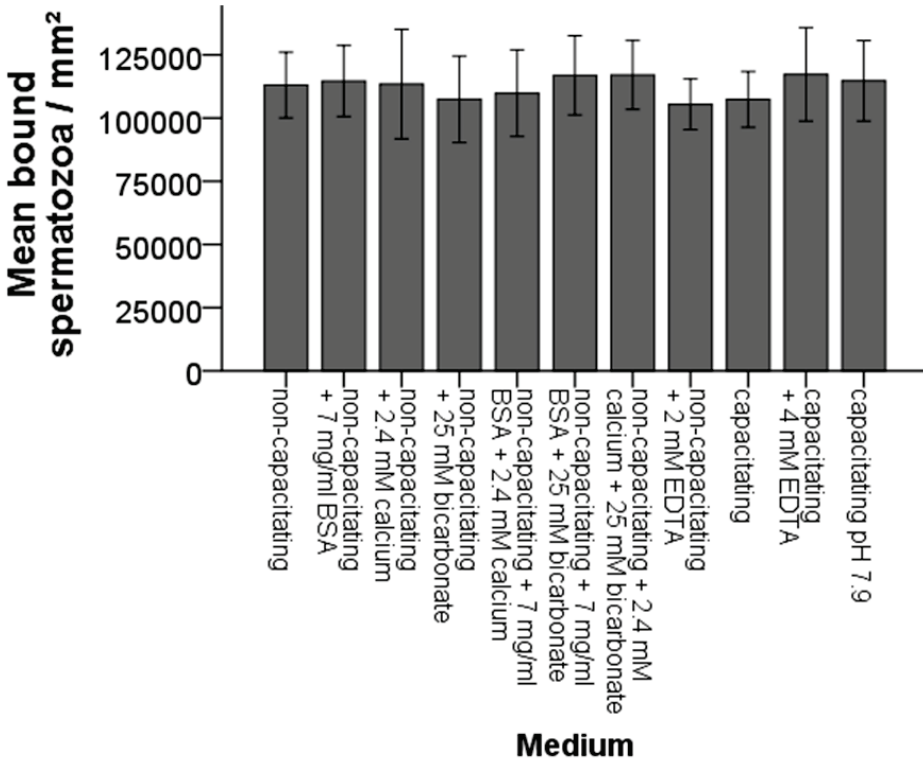


**Figure 12:** The effect of different glycosaminoglycans (chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate) on sperm release from oviduct explants under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions was assessed. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over five replicates. None of these glycosaminoglycans induced release of spermatozoa from the oviduct explants. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.



**Figure 13:** The effect of different concentrations of penicillamine (0, 0.125, 0.25, 0.5, 1, 5 and 10 mM) on density of sperm binding to oviduct explants after attempts to trigger release under non-capacitating (dark grey bars) and capacitating (light grey bars) conditions. No effect of penicillamine on sperm-oviduct release was evident in either condition. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over five replicates. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison.

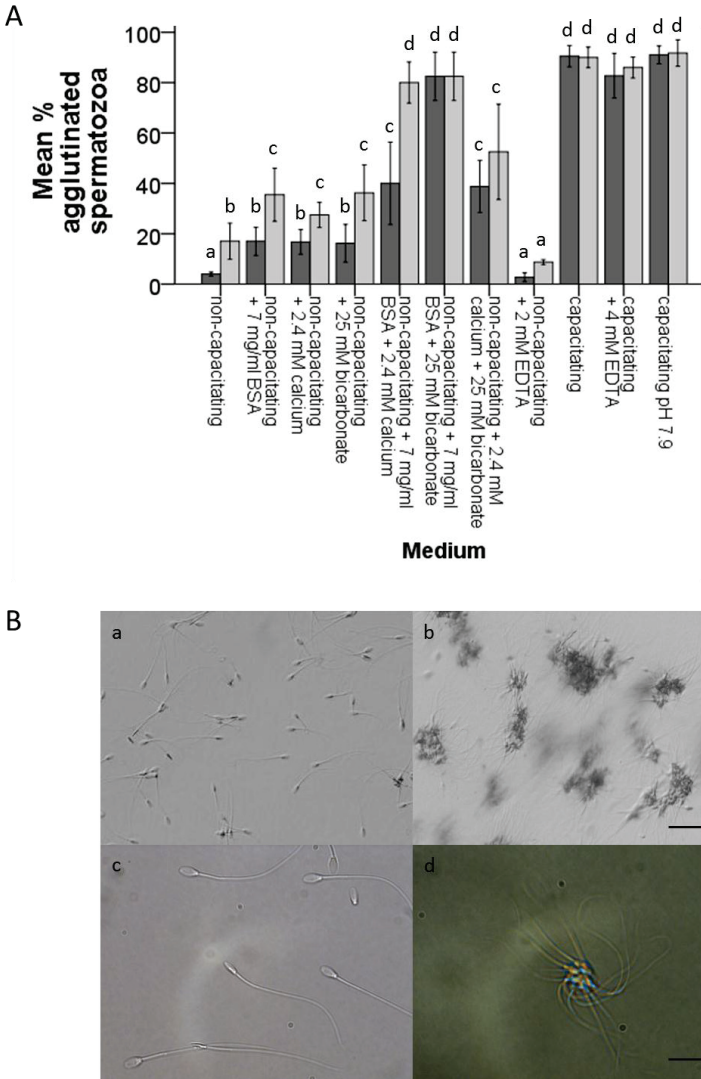




**Figure 14.** Individual and combined effects of albumin,  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and EDTA on binding density after incubation to trigger sperm release. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) per  $\text{mm}^2$  over three replicates. None of the tested conditions caused marked release of spermatozoa from the oviduct explants. One-way ANOVA was followed by Bonferroni post hoc tests for pairwise comparison.

**Exposure of stallion sperm to  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and albumin in combination induces head-to-head agglutination independent of external  $\text{Ca}^{2+}$** 

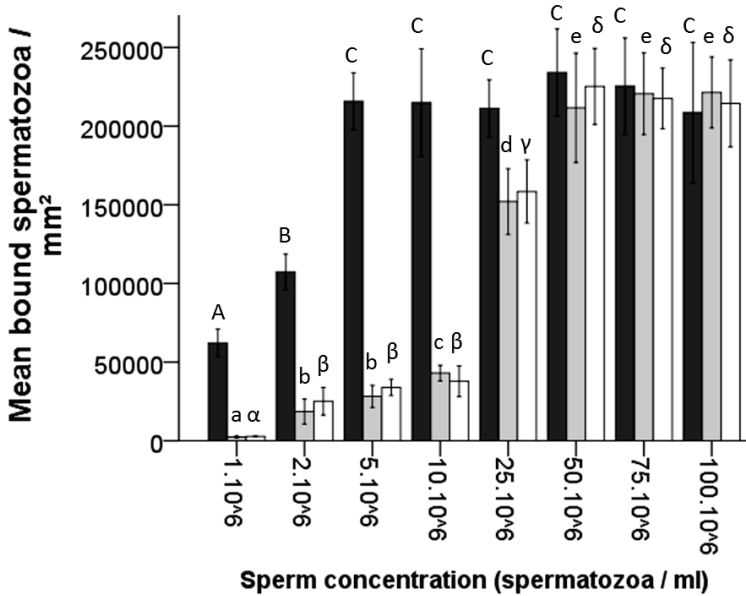
As previously shown for bull [50] and boar [51, 52] spermatozoa, contact with  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and albumin induced rapid head-to-head agglutination in suspended sperm. For this reason, we hypothesized that reduced sperm-oviduct binding in combined  $\text{HCO}_3^-$  and albumin conditions was a product of rapid head-to-head agglutination of stallion sperm. Therefore, we examined sperm agglutination in the presence of the three capacitation agents (BSA,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ ) added individually or in combinations. In comparison to non-capacitating medium ( $4 \pm 1\%$ ), we observed a small but significant increase after 0.5 h incubation in non-capacitating medium enriched with albumin ( $17 \pm 6\%$ ;  $P=0.04$ ),  $\text{Ca}^{2+}$  ( $17 \pm 5\%$ ;  $P=0.04$ ) or  $\text{HCO}_3^-$  ( $16 \pm 8\%$ ;  $P=0.03$ ). Agglutination was even more pronounced when capacitating factors were combined (albumin +  $\text{Ca}^{2+}$ :  $40 \pm 16\%$ ;  $P=0.001$  and  $\text{Ca}^{2+}$  +  $\text{HCO}_3^-$ :  $39 \pm 10\%$ ;  $P=0.002$ ). Surprisingly, incubation in combined albumin +  $\text{HCO}_3^-$  ( $83 \pm 10\%$ ) showed similar agglutination rates to those observed when all three capacitating factors were combined (capacitating medium;  $90 \pm 4\%$ ;  $P=0.99$ ). Moreover, sperm incubated in capacitating medium (albumin +  $\text{Ca}^{2+}$  +  $\text{HCO}_3^-$ ) at elevated pH 7.9 showed a similar agglutination rate ( $91 \pm 3\%$ ;  $P=0.99$ ) (Figure 15A and Figure 15B). After 2 h incubation, similar trends were observed though agglutination rates were generally higher than at 0.5 h (non-capacitating,  $17 \pm 7\%$ ; albumin,  $36 \pm 11\%$ ;  $\text{Ca}^{2+}$ ,  $28 \pm 5\%$ ;  $\text{HCO}_3^-$  enriched medium  $36 \pm 11\%$ , and combined albumin +  $\text{Ca}^{2+}$  medium  $80 \pm 8\%$ ;  $P<0.01$  for all comparisons) with the exceptions of (1) albumin +  $\text{HCO}_3^-$  ( $83 \pm 10\%$ ;  $P=0.98$ ) and (2)  $\text{Ca}^{2+}$  +  $\text{HCO}_3^-$  ( $39 \pm 10\%$ ;  $P=0.24$ ), and capacitating medium at both (3) pH 7.4 ( $39 \pm 10\%$ ;  $P=0.87$ ) and (4) 7.9 ( $39 \pm 10\%$ ;  $P=0.82$ ) (Figure 15A). Interestingly, stallion sperm agglutination in our studies was  $\text{Ca}^{2+}$  independent, and there was no effect on agglutination rates of adding EDTA to non-capacitating (at 0.5 h:  $3 \pm 2\%$ ,  $P=0.99$  and at 2 h:  $8 \pm 1\%$ ,  $P=0.96$ ) or capacitating medium (at 0.5 h:  $83 \pm 9\%$ ,  $P=0.97$  and at 2 h:  $86 \pm 4\%$ ,  $P=0.98$ ) (Figure 15A). In summary, combined  $\text{HCO}_3^-$  and albumin supplementation facilitated rapid sperm agglutination which prevented sperm-oviduct binding.



**Figure 15.** (A) Individual and combined effects of albumin, Ca<sup>2+</sup>, HCO<sub>3</sub><sup>-</sup> and EDTA on head-to-head stallion sperm agglutination after 0.5 h (dark grey) and 2 h (light grey) incubation. Data represent mean (± SD) percentages of agglutinated spermatozoa (n=200 spermatozoa per group; three replicates). In general, the combination of Ca<sup>2+</sup>, HCO<sub>3</sub><sup>-</sup> and albumin induced very high rates of sperm agglutination whereas the effects of individual capacitating factors was much lower. Moreover, sperm agglutination was shown to be Ca<sup>2+</sup> independent. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparison. (B) Light microscope images illustrate that pre-incubated stallion sperm (2 x 10<sup>6</sup> spermatozoa / ml) did not agglutinate in (a,c) medium without capacitating factors (non-capacitating medium) whereas in medium containing albumin, Ca<sup>2+</sup> and HCO<sub>3</sub><sup>-</sup> (capacitating conditions: b,d) sperm rapidly agglutinated (a, b: original magnification, 400x, Bar = 62.5 µm; c, d: original magnification, 1000x, Bar = 25 µm).

### **Role of combined $\text{HCO}_3^-$ and albumin in sperm concentration dependent binding to equine oviduct explants**

To assess the effects of combined  $\text{HCO}_3^-$  and albumin or capacitating medium ( $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and albumin) on sperm binding to oviduct explants, we additionally evaluated the effect of increasing sperm concentrations (1, 2, 5, 10, 25, 50, 75 and  $100 \times 10^6$  spermatozoa / ml) on binding to oviduct explants in non-capacitating, non-capacitating enriched with albumin and  $\text{HCO}_3^-$ , and capacitating medium. Saturation for sperm binding after 20 min pre-incubation in both combined albumin /  $\text{HCO}_3^-$  enriched non-capacitating and capacitating medium was reached at  $50 \times 10^6$  spermatozoa / ml (combined albumin /  $\text{HCO}_3^-$  enriched non-capacitating medium;  $2.1 \pm 0.3 \times 10^5$  spermatozoa /  $\text{mm}^2$ : capacitating medium;  $2.3 \pm 0.2 \times 10^5$  spermatozoa /  $\text{mm}^2$ : Figure 16) whereas in non-capacitating medium a similar saturation was already reached at  $5 \times 10^6$  spermatozoa / ml ( $2.2 \pm 0.2 \times 10^5$  spermatozoa /  $\text{mm}^2$ : Figure 16). These results suggest that media enriched with both albumin and  $\text{HCO}_3^-$  contain insufficient albumin and / or  $\text{HCO}_3^-$  to reduce the affinity of sperm for oviduct epithelium in suspensions with a sperm concentration of  $50 \times 10^6$  / ml or higher. Above this sperm concentration, sperm agglutination is presumably saturated and remaining sperm are free to bind to oviduct epithelium.



**Figure 16.** Effect of sperm concentration on density of stallion sperm binding to equine oviduct explants under (1) non-capacitating (without albumin; dark grey bars), (2) capacitating (light grey bars) and (3)  $\text{HCO}_3^-$  / albumin enriched non-capacitating conditions (white bars). Saturation of sperm-oviduct explant binding was detected in non-capacitating conditions at  $5 \times 10^6$  spermatozoa / ml whereas in capacitating and  $\text{HCO}_3^-$  / albumin enriched non-capacitating conditions saturation was not evident until  $50 \times 10^6$  spermatozoa / ml. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n = 20$  / group) in three replicates. For non-capacitating conditions, values that differ significantly are indicated by different capital letters. For capacitating conditions, values that differ significantly are indicated by different small letters. For non-capacitating conditions enriched with  $\text{HCO}_3^-$  and albumin, values that differ significantly are indicated by different greek symbols. ANOVA with Greenhouse-Heisser correction, followed by post hoc tests after Bonferroni correction.

## DISCUSSION

In this study, we found that binding of stallion spermatozoa to oviduct epithelium is not influenced by a range of carbohydrates, glycosaminoglycans, S-S reductants, lectins,  $\text{Ca}^{2+}$  or alkalinity (pH 7.9). On the other hand, we found that the density of stallion sperm binding to the oviduct decreased when the spermatozoa had previously been exposed to  $\text{HCO}_3^-$  and albumin combined. These results suggest that, in contrast to many other mammalian species, equine sperm-oviduct binding is not regulated by  $\text{Ca}^{2+}$ -dependent lectin or disulphide binding. The presence of  $\text{HCO}_3^-$  and albumin altered the capacity of stallion spermatozoa to bind to

oviduct epithelium primarily by rapid stimulation of head-to-head sperm agglutination, whereas sperm release from oviduct binding could not be provoked under the various tested capacitation inducing conditions.

Lectin histochemistry of the different regions of the mare's oviduct revealed that oligosaccharides with terminal *N*-acetylgalactosamine moieties were the most abundant carbohydrates in the isthmus [30, 31, 53]. The expression of *N*-acetylneuraminic (sialic) acid moieties on the luminal surface was lower. Desantis *et al.* [30, 31] demonstrated that these lectin-labeling patterns were very similar in both the isthmus and the ampulla, indicating that carbohydrate moieties are fairly uniformly distributed throughout the complete oviduct where they act as potential receptors for sperm binding. Moreover, in cattle studies it has been shown that the sperm binding capacity of the isthmic and ampullary epithelium is almost identical [16, 54]. For these reasons, we hypothesized that sperm-oviduct binding is not determined by oviduct location. We subsequently used equine oviduct epithelial explants and APM harvested from the ampullary-isthmic junction as an *in vitro* model for the sperm oviduct reservoir as it is not practically feasible to collect epithelial cells exclusively from the tiny and very tortuous isthmus.

Oviduct monolayers have been used extensively in earlier *in vitro* studies of sperm-oviduct binding [27, 55-57]. However, because morphological and ultrastructural features, and consequently the membrane molecular expression patterns are much better preserved, we preferred oviduct explants [33]. With regard to the quantification of sperm-oviduct binding, we were aware that standardization was a challenge when using equine oviduct explants because of the invaginated and irregular oviductal surface, which is very different from the flattened surface of an oviduct monolayer [56, 58]. To improve accuracy in the competitive sperm-oviduct binding assay, we developed a more standardized oviduct APM assay to quantify the binding densities of pre-treated sperm. The oviduct apical membranes still displayed the salient surface organelles (e.g. cilia, microvilli) of the oviduct plasma membrane while a more flattened surface was achieved. However, this model required many oviducts whereas the supply was limited. Therefore, only competitive carbohydrate / glycosaminoglycan inhibition of sperm-oviduct binding and the effect of N-glycosidase activity were tested using this APM assay. Nevertheless, concordance between the two assays

was high, and the results of the oviduct explant assay can be considered as a validation of the APM assay.

Carbohydrate moiety expression on oviduct explants was unchanged after overnight culture. In accordance with Desantis *et al.* [30, 31] who examined oviduct tissue sections, *N*-acetylgalactosamine moieties were highly expressed on the surface of oviduct explants (DBA and HPA +++). Interestingly, our study revealed considerable expression of *N*-acetylneuraminic (sialic) acid (SNA +++),  $\alpha$ -D-mannose and / or  $\alpha$ -D-glucose moieties (Con A, LCA and PSA +++)) on epithelium of oviduct explants. The negligible histochemical PNA signal on oviduct sections [30, 31] and explants indicates that D-galactose is not the ultimate critical carbohydrate mediating sperm-oviduct binding in the horse, as previously suggested [27, 29].

The potential participation of a broad range of carbohydrates and polysaccharides (D-galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, asialofetuin, fetuin, D-fucose, fucoidan, D-mannose, mannan and *N*-acetylneuraminic (sialic) acid) in sperm-oviduct interaction was tested in the current study using concentrations similar to those used by Dobrinski *et al.* [27]. Fetuin is a polysaccharide that expresses several terminal carbohydrates: *N*-acetylneuraminic (sialic) acid, *N*-acetylgalactosamine, D-galactose, *N*-acetylglucosamine and D-fucose [57] while asialofetuin expresses identical carbohydrates but has a higher ratio of galactosyl residues [59, 60]. Pre-incubating sperm suspensions with any of these carbohydrates, including D-galactose, did not inhibit sperm-oviduct binding in either non-capacitating ( $\text{Ca}^{2+}$  free) or capacitating ( $\text{Ca}^{2+}$  containing) conditions, as evaluated by both assays (oviduct explant or oviduct APM). Using the converse approach, i.e. inhibition of sperm-oviduct binding by pre-incubating oviduct explants with various lectins, at concentrations similar to those described by Desantis *et al.* [30, 31], also failed to inhibit sperm binding. In addition, sperm-oviduct binding was not decreased when *N*-linked carbohydrates were removed by *N*-glycosidase F (PNGase). When interpreting these results, a few points need to be considered: (1) not only the type of carbohydrate, but also the carbohydrate conformation / isomer is important; (2) a secondary binding can possibly be facilitated after competing with a primary carbohydrate receptor; (3) the cooperation of carbohydrates to facilitate sperm-oviduct binding and (4) a higher sperm-oviduct binding density was observed in  $\text{Ca}^{2+}$ -free or non-capacitating conditions than in capacitating medium

containing  $\text{Ca}^{2+}$ . Although EDTA addition reduced sperm-oviduct binding, the number of spermatozoa bound to oviduct explant epithelium was still high. The change in sperm binding capacity in the presence of EDTA appears to be due primarily to a depression of sperm motility by  $\text{Ca}^{2+}$  removal after 30 min of co-incubation (data not shown). As a result, spermatozoa exposed to EDTA may have had less time to reach the oviduct explants and establish binding in the 50  $\mu\text{l}$  medium droplet. Importantly, the supposed  $\text{Ca}^{2+}$  independency supports the carbohydrate insensitivity of sperm-oviduct binding as lectin binding is mainly regulated by  $\text{Ca}^{2+}$ . This in turn supports the hypothesis that equine sperm-oviduct affinity is not primarily a factor of  $\text{Ca}^{2+}$ -dependent lectin binding. Considering the findings above, one could question whether the  $\text{Ca}^{2+}$ -dependent carbohydrate-protein (lectin) interactions demonstrated between sperm and oviduct epithelial cells *in vitro* in a few mammalian species represent (1) the totality of the pre-ovulatory sperm-oviduct binding reaction or (2) only a part of that reaction. Hunter [61] previously hypothesized the latter and suggested that there may be considerable non-specific binding *in vivo*. This hypothesis was illustrated by studies in the pig that showed similar sperm binding density to tracheal epithelium, containing another type of ciliary epithelial cell [61, 62]. In contrast, in man a sequence of three amino acids (Arg-Gly-Asp) is thought to play an important regulatory role in sperm-oviduct interaction [63].

We also found that sperm-oviduct binding could not be inhibited by pre-incubating sperm with different glycosaminoglycans: chondroitin sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid and keratan sulfate, at concentrations based on the study of Sostaric *et al.* [11]. Neither could these molecules induce sperm release from oviduct explant binding. In cattle, an obvious effect of sulfated glycosaminoglycans on sperm-oviduct release [12, 26], capacitation / protein tyrosine phosphorylation [12, 36, 64, 65] and fertilization [36, 65] has been shown. In the mare, a high concentration of these molecules has been reported in follicular fluid and oviductal fluid, but in our study different types of glycosaminoglycans were unable to reduce density of stallion spermatozoa binding to oviduct epithelia. Moreover, the tested glycosaminoglycans also had no effect on sperm capacitation parameters such as tail-associated protein tyrosine phosphorylation, and presumably play no role in equine sperm capacitation. Penicillamine also failed to inhibit sperm-oviduct binding or induce sperm release from oviduct epithelium at concentrations based on Aitken *et al.*'s [66] report that *in vitro* motility of stallion sperm could be preserved by disulfide-reductants like penicillamine. In cattle, disulfide-reductants did facilitate sperm release from oviduct epithelium by reducing



disulphide covalent bonds to sulphhydryl groups [26]. Our results clearly demonstrate that sperm-oviduct binding in the horse is not dependent on disulphide covalent bonds.

Combined  $\text{HCO}_3^-$  and albumin enrichment of incubation media did cause a clear decline in density of sperm binding to oviduct epithelium (>10 fold), associated with rapid induction of sperm head-to-head agglutination. Both characteristics were independent of  $\text{Ca}^{2+}$  and alkalinity (pH 7.9). In general, it is thought that a sperm's capacitation status determines its ability to interact with oviduct epithelium [10]. In pig spermatozoa,  $\text{HCO}_3^-$  initiates rapid cAMP-driven membrane changes [67] that permit albumin-dependent cholesterol removal, followed by a series of functional membrane changes required for the acquisition of fertilizing potential [40, 68-70]. These membrane changes are important steps in sperm capacitation *in vivo* and, possibly underlie reduced affinity of sperm for oviduct epithelium in the horse. In support of this hypothesis, it was previously reported that exposure of bull sperm to  $\text{HCO}_3^-$  resulted in decreased affinity for the D-fucose moieties involved in sperm-oviduct interaction [11]. To better understand the role of capacitation in sperm affinity for oviduct binding, it would be useful to elucidate whether and which early plasma membrane changes and / or cholesterol removal are induced under the combined  $\text{HCO}_3^-$  and albumin conditions.

Sperm agglutination is a common event during manipulation of sperm from many mammalian species and is a problem during *in vitro* sperm studies because it interferes with accurate sperm assessment. Sperm agglutination occurs between spermatozoa with intact plasma and acrosome membranes [50]. Typically head-to-head agglutination takes place when sperm is exposed *in vitro* to: (1) washing media (ram: [71]); (2) fluids from the female genital tract (bull: [72]); (3) divalent cations (rabbit: [73]; bull: [74]); (4) bovine serum in combination with semen extender (bull: [75]); and (5) IVF medium (boar: [51, 52]). In general, head-to-head sperm agglutination is triggered by an ATP-dependent surface reaction activated by divalent cations (including  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$ ) [50-52]. Alternatively, sperm head-to-head agglutination has been reported to result from removal of anti-agglutinin from the sperm surface at an early stage of the capacitation process *in vitro* [76, 77]. Since sperm agglutination acts independently from  $\text{Ca}^{2+}$  we propose that the combination of  $\text{HCO}_3^-$  and albumin leads to the release of anti-agglutinin from the sperm plasma membrane over the acrosomal region of stallion sperm whereby previously 'hidden' receptors on the sperm plasma membrane become available for sperm specific head-to-head agglutination

interactions. In the pig, Harayama *et al.* [78, 79] observed similar anti-agglutinin release from boar sperm exposed to capacitating media. It has been hypothesized that the loss of anti-agglutinin from sperm is a product of the  $\text{HCO}_3^-$  induced adenylyl cyclase-cAMP-protein kinase pathway initiating changes in the plasma membrane that are aided by serum albumin contained in the medium [51]. As a consequence of sperm head-to-head agglutination, in our experiments putative sperm plasma membrane receptors in the apical ridge would no longer have been available for oviduct binding.

Unfortunately, under none of the tested conditions, we were able to achieve release of spermatozoa from oviduct explants. In cattle, in addition to the  $\text{HCO}_3^-$  and albumin induced plasma membrane changes, the ability to provoke hyperactivated motility was considered to be essential for sperm release [80]. We therefore assume that the failure to induce sperm release from oviduct binding is in part a result of the currently defined capacitation media for stallion sperm being insufficient to induce full sperm capacitation, including the hyperactivated motility required to initiate sperm release from the oviduct epithelium.

In conclusion, equine sperm-oviduct binding is independent of a range of carbohydrates, S-S reductants,  $\text{Ca}^{2+}$  and alkalinity (pH 7.9) whereas pre-incubation in media containing both  $\text{HCO}_3^-$  and albumin reduced sperm-oviduct binding. We suggest that the requirement for sperm-oviduct binding is dependent on the timing of insemination. If mating or insemination occurs during early estrus, sperm may need to survive several days until ovulation is imminent, and sperm-oviduct interaction may be critical to adequate longevity of sperm viability [19, 81]. By contrast, if sperm enters the oviduct during the peri-ovulatory period, rapid induction of capacitation events may decrease sperm affinity for oviduct binding or *vice versa*. This would allow capacitation triggered sperm to proceed immediately along the mare's oviduct to the site of fertilization without an obligatory period of epithelial binding. Nevertheless, sperm bound to oviduct epithelium should be able to react to capacitation triggers in the oviduct, such as elevated pH [39], to achieve full fertilizing capability. Full and proper initiation of capacitation will presumably release sperm optimally primed to fertilize from both oviduct epithelium.

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# CHAPTER 4

## OVIDUCT BINDING AND ELEVATED PH INDUCE PROTEIN TYROSINE PHOSPHORYLATION IN STALLION SPERMATOZOA

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*Oviduct Binding and Elevated Environmental pH Induce Protein Tyrosine Phosphorylation in Stallion Spermatozoa. Biology of Reproduction 2014; 91(1): 1–12.*



## ABSTRACT

Sperm-oviduct binding is an essential step in the capacitation process preparing the sperm for fertilization in several mammalian species. In many species, capacitation can be induced *in vitro* by exposing spermatozoa to bicarbonate,  $\text{Ca}^{2+}$  and albumin; however, these conditions are insufficient in the horse. We hypothesized that binding to the oviduct epithelium is an essential requirement for the induction of capacitation in stallion spermatozoa. Sperm-oviduct binding was established by co-incubating equine oviduct explants for 2 h with stallion spermatozoa ( $2 \times 10^6$  spermatozoa / ml), during which it transpired that the highest density (per  $\text{mm}^2$ ) of oviduct-bound spermatozoa was achieved under non-capacitating conditions. In subsequent experiments, sperm-oviduct incubations were performed for 6 h under non-capacitating versus capacitating conditions. The oviduct-bound spermatozoa showed a time-dependent protein tyrosine phosphorylation response, that was not observed in unbound spermatozoa or spermatozoa incubated in oviduct explant conditioned medium. Both oviduct-bound and unbound sperm remained motile with intact plasma membrane and acrosome. Since protein tyrosine phosphorylation can be induced in equine spermatozoa by media with high pH, the intracellular pH of oviduct explant cells and bound spermatozoa was monitored fluorometrically after staining with BCECF-AM dye. The epithelial secretory cells contained large, alkaline vesicles. Moreover, oviduct-bound spermatozoa showed a gradual increase in intracellular pH, presumably due to an alkaline local microenvironment created by the secretory epithelial cells, given that unbound spermatozoa did not show intracellular pH changes. Thus, sperm-oviduct interaction appears to facilitate equine sperm capacitation by creating an alkaline local environment that triggers intracellular protein tyrosine phosphorylation in bound sperm.

## INTRODUCTION

During natural mating, mammalian spermatozoa are deposited in the female reproductive tract and subsequently migrate to a sperm reservoir which in many species is located in the isthmus of the oviduct [1-3], although there are exceptions such as the bitch [4, 5]. In the sperm reservoir, spermatozoa bind via the apical region of their head to oviduct epithelial cells [6-8]. In cattle and pigs, only spermatozoa with intact acrosomal and plasma membranes are able to bind to the oviduct [9-11]. After a period of association with the oviduct epithelium, the bound spermatozoa undergo essential capacitation steps and are released from the reservoir. The released spermatozoa have thereby acquired a hyperactive motility pattern that helps them to move through the extracellular matrices of the cumulus complex and zona pellucida in order to reach and fertilize the mature oocyte [1-3]. Despite being first described in 1951 [12, 13], the capacitation process is still not fully understood, although it is known to involve a series of changes including reorganization of the sperm plasma membrane that facilitate cholesterol loss. These membrane events are induced by an increase in intracellular  $\text{HCO}_3^-$  concentrations and activation of second messenger systems, including a soluble adenylyl cyclase (sAC) and a rise in intracellular  $\text{Ca}^{2+}$  [14-16]. The activation of sAC and concomitant production of cAMP also result in the activation of protein kinase A which in turn phosphorylates tyrosine residues on sperm proteins [15, 17, 18]. The necessity for cAMP dependent protein tyrosine phosphorylation, especially in the sperm tail, is in various species related to the acquisition of hyperactivated sperm motility and is considered to be a marker for some essential elements of the capacitation process [19-24]. The rapid cAMP-driven membrane changes also enable depletion of cholesterol, which in turn allows aggregation of lipid ordered microdomains at the apical ridge area of the sperm head [25]. These microdomains contain functional zona pellucida binding protein complexes [26] and the SNARE proteins required to form the trans-SNARE complexes required for the docking of the outer acrosomal membrane to the apical sperm plasma membrane [27, 28]. In short, the cAMP-driven membrane changes allow albumin dependent removal of cholesterol which is followed by a slower series of functional membrane changes required for the acquisition of fertilizing potential.

While *in vitro* capacitation and fertilization are considered to be routine procedures in many species, and despite promising results in equine intracellular sperm injection (ICSI)

programmes [29-31], there are still no reliably successful conventional *in vitro* fertilization (IVF) protocols for equids [32-34]. Most likely, this is because functional stallion sperm capacitation is not induced *in vitro* using routine capacitation media, i.e. media containing BSA, bicarbonate and  $\text{Ca}^{2+}$  to promote sperm capacitation as described for various other mammalian species including the mouse [19, 20], pig [14] and man [35]. In cattle, induction of capacitation is enhanced by additional use of heparin and phosphodiesterase inhibitors [36, 37]. The disappointing protein tyrosine phosphorylation response after incubating stallion spermatozoa in simple *in vitro* capacitation media can be compensated by addition of membrane soluble cAMP analogues in combination with phosphodiesterase inhibitors which combine to increase protein kinase A (PKA) activity and induce protein tyrosine phosphorylation in the tail of approximately 50% of stallion spermatozoa [38]. Alternatively, reactive oxygen species will induce protein tyrosine phosphorylation in stallion spermatozoa [39]. Recently, an increase in the alkalinity of the capacitation medium (to approx. pH values of 8) has been reported to induce protein tyrosine phosphorylation [40, 41]. However, it is unlikely that such a high pH is the physiological trigger involved in sperm capacitation *in vivo* and it remains unclear how protein tyrosine phosphorylation and related sperm hyperactive motility are elicited physiologically in stallion sperm. The current consensus is that the central event in sperm capacitation *in vivo* is binding of the sperm to the oviduct. After a period of sperm-oviduct binding, the spermatozoa are released from the epithelium, by which time they have acquired the competence for hyperactive motility [42]. Despite the likely involvement of oviduct interaction in sperm capacitation *in situ*, during *in vitro* fertilization the treatments commonly used to trigger capacitation are limited to removal of seminal plasma and incubation in a bicarbonate,  $\text{Ca}^{2+}$  and albumin enriched medium; this condition fails to elicit reliable *in vitro* fertilization using equine gametes. It is, therefore, tempting to speculate that equine sperm-oviduct interaction is an essential requirement for equine sperm capacitation. While *in situ* sperm oviduct interactions are difficult to monitor and interpret, oviduct epithelial cell (OEC) monolayers have been used as a model system to study sperm-oviduct binding. Bovine, equine and porcine spermatozoa have all been shown to exhibit a capacitation-specific chlortetracycline (CTC) staining pattern when released from homologous OEC monolayers [9, 43, 44]. However, the utility of oviduct monolayers to induce a more physiological sperm capacitation has been questioned because OECs rapidly dedifferentiate during culture [11] and it has, therefore, been suggested that an equine oviduct explant model may be more representative of the *in vivo* situation [45]. In cattle, a similar

system has been shown to activate spermatozoa which, when released, have acquired the competence to fertilize an oocyte [46]. It has been further suggested that spermatozoa are activated / capacitated during the late pre-ovulatory period when the oviductal microenvironment changes in a way thought to be relevant for inducing sperm capacitation and the release of activated spermatozoa [1]. The aim of the current study was therefore to develop an equine oviduct explant system and determine its ability to trigger essential steps in the capacitation of stallion spermatozoa. We hypothesized that binding to the oviduct epithelium is an essential requirement for the induction of capacitation in stallion spermatozoa. To this end we investigated whether stallion spermatozoa have affinity for mare oviduct epithelial explants and whether this results in intracellular pH changes, protein tyrosine phosphorylation and subsequent release of spermatozoa with hyperactivated motility. These new insights may help to explain why conventional *in vitro* fertilization still fails in the horse.

## **MATERIALS AND METHODS**

### **Chemicals and reagents**

Propidium iodide (PI), SYBR14 (LIVE / DEAD Sperm Viability Kit), JC-1, Hoechst 33342, BCECF-AM, Alexa Fluor 488-conjugated goat anti-mouse antibody and Texas red-conjugated goat anti-rabbit antibody were obtained from Molecular Probes (Ghent, Belgium). Monoclonal 4G10®Platinum, anti-phosphotyrosine mouse antibodies were obtained from Millipore (Overijse, Belgium). Triton X-100, PNA-FITC, the rabbit anti-tubulin antibody, fatty acid-free bovine serum albumin (A9418; cell culture tested) and all other chemicals not otherwise listed were obtained from Sigma-Aldrich (Bornem, Belgium).

### **Animals**

Oviducts were collected at a slaughterhouse soon after the slaughter of healthy Warmblood mares aged between 5 and 22 years and without any visible reproductive tract pathologies. Only oviducts from mares with a large follicle (>35 mm diameter) in combination with estrous oedema in the uterine wall, indicating imminent ovulation, were used for this study.

### **Preparation of oviduct explants**

Five oviducts per experiment were prepared as previously described by Nelis *et al.* [45]. Briefly, oviducts from pre-ovulatory mares were dissected free of excess connective tissue, clamped at both ends and transported on ice in sterile 0.9% saline containing 50 µg / ml gentamycin. On arrival at the lab, the oviducts were washed in DPBS and the epithelial cells were harvested by scraping the mucosa at the ampullary-isthmic junction of the longitudinally incised oviduct. The harvested cellular material was transferred to a tube containing HEPES buffered TALP medium and left to settle for 10 min, after which the cell pellet was resuspended in 3 ml of fresh HEPES-buffered TALP washing medium. The process of sedimentation was repeated twice. Afterwards, the harvested cellular material was washed and cultured overnight in Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) at 38.5 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The timespan from slaughter of mares to seeding of the cells was approximately 3 to 4 h.

### **Semen collection and preparation**

Semen was collected using an artificial vagina (Colorado State University AV) from three adult stallions of proven good fertility. The raw ejaculate was filtered through gauze to remove the gel fraction and any debris, before visual evaluation of sperm motility by light microscopy (200x) on a heated stage at 37.0°C; assuming good motility, the semen was immediately transported to the laboratory for further processing. One ml of fresh semen with a concentration of 100 to 300 x 10<sup>6</sup> spermatozoa / ml was then washed using a 45 / 90% Percoll® gradient [36, 47]. Next, the sperm pellet was diluted to a concentration of 20 x 10<sup>6</sup> spermatozoa / ml. At least 3 replicates of each experiment were performed using one ejaculate from each of the three stallions. The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/175 and EC2013/176).

### **Sperm oviduct binding**

Oviduct explants were cultured in DMEM/F12 medium supplemented with 10% FBS, equilibrated with 5% CO<sub>2</sub> in a humidified atmosphere at 38.5 °C, as described by Nelis *et al.*

[45]. After overnight incubation, oviduct explants with a diameter of less than 200  $\mu\text{m}$  were selected and washed; 5 oviduct explants per droplet were then transferred to 45  $\mu\text{l}$  droplets of different media. Sperm binding to oviduct explants was performed in Whitten's medium (100 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{MgCl}_2$ , 5.5 mM glucose, 22 mM HEPES, 2.4 mM sodium lactate pentahydrate and 1.0 mM pyruvic acid; pH=7.4 and 280-300 mOsm / kg) at 38.5 °C in air; further referred to as non-capacitating medium / conditions. To provide sperm capacitation supporting conditions, Whitten's medium was modified by replacing the sodium lactate pentahydrate with 2.4 mM calcium lactate pentahydrate and adding 25 mM  $\text{NaHCO}_3$  and 7 mg / ml BSA (pH=7.4 and 280-300 mOsm / kg; osmolality was adjusted by reducing NaCl); this medium was pre-equilibrated for at least 2 h in a humidified atmosphere containing 5%  $\text{CO}_2$  at 38.5°C and is further referred to as capacitation medium (adapted from McPartlin *et al.* [48]). In general, a final concentration of  $2 \times 10^6$  spermatozoa / ml was obtained by adding 5  $\mu\text{l}$  Percoll® washed and diluted sperm ( $20 \times 10^6$  / ml spermatozoa) to the 45  $\mu\text{l}$  oviduct explant-containing droplet. The droplets were cultured under mineral oil to prevent evaporation. Three different incubation conditions were applied; non-capacitating conditions (38.5°C in air), DMEM/F12 with 10% FBS and capacitating medium (38.5°C, in a humidified atmosphere equilibrated with 5%  $\text{CO}_2$ ). Each replicate was performed with a different ejaculate.

### **Oviduct ciliary activity and sperm binding**

Oviduct explant viability was tested in both non-capacitating and capacitating medium at various durations of culture (0, 2, 4, 6, 12 and 24 h). At each time point, viability of oviduct explants was evaluated by assessing ciliary activity using a phase contrast microscope (magnification of 1000x). The effect of sperm concentration on sperm binding to oviduct explants was assessed in non-capacitating medium using 0.5, 1, 2, 5 or  $10 \times 10^6$  spermatozoa / ml. After 2 h of co-incubation, the sperm-oviduct explants were washed twice in non-capacitating medium and evaluated for sperm-oviduct binding. Subsequently, sperm-oviduct binding was tested in three different co-incubation media: DMEM/F12 with 10% FBS (basic culture medium for oviduct explants), non-capacitating medium and capacitating medium. For each condition, stallion sperm were added to oviduct explants at a concentration of  $2 \times 10^6$  spermatozoa / ml and incubated for 0.5, 2, 4 or 6 h. In some cases, after a 2 h co-incubation in non-capacitating medium, sperm-oviduct explants were washed and transferred to non-



capacitating or to capacitating medium for a further 0.5, 3 or 6 h.

### **Sperm and oviduct explant staining**

The number of spermatozoa bound to oviduct epithelium was determined by visualizing the live sperm using 2  $\mu$ M JC-1 in HEPES-buffered washing medium for 15 minutes at 37 °C in 5% CO<sub>2</sub> in air, to stain the mitochondria in the sperm mid-piece [49]. This fluorophore can reversibly change its emission from yellow-red (aerobic sperm metabolism) to green (anaerobic metabolism accompanied by depolarization of the inner mitochondrial membrane [50]). JC-1 was combined with a live / dead cell nucleus stain combination, SYBR14 (20  $\mu$ M) and propidium iodide (PI; 50 nM) (LIVE / DEAD Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands) [49]. Stained oviduct explants and sperm-oviduct explant complexes were washed twice in HEPES buffered washing medium and mounted on siliconized glass slides (Marienfeld, Germany) using 1.4-Diazabicyclo[2.2.2]octane (DABCO) as antifade, and sealed with nail polish. Green fluorescence-labelled oviduct cells and sperm heads were considered viable, whereas red oviduct cells and sperm heads were considered dead. Finally, the relative percentages of viable, aerobically metabolizing oviduct epithelial cells and spermatozoa were scored by counting the viable cells in 5 microscopic fields. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400x.

### **Acrosome status**

The acrosome status of spermatozoa that had been incubated with oviduct epithelial explants for a period of 6 h in non-capacitating and capacitating media was assessed using fluorescein conjugated peanut agglutinin (PNA-FITC) to discriminate acrosome-intact from acrosome deteriorated spermatozoa [51]. Briefly, after fixation in 4% (w/v) paraformaldehyde in DPBS for 15 min at room temperature, sperm oviduct explants were washed in DPBS and further permeabilized by incubating in 0.1% Triton X-100 in DPBS for 10 min at room temperature. After washing in DPBS, the sperm-oviduct explants were stained for 15 min at room temperature with 1  $\mu$ g / ml PNA-FITC. After two extra washes with DPBS, the stained sperm-oviduct explants were mounted as described above. Spermatozoa with PNA-FITC-labelled acrosome regions were considered acrosome-intact, whereas spermatozoa with no fluorescence over the acrosomal region were considered to be acrosome reacted. The relative

percentages of acrosome-intact spermatozoa were scored by counting 200 spermatozoa per sperm-oviduct explant incubation. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400x.

### **Sperm tail-associated protein tyrosine phosphorylation**

After co-incubation, sperm-oviduct explant complexes were washed twice and fixed in 4% paraformaldehyde in DPBS at room temperature for 15 min. The fixative was removed by three washing steps using DPBS. The washed sperm-oviduct explant complexes were subsequently incubated in 0.1% Triton X-100 in DPBS for 10 min at room temperature to ensure full permeabilization of membranes. The immobilized and permeabilized sperm-oviduct explant complexes were then incubated in blocking buffer (DPBS containing 1% BSA) for 10 min at room temperature. After this step, spermatozoa were incubated in buffer containing 0.1% BSA and supplemented with a mix of mouse monoclonal 4G10@Platinum IgG<sub>2b</sub> protein anti-phosphotyrosine antibody (diluted 1:500) and rabbit anti-tubulin antibody (diluted 1:80) at 4°C. After overnight incubation, unbound antibody was removed by gently washing the sperm-oviduct explant complexes twice using 1 ml of PBS containing 0.1% BSA. The resulting sperm-oviduct explant complexes were then stained with a mix of a monoclonal goat anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Molecular Probes, Ghent, Belgium) and a goat anti-rabbit antibody conjugated to Texas red for 1 h at room temperature. After immunolabeling, the two non-bound antibody conjugates were removed by washing three times using DPBS containing 0.1% BSA, and once using DPBS. The immunolabelled sperm-oviduct explant complexes were mounted on glass slides as described above and sealed with nail polish. The proportion of spermatozoa with green fluorescent tails among the total sperm population (with red fluorescent tails) was determined by counting 200 spermatozoa per sperm-oviduct explant complex. Samples were examined using an Eclipse Ti microscope (Nikon, Tokyo, Japan) equipped with a mercury lamp and appropriate filters at a minimum magnification of 400×.

### **Assessing intracellular pH of oviduct epithelial cells and spermatozoa**

Oviduct explants and sperm-oviduct explant complexes were washed twice using HBS (HEPES buffered saline) and subsequently stained with 5 μM of the pH-sensitive dye

BCECF-AM. After a 30 minute incubation at 38.5 °C, the non-cellular dye was removed by washing the complexes twice in HBS. The complexes were then resuspended in medium and incubated for an additional 20 min to allow de-esterification of the dyes, and mounted on glass slides using DABCO as anti-fading agent and to help immobilize spermatozoa for assessing intracellular pH. To determine the pHi of sperm cells, a calibration was first performed using BCECF-AM equilibrated spermatozoa in the presence of 0.1% Triton X-100 and by adjusting the pH with HCl and NaOH [52, 53]. Increasing relative green fluorescence was related to increasing intracellular pH [54]. The BCECF signal was measured in oviduct epithelial cells and during 6 h sperm-oviduct explant co-incubations in bound as well as unbound spermatozoa. Samples were examined using a Leica DMR microscope equipped with a Hg-lamp and appropriate filters, at a magnification of 400x and 1000x.

### **Sperm motility assessment**

Motility patterns during sperm-oviduct explant binding and after sperm release from the oviduct explants were assessed using a CCD ICD-46E camera (Ikegami Tsushinki Co. Ltd., Japan) attached to an Olympus IX70 inverted microscope (Olympus Belgium N.V., Aartselaar, Belgium). Images were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium).

### **Microscopic imaging of sperm-oviduct binding**

Density of spermatozoa bound to the oviduct explants, along with membrane integrity, acrosome status and intracellular pH of bound spermatozoa during the 6 h co-incubation were determined in five microscopic fields (400x magnification) by means of fluorescence microscopy using a Leica DMR microscope equipped with excitation filters BP 340/380 nm, BP 450/490 nm, BP 560/40 nm and a 100 W mercury lamp. Alexa Fluor 488-conjugated goat anti-mouse antibody, SYBR14, PNA-FITC, propidium iodide (PI), Texas red-conjugated goat anti-rabbit antibody, JC-1, Hoechst 33342 and BCECF-AM ester were sequentially excited using 499 nm, 498 nm, 495 nm, 536 nm, 589 nm, 592 nm, 345 nm and 490 nm wavelengths. The emission spectra were detected by Blue (BP 470/40 nm), Green (LP 515 nm) and Red (BP 645/75 nm) filters corresponding to the emission peaks of the dyes of respectively 519 nm, 522 nm, 519 nm, 617 nm, 615 nm, 595 nm, 478 nm and 530 nm. Images were acquired

using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium). In addition, the surface area of the oviduct explants in each microscopic field (at a magnification of 400×) was measured using this program. The percentage of spermatozoa with tail-associated protein tyrosine phosphorylation (PY+ / TUB+) was determined by evaluating 200 randomly selected spermatozoa (TUB+); the acrosome status of bound spermatozoa (PNA-FITC+ / TUB+) at each time point of co-incubation was similarly evaluated by confocal microscopy using a Bio-Rad Radiance 2100 MP system (Zeiss / Bio-Rad, Hertfordshire, U.K.) attached to a Nikon Eclipse TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) at a magnification of 400x or 1000x. The fluorescent dyes were excited using an argon laser and analyzed using the same filters as described for fluorescence microscopy. Images were acquired using LaserSharp 2000 software (Zeiss / Bio-Rad) after background corrections. For each wavelength, digital optical sections were collected using Z-series acquisition at intervals of 0.35 µm. In both systems, the various fluorophores were checked for signal overlap; no leakage of signals was detected.

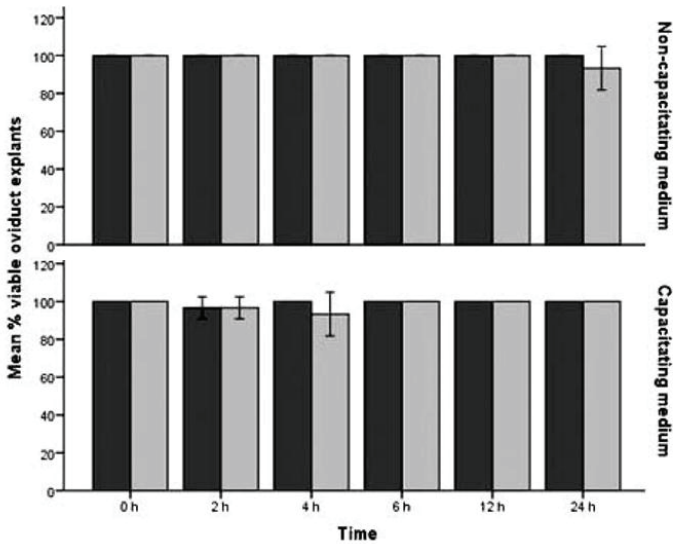
### **Statistical analysis**

The effects of treatments on sperm parameters were assessed by analysis of variance (ANOVA) using the general linear model procedure of SPSS version 20 for Windows (SPSS IBM, Brussels, Belgium). The number of spermatozoa bound to oviduct explants and the percentages of PI- and SYBR+ spermatozoa as well as the change in the percentage of tyrosine phosphorylated (PY+) spermatozoa over time were calculated as repeated measures with Greenhouse-Geisser correction by the general linear model and a Bonferroni correction. Post hoc tests were performed by Sheffé analysis. Differences were considered significant if  $P < 0.05$ .

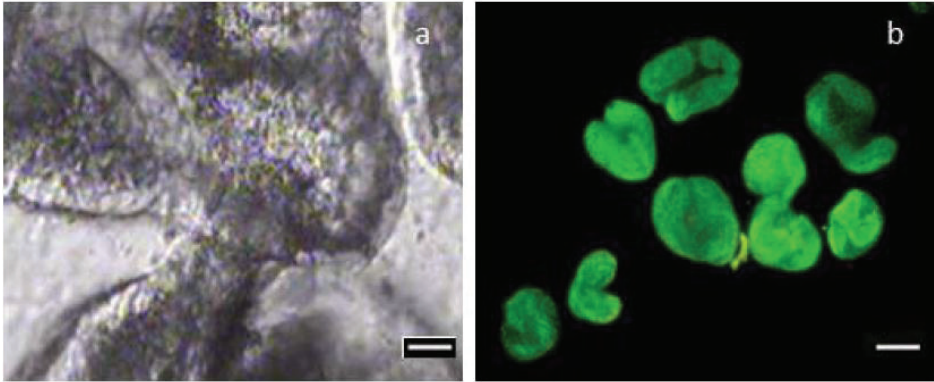
## RESULTS

### Viability of oviductal explants

We previously demonstrated that oviduct explants remained viable for up to 6 days when incubated in DMEM/F12 medium with 10% FBS [45]. In order to assess aspects of sperm-oviduct binding however, the possibility that the relatively simple sperm incubation media may compromise oviduct explant viability was examined by assessing changes in morphological features (ciliary activity and membrane-integrity) of explants incubated for 24 h in non-capacitating and capacitating media. We were able to demonstrate that oviduct explants remained viable for at least 24 h when incubated in these media (Figure 1); indeed, the vast majority of oviduct explants displayed ciliary activity which was very similar between media and changed little over time ( $99 \pm 2\%$ ;  $P=0.44$ : supplementary video clip; Figure 2a) while all cells ( $100 \pm 0\%$ ;  $P=0.67$ ; Figure 2b) of nearly all explants (>99%) remained membrane-intact.



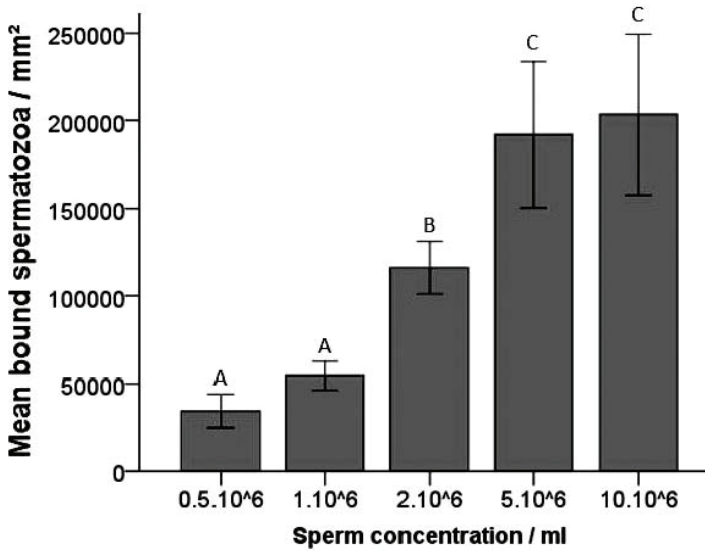
**Figure 1.** Oviduct explant viability during 24 h incubation in non-capacitating and capacitating media. Data represent mean ( $\pm$  SD) % of oviduct explants ( $n=30$  per group) showing ciliary activity (dark grey bars) and membrane integrity (light grey bars) over three replicates. ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.



**Figure 2.** (a) Representative image from a supplementary video clip of oviduct explants after 24 h incubation in non-capacitating Whitten’s medium or capacitating modified-Whitten’s medium. In 99% of the oviduct explants, ciliary activity was evident after 24 h incubation. (b) Fluorescence microscopic image of oviduct explants after 24 h incubation in non-capacitating Whitten’s medium or capacitating modified-Whitten’s medium. SYBR14 / PI staining demonstrated that more than 99% of the cells were membrane intact after 24 h incubation (green = membrane-intact) (a: original magnification, 1000x; scale bar = 6.25  $\mu\text{m}$ ; b: original magnification, 100x; scale bar = 62.5  $\mu\text{m}$ ).

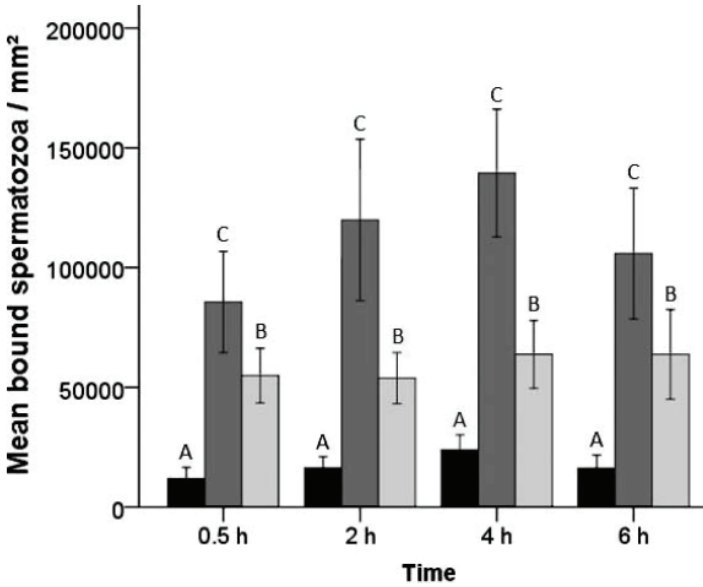
### **Sperm binding capacity of equine oviduct explants**

To standardize the sperm-oviduct binding assay, the saturation concentration for sperm binding to oviduct explants was evaluated by exposing oviduct explants to increasing sperm concentrations (0.5, 1, 2, 5 and 10 x 10<sup>6</sup> spermatozoa / ml) in non-capacitating medium. Saturation for sperm binding was reached at 5 x 10<sup>6</sup> spermatozoa / ml (see Figure 3: 1.9  $\pm$  0.4 x 10<sup>5</sup> spermatozoa / mm<sup>2</sup>).



**Figure 3.** Effect of sperm concentration on binding capacity of equine oviduct explants for stallion sperm under non-capacitating conditions. Saturation of sperm-oviduct explant binding was detected at  $5 \times 10^6$  spermatozoa / ml. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=30$  per group) in four replicates. Values that differ significantly are indicated by different capitals. ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

At different time points (0.5, 2, 4, 6 h), sperm-oviduct binding was quantified by using a sperm concentration of  $2 \times 10^6$  spermatozoa / ml in 45  $\mu$ l droplets of non-capacitating medium and compared to both capacitating medium and the previously described DMEM/F12 with 10% FBS. The mean  $\pm$  SD number of bound spermatozoa at different time points (0.5, 2, 4, 6 h) was  $10.0 \pm 2.7 \times 10^5$  spermatozoa / mm<sup>2</sup> in non-capacitating medium, compared to  $6.0 \pm 1.1 \times 10^5$  spermatozoa / mm<sup>2</sup> in capacitating medium and  $1.4 \pm 0.4 \times 10^5$  spermatozoa / mm<sup>2</sup> in DMEM/F12 based culture medium; these sperm-binding densities differed significantly between media ( $P < 0.001$ ). Within a medium, there was no significant effect of incubation time on the number of bound spermatozoa (DMEM/F12 based culture medium:  $P=0.25$ ; non-capacitating medium:  $P=0.07$ ; capacitating medium:  $P=0.80$ ) (Figure 4).

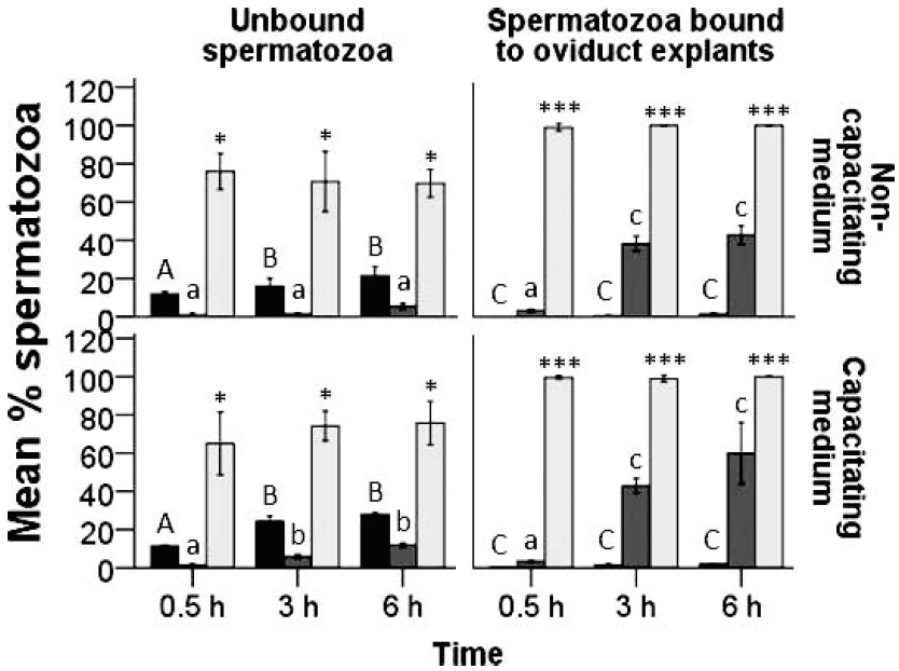


**Figure 4.** Effect of media on sperm binding capacity of oviduct explants over time. Data represent mean ( $\pm$  SD) number of spermatozoa bound to oviduct explants ( $n=10$  per group) over four replicates. Non-capacitating medium (dark grey bars) supported sperm-oviduct binding at a higher level than capacitating medium (light grey bars). DMEM/F12 basic culture medium supplemented with 10% FBS (black bars) supported the lowest sperm-oviduct binding. Within each time point, values that differ significantly are indicated by different capitals. Repeated measures ANOVA with Greenhouse-Heisser correction; post hoc tests with Bonferroni correction was performed.

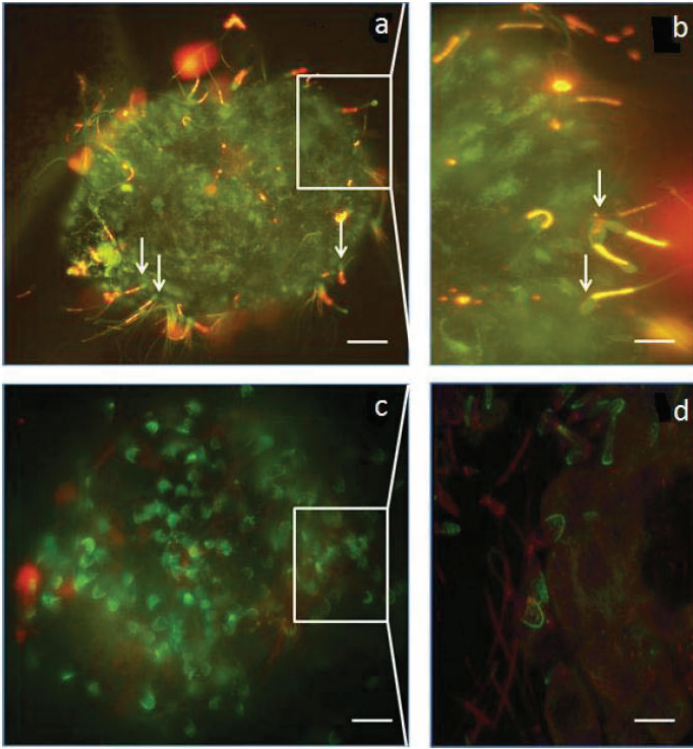
### Selective binding of intact sperm to oviduct explants

Plasma membrane and acrosome integrity of sperm bound to oviduct explants were assessed during 6 h co-incubation and compared to those of the unbound sperm fraction. It transpired that membrane-intact spermatozoa were more prevalent among the oviduct-bound sperm population ( $99.0 \pm 0.5\%$ ) compared to the unbound sperm fraction ( $80 \pm 2.5\%$ ) ( $P<0.001$ ). (Figures 5, 6a, 6b). The same was true for acrosome integrity ( $99.6 \pm 0.8\%$  for oviduct explant bound spermatozoa versus  $72 \pm 11\%$  for non-bound spermatozoa) (Figures 5, 6c, 6d); again these differences ( $P<0.001$ ) were not time-dependent ( $P=0.63$ ) but already apparent at time-point 0.5 h.





**Figure 5:** Percentage of plasma membrane-intact, protein tyrosine phosphorylated and membrane-intact, or acrosome-intact spermatozoa in non-capacitating and capacitating media over time, for sperm in suspension or for sperm bound to oviduct explants. In the oviduct explant bound sperm population, a time-dependent increase in tail-associated protein tyrosine phosphorylation was observed in both conditions. Plasma membrane and acrosome integrity were conserved during sperm-oviduct binding. Data represent mean ( $\pm$  SD) percentages of membrane-damaged (black bars), membrane-intact and protein tyrosine phosphorylated (dark grey bars) and membrane-intact and acrosome-intact spermatozoa (light grey bars) ( $n=10$  oviduct explants in each group; three replicates). For the percentage of membrane-damaged spermatozoa, values that differ significantly are indicated by different capitals. For the percentage of membrane-intact and protein tyrosine phosphorylated spermatozoa, values that differ significantly are indicated by different small letters. For the percentage of membrane-intact plus acrosome-intact spermatozoa, values that differ significantly are indicated by asterisks. Repeated measures ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

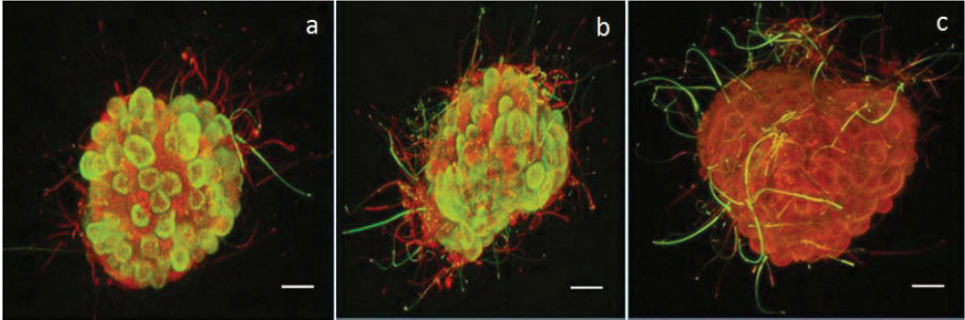


**Figure 6:** (a,b) Fluorescence micrographs of spermatozoa bound to oviduct explants. As demonstrated by SYBR14 / PI / JC-1 staining, the bound spermatozoa remained membrane-intact (green) for at least 6 h in non-capacitating and capacitating media (white arrows). (c) Acrosome integrity of spermatozoa bound to oviduct explants after 6 h co-incubation in non-capacitating and capacitating media evaluated by PNA-FITC using fluorescence and (d) confocal microscopy (a, c: original magnification, 400x, scale bar = 25  $\mu\text{m}$ ; b, d: original magnification, 1000x; scale bar = 6.25  $\mu\text{m}$ ).

### Sperm-oviduct binding induced protein tyrosine phosphorylation in sperm tails

The hypothesis that *in vivo* sperm capacitation is initiated during residence in the sperm reservoir [2] led us to investigate the effect of sperm-oviduct binding on tail-associated protein tyrosine phosphorylation, which is thought to be an essential step in capacitation. The percentage of membrane-intact spermatozoa with tail-associated protein tyrosine phosphorylation was significantly higher for the oviduct explant-bound sperm fraction than among the unbound population ( $P < 0.001$ ). After 6 h co-incubation, the percentage of protein tyrosine phosphorylated sperm increased to  $43 \pm 5\%$  of the bound sperm population in non-capacitating medium compared to  $60 \pm 16\%$  in capacitating medium (Figure 5, 7a, 7b, 7c). By

comparison, only a small percentage of unbound sperm in the same media showed evidence of protein tyrosine phosphorylation after 6 h ( $6 \pm 2\%$  and  $12 \pm 1\%$ , respectively).

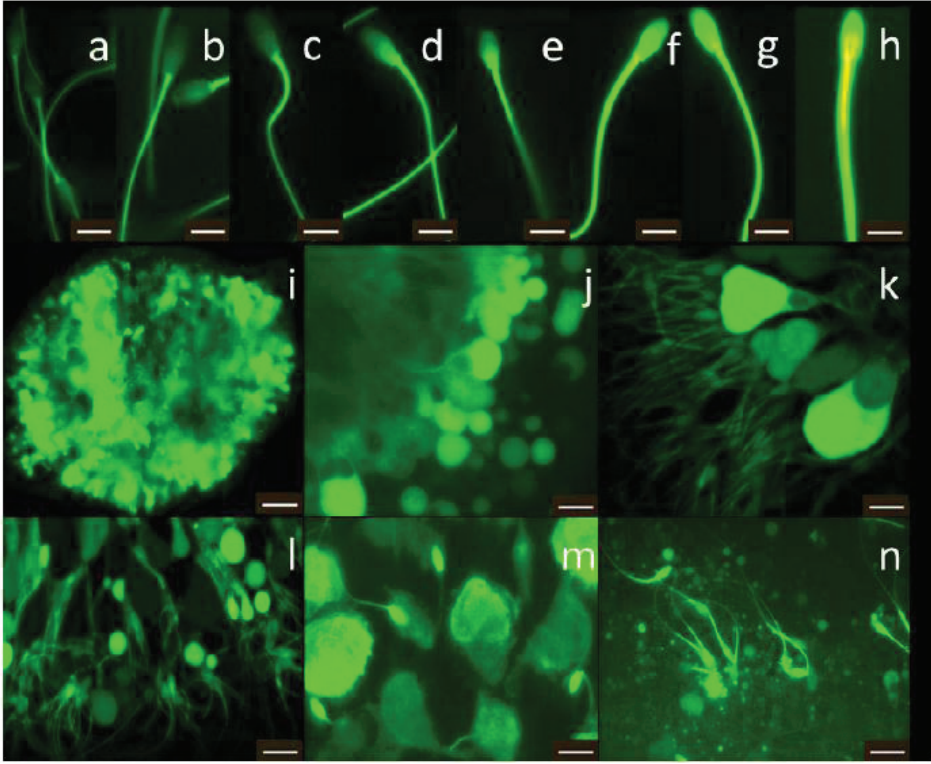


**Figure 7:** Fluorescence micrographs of protein tyrosine phosphorylated spermatozoa bound to oviduct explants after 6 h co-incubation in capacitating medium. Bound spermatozoa were identified by means of confocal microscopy after double indirect immunofluorescence staining (tubulin = red; protein tyrosine phosphorylation = green) at 30 min (a), 3 h (b), and 6 h (c) of co-incubation in capacitating medium. Tyrosine phosphorylation increased over time (a, b, c: original magnification, 400x; scale bar =25  $\mu\text{m}$ ).

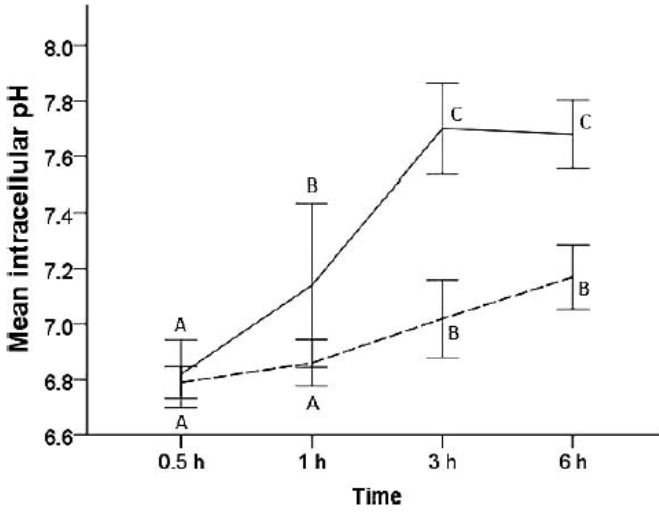
#### Alkaline secretory activity of oviduct explants and the effects on spermatozoa pH<sub>i</sub>

It has been reported that equine spermatozoa become protein tyrosine phosphorylated after incubation in medium with an external  $\text{pH} \approx 8$ , achieved by incubation in air [40, 41]. In order to explain the increased protein tyrosine phosphorylation observed in oviduct bound spermatozoa we investigated whether this interaction induces alkalization of spermatozoa. BCECF-AM staining was performed on oviduct explants and oviduct-bound spermatozoa and intracellular pH of both types of cells was assessed over time. Oviduct explants recovered from mares at the late pre-ovulatory stage of the cycle contained secretory cells with a mildly alkaline apical segment ( $\text{pH} = 7.5\text{--}7.8$ ) (Figure 8i, 8j). As incubation progressed, the incidence of oviductal cells with an alkaline content decreased ( $121 \pm 23$ ,  $112 \pm 25$ ,  $76 \pm 19$  and  $23 \pm 11$  cells per  $\text{mm}^2$  at 0.5, 1, 3 and 6 h, respectively). By contrast, the intracellular pH of spermatozoa bound to the oviduct cells increased over time ( $6.82 \pm 0.12$ ,  $7.14 \pm 0.30$ ,  $7.70 \pm 0.16$ ;  $7.68 \pm 0.12$  at 0.5, 1, 3 and 6 h) (Figure 8k, 8l, 8m, 8n). Unbound sperm also exhibited a significant intracellular pH rise over time ( $6.79 \pm 0.57$ ,  $6.86 \pm 0.84$ ,  $7.02 \pm 0.14$ ,  $7.17 \pm 0.12$  at 0.5, 1, 3 and 6 h) but the pH values were consistently lower than for oviduct explant-bound spermatozoa ( $P < 0.001$ ). Comparing the timing of intracellular alkalization and protein

tyrosine phosphorylation in oviduct explant-bound sperm, indicated that alkalization preceded protein tyrosine phosphorylation (Figure 5 versus Figure 9:  $P < 0.01$ ).



**Figure 8:** Fluorometric recordings of intracellular pH of (a-h) pH-calibrated spermatozoa (pH 6.7, 6.8, 6.9, 7.1, 7.3, 7.5, 7.9, 8.3), (i-j) oviduct explants and (k-n) oviduct-bound spermatozoa after 0.5 h, 1 h, 3 h and 6 h. As demonstrated by BCECF-AM staining, secretory epithelial cells of oviduct explants contained intracellular content with increased pH. During 6 h sperm-oviduct explant binding, the intracellular pH of bound spermatozoa reached a maximum at 3 h which was maintained until 6 h. Additionally, the alkaline secretory content of epithelia was released gradually during the 6 h of sperm-oviduct binding. Images were recorded using fluorescence microscopy (i: original magnification, 400x, scale bar = 25  $\mu\text{m}$ ; a-h and j-n: original magnification, 1000x, scale bar = 6.25  $\mu\text{m}$ ).



**Figure 9:** Mean pH<sub>i</sub> recorded in oviduct bound spermatozoa (black full line) and sperm suspensions (black dotted line) over time. In both sperm populations, a time dependent increase in intracellular pH was observed. However, the intracellular pH of oviduct-bound spermatozoa tended to alkalization after 3 h sperm-oviduct binding whereas this did not occur in sperm in suspension. Data represent mean ( $\pm$  SD) intracellular pH per spermatozoa ( $n=50$  in each group) over three replicates. Within the oviduct-bound spermatozoa and suspended sperm populations, values that differ significantly are indicated by different capitals. Repeated measures ANOVA with Greenhouse-Heisser correction; post hoc tests were performed after Bonferroni correction.

## DISCUSSION

*In vivo*, mammalian spermatozoa follow an ordered sequence of events in preparation for the fertilization of an oocyte [55]. For equids, the proposed sequence of events includes; (1) stallion spermatozoa are ejaculated into the uterine body and transported to the utero-tubal junction (UTJ); (2) a ‘reservoir’ of non-capacitated spermatozoa is established at the UTJ; (3) spermatozoa within the reservoir become capacitated as the time of ovulation approaches; (4) the capacitated spermatozoa are released from the sperm reservoir having acquired hyperactivated motility, (5) the released spermatozoa meet the mature oocyte at the ampullary-isthmic junction (6) after recognition, the spermatozoa bind to the zona pellucida or the intercellular matrix of the cumulus cell complex [56, 57], (7) the acrosome reaction is subsequently triggered allowing the sperm to (8) penetrate the cumulus and zona pellucida

and enter the perivitelline space, from where (9) the fertilizing spermatozoon can bind and fuse with the oolemma. The effects of the oviduct on equine sperm physiology is the subject of the current study.

Conventional IVF with equine gametes is not successful [32-34], whereas oocyte transfer [58] and ICSI [31, 59, 60] can be applied successfully in horses. This implies that stallion spermatozoa are able to fulfill their role in fertilization *in vivo* or after injection into an oocyte. In contrast to most other mammalian species, stallion spermatozoa are not efficiently activated by conventional IVF or *in vitro* capacitation media (i.e. media containing bicarbonate,  $\text{Ca}^{2+}$  and defatted albumin as capacitation supporting factors, with or without an additional protein kinase A inducer). *In vivo* the oviduct almost certainly plays a critical role in regulating sperm activation, and it is tempting to speculate that oviduct-sperm interactions are essential to eliciting capacitation of stallion spermatozoa. Of course, it is difficult to monitor sperm-oviduct interactions *in situ*. Therefore, in the current study, an *ex vivo* equine sperm-oviduct binding model was used to mimic the events that take place in the oviduct during the late pre-ovulatory stage.

Equine oviductal explants in which morphological and ultrastructural features, such as ciliary beating, are well preserved [45] were used. This system was selected primarily because ciliation in bovine oviductal epithelial cells is thought to be a terminal differentiation event that is difficult to induce or maintain *in vitro* [61]. Moreover, because bull [46] and boar [62] sperm bind preferentially to the cilia or to deeper regions of ciliated, and not to the secretory, epithelial cells; oviduct epithelial explants are preferred to less differentiated oviduct monolayers. Baillie *et al.* [63] reported that, in man, spermatozoa bind more avidly to explants than monolayers. We did not investigate this preference further in the current study but found the oviduct explant system to be very practical because, as for cattle [64], the explants could be used within 6 to 12 h of harvesting, whereas monolayers can only be used several days later [45].

To establish the “*in vivo*-like” equine oviduct explant culture, epithelial cells were recovered from the ampullary-isthmic junction. *In vivo* on the other hand, the sperm reservoir is not thought to extend beyond the first few centimeters of the isthmus proximal to the UTJ. However, it is technically almost impossible to reliably and repeatedly collect oviduct

epithelial cells exclusively from the equine isthmus because of its very narrow lumen and tortuous anatomy [6, 65]. Moreover, because sperm-oviduct epithelial cell binding characteristics have been shown to be independent of the oviductal site of origin in cattle [7, 11], we decided to harvest cells from the isthmic-ampullary junction where we could at least reliably harvest a comparable cell population (i.e. from the same location) for each replicate. While it is also likely that sperm in the oviductal reservoir are exposed to ampullary secretions *in vivo*, we do acknowledge that the absence of regional differences in oviduct epithelial cell binding properties has not been proven for the horse. In this respect, it remains possible that cells from the distal isthmus may display different sperm-oviduct interactions to those at the ampullary-isthmic junction which would affect interpretation of the outcome of the binding studies.

With regard to the quantification of sperm-oviduct binding, we were aware that standardization is the main challenge when using equine oviduct explants due to the invaginated and irregular oviductal surface, which is very different from the flattened surface of an oviduct monolayer [66, 67]. To achieve standardization in the oviduct-explant model, we decided that the ideal sperm concentration needed to be below the sperm-oviduct saturation level ( $5 \times 10^6$  spermatozoa / ml), and this was set in non-capacitating conditions at  $2 \times 10^6$  spermatozoa / ml.

Interestingly, the oviduct explants were able to selectively bind intact spermatozoa, which is in agreement with previous observations made in pigs [9] and cattle [10], where sperm-oviduct binding was shown to be a useful method for selecting and preserving plasma membrane and acrosome-intact spermatozoa. We confirmed that only membrane- and acrosome-intact spermatozoa bound to oviduct explants and that they maintained their intact status for at least 6 h. Oviduct explant binding was most efficient in incubation media devoid of capacitation supporting factors, in which sperm binding capacity was approximately twice that of incubation in capacitation supporting media. Similar results have been reported in cattle [44] and hamsters [68], and support Suarez' [1] hypothesis that only non-capacitated spermatozoa are able to bind to oviduct epithelium during estrus. The reduced sperm binding capacity observed under capacitating conditions may be a factor of a substantial population of spermatozoa undergoing membrane changes that decrease their affinity for oviduct epithelia. Sperm plasma membrane changes are an early and integral part of the stallion sperm

capacitation process [69] and are precursors to critical intracellular changes such as protein kinase A (PKA) activation and subsequent protein tyrosine phosphorylation. We showed that incubation of spermatozoa in capacitation supporting conditions ( $\text{HCO}_3^-$ , BSA and  $\text{Ca}^{2+}$ ) was a poor stimulator of protein tyrosine phosphorylation, whereas binding of spermatozoa to oviduct explants caused a spectacular increase in the percentage of sperm displaying protein tyrosine phosphorylation. On the other hand, significant release of spermatozoa from oviduct epithelia was not observed in any of the media tested. The fact that spermatozoa showed the lowest binding in the DMEM/F12 based oviduct explant culture medium that contained 10% FBS may be explained by the presence of many blocking proteins in FBS that could conceivably saturate oviduct binding receptors [70, 71].

One of the central changes triggered during sperm capacitation is the generation of hyperactivated sperm motility, which in boar sperm is a process induced by massive protein tyrosine phosphorylation [23]. Only a small proportion of the spermatozoa suspended in *in vitro* capacitation media showed a visible protein tyrosine phosphorylation response. However, in both the absence and, in particular, the presence of capacitation supporting factors, binding to pre-ovulatory oviduct explants induced protein tyrosine phosphorylation in approximately half of the spermatozoa, although hyperactivated motility was not observed. Moreover, the non-bound sperm showed no such response, indicating that prolonged direct contact with the oviduct epithelium is required for the protein tyrosine phosphorylation response. Sperm-oviduct binding is regarded in many species (pigs [9] and cattle [10]) as a mechanism to select membrane-intact, acrosome-intact and non-capacitated spermatozoa. During the 2 h co-incubation of spermatozoa with oviduct explants, a gradual increase in oviduct binding was evident. After 2 washing steps, established sperm-oviduct explants were transferred to either non-capacitating or capacitating medium. In both conditions, only minimal release of bound spermatozoa was observed during the subsequent 6 h incubation. Possibly, unknown *in vivo* factors (such as the influx of follicular fluid post-ovulation, chemo-attractant components derived from the cumulus oocyte complex entering the oviduct or simply the stream of fluids through the oviduct) may be required for sperm release. In any case, the protein tyrosine phosphorylation induced *in vitro* by sperm binding to oviduct explants was insufficient to allow sperm release from the oviduct cells. This also suggests that the unbound fraction almost certainly did not first bind to the oviduct epithelium and then detach soon after but within the 2 h co-incubation period. This conclusion is supported by the following



observations: (i) In the unbound sperm population, a minimal percentage of spermatozoa showed protein tyrosine phosphorylation. If this population had previously bound, we assume that the rates of protein tyrosine phosphorylation would have been higher. (ii) Stallion sperm incubated in capacitation media in the absence of oviduct epithelium showed similar minimal protein tyrosine phosphorylation responses rates (<10% after 6 h; data not shown).

Interestingly, a similar protein tyrosine phosphorylation response has been reported for stallion sperm suspensions when the pH of the capacitation medium was alkaline [40, 41]. It may therefore be significant that the secretory cells in the oviduct explants contained large alkaline vesicles, although these only marginally raised the pH of the incubation medium. *In vivo* however, the alkaline secretions may be diluted to a much lesser extent by oviduct fluid and may therefore induce an alkaline local microenvironment that is sufficient to induce sperm protein tyrosine phosphorylation without the need for the sperm to bind to the oviduct. Certainly, in our *in vitro* oviduct explant system, direct contact between spermatozoa and oviduct explants was necessary. Indeed, the pH of the adhered spermatozoa reached levels similar to those described in alkalinized capacitation media [40]. The direct contact of stallion spermatozoa to the oviduct epithelium was thus sufficient to induce intracellular alkalization consistent with capacitation induction. In the female reproductive tract the pH has been reported to exceed 7, reaching values of up to pH 8.0 in cervical mucus and pH 7.4 in the equine oviduct [72], although oviductal pH is also affected by the stage of the oestrous cycle [72]. Combining the various observations, it is hypothesized that a local increase in the  $\text{HCO}_3^-$  concentration is essential for stallion sperm to capacitate and subsequently fertilize [73].

During sperm transport through the female reproductive tract, the intracellular pH of a murine or human spermatozoon increases, accompanied by an additional decrease in extracellular  $\text{H}^+$  concentration, and leading to a slightly alkaline pH which is consistently lower than the extracellular pH, due to the presence of transmembrane ion pumps [74]. Our finding supports and extends previous studies describing the necessity of an alkaline environment for stallion sperm capacitation, at the level of protein tyrosine phosphorylation. The novel aspect of this study is the finding that this process can be elicited by binding to pre-ovulatory oviduct explants, which makes it likely that an analogous process takes place *in vivo*. Further research should focus on how the oviduct epithelial cell-sperm contact results in induction of protein tyrosine phosphorylation.

In contradiction to the present study, it has been shown that horse sperm binding to oviduct epithelial cell monolayers reduces  $\text{Ca}^{2+}$  metabolism leading to a temporary decrease in motility, inhibition of capacitation and increased sperm survival time [75, 76]. Besides a low intracellular  $\text{Ca}^{2+}$  concentration, Kirichok and Lishko [74] reported a high intracellular  $\text{H}^+$  concentration during this phase that they proposed to have a complementary effect on mammalian sperm survival. To date, it is clear that stallion spermatozoa can remain viable in the mare's sperm reservoir for at least 6 days [72]. In contrast to oviduct explants, monolayers do not typically retain their morphological and physiological characteristics during culture. In particular, the number of secretory cells decreases dramatically, such that the release of molecules from secretory granules is likely to be marginal when monolayers are used, whereas it is much better conserved in equine oviduct explants [45]. Due to this maintenance of secretory activity, the equine oviduct explant model almost certainly mimics *in vivo* conditions better than an epithelial cell monolayer [45]. Moreover, they loss of the alkaline secretory vesicles in cells cultured in monolayers, but their retention in oviduct explant vesicles probably explains the divergence in their effects on the physiology of bound sperm.

Our findings suggest that physical sperm-oviduct epithelium contact during the late pre-ovulatory period is important to switch on intracellular processes involved in sperm capacitation. This enhancement of capacitation may involve interaction with oviduct plasma membrane molecules or with capacitating factors derived from the secretory oviduct epithelial cells. As demonstrated by Suarez [2], species-specific carbohydrate moieties expressed on the epithelium are responsible for oviduct epithelium binding to the sperm head. This specific cell contact may prepare spermatozoa for capacitation, where the importance of physical contact is underlined by the failure of oviduct explant-conditioned medium to stimulate enhanced sperm tail protein tyrosine phosphorylation. This rules out the possibility that any "pro-capacitation factors" are simply released by the secretory epithelial cells of pre-ovulatory oviduct and are active in the surrounding milieu. We therefore hypothesize that a rise in alkaline secretory activity takes place in the mare's oviduct at the late follicular stage and that physical interaction of stallion spermatozoa with oviduct epithelial cells induces alkalization and the first stages of capacitation (membrane changes and tail-associated protein tyrosine phosphorylation).

One note of caution is that, despite the alkalization and protein tyrosine phosphorylation responses of stallion spermatozoa bound to the oviductal explants, there was little or no spontaneous release of activated spermatozoa. In comparison to pigs [23], our study did not show a correlation between protein tyrosine phosphorylation and sperm release by hyperactivation. This release is a prerequisite for fertilization; to date, only sperm incubation with procaine has been shown to reliably induce hyperactivated motility in equine sperm [77, 78].

In conclusion, we showed that oviduct explants harvested during the pre-ovulatory period selectively bound membrane-intact sperm and induced protein tyrosine phosphorylation, probably by increasing intracellular pH via direct sperm-oviduct epithelial contact. Other capacitation tests (membrane lipid order, Ca-assay, acrosome integrity and oocyte penetration) and subsequent demonstration of the triggering of the release from oviduct epithelium of spermatozoa exhibiting hyperactivated motility would greatly complement this study.

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# CHAPTER 5

## AN ALKALINE FOLLICULAR FLUID FRACTION INDUCES CAPACITATION AND LIMITED RELEASE OF OVIDUCT EPITHELIUM BOUND STALLION SPERM

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*An alkaline follicular fluid fraction induces capacitation and limited release of oviduct epithelium bound stallion sperm. Reproduction 2015; 150(3): 193–208.*



## ABSTRACT

Induction of hyperactivated motility is considered essential to trigger release of oviduct-bound mammalian spermatozoa, in preparation for fertilization. In this study, oviduct-bound stallion spermatozoa were exposed for 2 h to: (1) pre-ovulatory and (2) post-ovulatory oviductal fluid; (3) 100% and (4) 10% follicular fluid; (5) cumulus cells, (6) mature equine oocytes, (7) capacitating and (8) non-capacitating medium; none of which triggered sperm release or hyperactivated motility. Interestingly, native follicular fluid was detrimental to sperm viability, an effect that was negated by heat inactivation, charcoal treatment and 30 kDa filtration alone or in combination. Moreover, sperm suspensions exposed to treated follicular fluid at pH7.9 but not pH7.4 showed  $\text{Ca}^{2+}$ -dependent hypermotility. Fluo-4 AM staining of sperm showed elevated cytoplasmic  $\text{Ca}^{2+}$  in hyperactivated stallion spermatozoa exposed to treated follicular fluid at pH7.9 compared to a modest response in defined capacitating conditions at pH7.9 and no response in treated follicular fluid at pH7.4. Moreover, 1 h incubation in alkaline, treated follicular fluid induced protein tyrosine phosphorylation in 20% of spermatozoa. None of the conditions tested induced widespread release of sperm pre-bound to oviduct epithelium. However, the hyperactivating conditions did induce release of 70-120 spermatozoa per oviduct explant, of which 48% showed protein tyrosine phosphorylation and all were acrosome intact, but capable of acrosomal exocytosis in response to  $\text{Ca}^{2+}$  ionophore. We conclude that, in the presence of elevated pH and extracellular  $\text{Ca}^{2+}$ , a heat resistant, hydrophilic, <30 kDa component of follicular fluid can trigger protein tyrosine phosphorylation, elevated cytoplasmic  $\text{Ca}^{2+}$  and hyperactivated motility in stallion sperm, but infrequent release of sperm pre-bound to oviduct epithelium.

## INTRODUCTION

A 'sperm reservoir' is established in the oviductal isthmus of female mammals after mating and in anticipation of ovulation, and involves sperm binding via their head to oviduct epithelial cells, primarily at the level of the luminal cilia but also possibly via microvilli [1-3]. Species-specific carbohydrate moieties expressed on the epithelium have been shown to underlie the binding with the sperm head [4], while the specific nature of the cell-cell contact triggers maturation of competent spermatozoa in a series of events collectively known as capacitation [5, 6]. Recently, we and others [7-9] have reported that an alkaline micro-environment within the equine oviduct leads to a gradual increase in sperm cytoplasmic pH which, in turn, correlates with increased phosphorylation of protein tyrosine residues. *In vivo*, it is hypothesized that these events are strictly coordinated to occur just before ovulation, when the pre-ovulatory follicle is preparing to release a mature oocyte [2, 10].

Kirichok and Lishko [11] reported that both the low intracellular  $\text{Ca}^{2+}$  concentration and the high intracellular  $\text{H}^+$  concentration, that prevail inside mouse and human spermatozoa, have a complementary, positive effect on sperm survival. It was hypothesized that these events occur during the oviductal binding phase. In the peri-ovulatory period, changes in the oviductal microenvironment provoke the final step in the capacitation process, predominantly by triggering  $\text{Ca}^{2+}$  influx into oviduct-bound spermatozoa. As a result of  $\text{Ca}^{2+}$  influx, a small subpopulation of spermatozoa resumes motility, which rapidly progresses to hyperactivated motility [12] and generates sufficient force to detach the sperm from the oviduct epithelium such that they can escape from the mucosal pockets [13-15]. Supported by this newly generated power, the released and hyperactivated sperm are able to progress through the viscous fluid of the oviduct and up to the site of fertilization at the ampullary-isthmic junction [4, 13, 15, 16]. Hyperactivation also assists the spermatozoa in penetrating the cumulus matrix and is essential for penetration of the zona pellucida to allow fertilization of the oocyte [17, 18].

At present, it is not entirely clear which biological factors trigger stallion sperm hyperactivation. Possible contributors are, however, likely to be present in oviduct epithelial secretions, or around the cumulus-oocyte-complex [4, 13, 15, 16]. The molecular basis of hyperactivation is also incompletely understood. Reports in mice suggest that opening of  $\text{H}^+$

and  $\text{Ca}^{2+}$  ion channels present in the sperm plasma membrane is sufficient to allow  $\text{H}^+$  efflux from and  $\text{Ca}^{2+}$  influx to the sperm cytosol, along their respective concentration gradients [11]. The primary source of  $\text{Ca}^{2+}$  for the spermatozoon is thus extracellular. An intracellular  $\text{Ca}^{2+}$  store is also available in the lumen of the redundant nuclear envelope (RNE) located at the base of the flagellum [13] but, while this internal  $\text{Ca}^{2+}$  store could provide sufficient  $\text{Ca}^{2+}$  for the induction of hyperactivation, external  $\text{Ca}^{2+}$  influx is required to maintain intracellular  $\text{Ca}^{2+}$  at a level sufficient to sustain hyperactivation [19]. The  $\text{Ca}^{2+}$  influx can be realized by activation of specific CatSper channels, located at the principal piece of the tail [13, 20-22]. Only spermatozoa with increased intracellular pH (due to  $\text{H}^+$  efflux) are able to activate their CatSper channels, and the resulting  $\text{Ca}^{2+}$  influx induces the high amplitude, asymmetrical flagellar beating of the axoneme that is characteristic of hyperactivated motility [22-24].

In contrast to mouse sperm, the trigger that activates  $\text{Ca}^{2+}$  influx into stallion spermatozoa has yet to be identified. On the other hand, the oviduct is the site where spermatozoa should acquire hyperactivated motility, physiologically [12]. It has been hypothesized that non-capacitated spermatozoa must first bind to the oviduct epithelium before they are able to be hyperactivated and released [25]. If release of hyperactivated sperm is specific to the late follicular development stage, oviductal secretions or sperm contact with oviduct cells exposed to specific endocrine stimuli might be involved in the acquisition of hyperactivity, whereas hyperactivation induced after ovulation could be driven by factors originating from the ovulating follicle (oocyte–cumulus mass and follicular fluid). Differences in the mechanism for triggering sperm hyperactivation exist between species (man: [26]; rabbit: [27]; mouse: [28]; cattle: [29]). The current study aimed to elucidate the conditions that trigger hyperactivated motility of stallion spermatozoa in the mare's oviduct and determine whether release of capacitated sperm from oviduct epithelial cells could be achieved under *in vitro* conditions.

## **MATERIALS AND METHODS**

### **Chemicals and reagents**

Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS) (Batch: 07G8814F) and Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 (DMEM/F12) were purchased from Gibco® Life Technologies (Merelbeke, Belgium). Propidium iodide (PI), SYBR14 (LIVE / DEAD Sperm Viability Kit), Hoechst 33342, Alexa Fluor 488-conjugated goat anti-mouse antibody and fluo-4 AM were obtained from Molecular Probes (Ghent, Belgium). Monoclonal 4G10@Platinum, anti-phosphotyrosine mouse antibody was obtained from Millipore (Overijse, Belgium). Triton X-100, PNA-FITC, fatty acid-free bovine serum albumin (A9418; cell culture tested), ethylenediaminetetraacetic acid (EDTA), procaine hydrochloride, Ca<sup>2+</sup> ionophore A23187 and all other chemicals not listed otherwise were obtained from Sigma-Aldrich (Bornem, Belgium).

### **Animals**

Oviducts were collected at a slaughterhouse (Euro Meat Group, Moeskroen, Belgium) soon after the slaughter of healthy warmblood mares aged between 5 and 22 years and without any visible reproductive tract pathologies [9]. Only oviducts from mares with a large follicle (>35 mm diameter) in combination with estrous oedema in the uterine wall, indicating imminent ovulation, were used to prepare oviduct explants.

### **Preparation of oviduct explants**

Five oviducts per experiment were prepared as previously described by Nelis *et al.* [30]. Briefly, epithelial cells were harvested from oviducts of preovulatory mares by scraping the mucosa at the ampullary-isthmic junction of the longitudinally incised oviduct. The harvested cellular material was washed and re-suspended twice in 3 ml fresh HEPES-buffered TALP washing medium [31]. Next, the cells were cultured overnight in DMEM/F12 with 10% FBS at 38.5 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The time from slaughter of mares to cell culture was approximately 3 to 4 h.

### **Semen collection and preparation**

Semen was collected from three stallions of proven fertility using a Colorado model artificial vagina (Animal Reproduction Systems; Chino, CA, USA). The raw ejaculate was filtered through gauze to remove the gel fraction and any debris. Samples with good motility (>70% motile sperm) were transported immediately to the laboratory for further processing. One ml of fresh semen was then centrifuged over a 45 / 90% Percoll® gradient [32, 33]. Next, the sperm pellet was washed once with Whitten's medium ('non-capacitating medium': 100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 5.5 mM glucose, 22 mM HEPES, 2.4 mM sodium lactate and 1.0 mM pyruvic acid; pH=7.4 and 280-300 mOsm / kg; [34] and subsequently diluted to a concentration of 20 x 10<sup>6</sup> spermatozoa / ml in non-capacitating medium. Each experiment was performed using one ejaculate from each of the three stallions. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/175 and EC2013/176).

### **Sperm-oviduct explant binding and induction of capacitation**

We previously described the establishment of sperm-oviduct explants in which bound spermatozoa can be induced to exhibit protein tyrosine phosphorylation in their tail [9]. In brief, oviduct explants were cultured in a DMEM/F12-based medium, and equilibrated with 5% CO<sub>2</sub> in a humidified atmosphere at 38.5°C, as described by Nelis *et al.* [30]. Sperm binding to oviduct explants (5 per 45 µl droplet) was performed in non-capacitating medium at 38.5°C in air. Capacitating medium was made by substituting the sodium lactate in non-capacitating medium with 2.4 mM CaCl<sub>2</sub> and adding 25 mM NaHCO<sub>3</sub> and 7 mg / ml BSA (pH=7.4 and 280-300 mOsm / kg; osmolality was adjusted by adding the NaCl in steps) (adapted from [34]). In general, a final concentration of 2 x 10<sup>6</sup> spermatozoa / ml was used. After a 2 h co-incubation in non-capacitating medium, sperm-oviduct explants were washed twice and transferred to capacitating / hyperactivating medium for 2 h. Each replicate was performed with one ejaculate from a different stallion.

### **Collection of fluids and cells that sperm may contact in the sperm reservoir**

For each experiment, pre- and post-ovulatory stage oviducts, and ovaries containing a pre-ovulatory follicle (diameter > 35mm), were collected at the slaughterhouse and immediately transported to the laboratory. Oviducts were trimmed and flushed successively with 1 ml of capacitating medium. Next, the oviduct flushing fluid was filtered through a 0.2 µm Acrodisc® Syringe Filter with a Supor® Membrane (Pall® Corporation Life Sciences, Ghent, Belgium).

Follicular fluid (FF; pH ≈ 7.2) was collected from five pre-ovulatory follicles (diameter > 35mm). Cumulus-oocyte complexes were recovered from the aspirated FF and matured by incubation in FF for 28 h at 38.5°C in 5% CO<sub>2</sub> in air. Subsequently, oocytes were denuded of their cumulus investment by gentle mouth pipetting. The remaining FF was centrifuged at 100xg for 10 min at 22°C before the supernatant was filtered as described for oviduct flushing fluids. Both cell suspensions and collected fluids were incubated for 2 h with sperm bound to oviduct explants to evaluate their effects on sperm hyperactivation and sperm release.

### **Treatment of FF**

Freshly filtered (2 µm diameter) FF (pH ≈ 7.2) was treated in 5 different ways: (1) centrifuged through a 100 kDa filter (10 min at 14000g; Amicon Ultra-0.5 ml Centrifugal Filters for Protein Purification and Concentration: Millipore, Overijse, Belgium) to see whether the effects of whole FF were elicited by molecules <100 kDa; (2) by heat inactivation in a 55°C water bath for 0.5 h [35-37] to examine whether the FF factor was heat resistant; (3) charcoal treatment as described by Cheng *et al.* [38] to examine whether a lipid or lipid-bound factor, including steroid hormones [39], was implicated. For charcoal treatment, pooled FF was stirred at ambient temperature for 45 minutes with 50 mg charcoal / ml (Norit: activated and neutralized; Sigma, Bornem, Belgium) and then centrifuged at 4500g for 1 h at 4°C. The supernatant was filtered through a 0.2 µm Acrodisc® Syringe Filter with a Supor® Membrane (Pall® Corporation Life Sciences, Ghent, Belgium), to remove any remaining charcoal particles; (4) centrifuged through a 30 kDa filter (30 min-3000g; Vivaspin 15R, Sartorius Biolab Products, Goettingen, Germany) to examine whether the FF factor MW was <30 kDa; (5) triple treated to see if the effects were still apparent after successive heat-



inactivation, charcoal treatment and 30 kDa filtration; and (6) some heat-inactivated, charcoal-treated, 30 kDa-filtered or triple-treated FF samples were incubated in air until the pH rose to 7.9. Similar treatments were performed with capacitating medium. All treated FF samples were stored in aliquots at -80°C until further use. To prevent pH changes in elevated pH treated FF and capacitating medium during sperm incubation, experiments were performed in closed 1 ml tubes (Greiner bio-one, Vilvoorde, Belgium) to prevent contact with air.

### **Sperm viability assay**

To examine the membrane integrity of spermatozoa attached to or released from oviduct explants, spermatozoa and sperm-oviduct explants were respectively washed by centrifugation (600g for 5 min) or transferred to pre-warmed DPBS (37°C) and stained with the nucleic acid stains SYBR14 (20 µM) and propidium iodide (PI; 50 nM) (LIVE / DEAD Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands) [40]. Stained spermatozoa and sperm-oviduct explants were washed and mounted on pre-warmed glass slides (Marienfeld, Lauda-Königshofen, Germany). Sperm with green fluorescence-labelled nuclei were considered viable, whereas sperm with red-stained nuclei were considered dead. Finally, the percentages of viable spermatozoa were scored by counting 200 randomly selected released spermatozoa. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters at a magnification of 400x.

### **Quantification of tail-associated protein tyrosine phosphorylation**

Incubated sperm preparations were washed twice and fixed in 4% paraformaldehyde in DPBS at room temperature for 15 min as described previously [9]. Fixed spermatozoa were washed and incubated in 0.1% Triton X-100 in DPBS for 10 min at room temperature. The permeabilized spermatozoa were then incubated in blocking buffer (DPBS containing 1% BSA) for 10 min at room temperature. Next, spermatozoa were incubated overnight at 4°C in buffer containing 0.1% BSA and the mouse monoclonal 4G10®Platinum IgG<sub>2b</sub> protein anti-phosphotyrosine antibody (diluted 1:500). After incubation, spermatozoa were washed and stained with a monoclonal goat anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Molecular Probes, Ghent, Belgium) for 1 h at room temperature. The immune

labelled spermatozoa were mounted on glass slides under a cover slip. The proportion of spermatozoa with green fluorescent tails among the total sperm population (with Hoechst 33342 fluorescent heads) was determined by randomly scoring 200 spermatozoa. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400x.

### **Sperm acrosome status**

The acrosome status of spermatozoa was assessed using fluorescein-conjugated peanut agglutinin (PNA-FITC) to discriminate acrosome-intact spermatozoa from acrosome deteriorated or reacted spermatozoa [41]. In brief, after fixation in 4% (w/v) paraformaldehyde in DPBS for 15 min at room temperature, spermatozoa were washed in DPBS and permeabilized in 0.1% Triton X-100 in DPBS for 10 min at room temperature [9]. After washing, spermatozoa were stained for 15 min at room temperature with 1  $\mu\text{g}$  / ml PNA-FITC and subsequently washed and mounted as described above. Spermatozoa with PNA-FITC-labelled acrosome regions were considered acrosome-intact, whereas spermatozoa with no fluorescence over the acrosomal region were considered acrosome reacted or deteriorated. The percentages of acrosome-intact spermatozoa were scored by examining 200 spermatozoa using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400x.

### **Cytoplasmic $\text{Ca}^{2+}$ imaging in stallion spermatozoa**

Stallion spermatozoa were washed in non-capacitating medium and stained for 30 min in 5  $\mu\text{M}$  of the  $\text{Ca}^{2+}$ -sensitive dye fluo-4 AM in non-capacitating medium at 38.5  $^{\circ}\text{C}$ . Spermatozoa were washed and incubated for an additional 20 min at 38.5  $^{\circ}\text{C}$  to allow de-esterification of the fluo-4 AM probe before analysis. Subsequently, fluo-4 AM loaded sperm suspensions ( $10 \times 10^6$  spermatozoa / ml) were incubated for 0.5 h in different capacitating or hyperactivating media. Next, the sperm suspensions were mounted on glass slides as described previously. The intensity of the Fluo-4 AM signal correlates with the cytoplasmic  $\text{Ca}^{2+}$  concentration. In this study, we used the cytoplasmic  $\text{Ca}^{2+}$  signal from sperm incubated in capacitating medium at pH 7.4 as the reference intensity. Increased cytoplasmic  $\text{Ca}^{2+}$  signal during incubation was taken to indicate external  $\text{Ca}^{2+}$  dependent capacitation or hypermotility.

The percentages of spermatozoa with increased cytoplasmic  $\text{Ca}^{2+}$  were scored by examining 200 spermatozoa and using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 1000x.

### **Sperm motility assessment**

Sperm motility patterns were assessed using a CCD ICD-46E camera (Ikegami Tsushinki Co. Ltd., Japan) attached to an Olympus IX70 inverted microscope (Olympus Belgium N.V., Aartselaar, Belgium). Images were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium).

Motility parameters of spermatozoa in suspension were evaluated using a computer-assisted sperm analyzer (CASA: Hamilton-Thorne Ceros 12.3). Under defined capacitating conditions, BSA was replaced with PVA to avoid the sperm agglutination observed after centrifugation in BSA-containing medium [42]. For each analysis, 10  $\mu\text{l}$  of sperm suspension diluted in the various treated FF or capacitating media was mounted on a pre-warmed glass slide (Marienfeld, Lauda-Königshofen, Germany) and maintained at 37°C using a Tokai Hit thermo plate. Five randomly selected microscopic fields in the center of the slide were scanned 4 times each, generating 20 scans for every sample. The mean of the 5 scans for each microscopic field was used for statistical analysis. The settings of the CASA-software HTR 12.3 for analyzing motility parameters of stallion sperm were based on Loomis and Graham [43] and described previously by Hoogewijs *et al.* [44]. To evaluate hyperactivated motility, 6 different parameters were initially evaluated: total motile sperm (TM; %), progressively motile sperm (PM; %), amplitude of lateral head displacement (ALH;  $\mu\text{m}$ ), curvilinear velocity (VCL;  $\mu\text{m} / \text{s}$ ), straightness (STR) and linearity (LIN). These parameters help detect increased vigour and asymmetry of flagellar movement during hyperactivation and have been associated with hyperactivated motility in bovine [29] and equine [41] sperm. Ultimately, the amplitude of lateral head displacement (ALH) and the curvilinear velocity (VCL) proved to be most useful for classifying hyperactivated motility in stallion sperm [42, 45].

### **Microscopic evaluation of stained spermatozoa**

Sperm stained for either membrane integrity, acrosome status or tail-associated protein tyrosine phosphorylation (PY+) were detected by means of fluorescence microscopy (400x) using a Leica DMR microscope equipped with excitation filters of 360–590 nm and a 100 W mercury lamp. Alexa Fluor 488-conjugated goat anti-mouse antibody, SYBR14, PNA-FITC, Fluo-4 AM, propidium iodide (PI) and Hoechst 33342 were excited using 495 nm, 488 nm, 495 nm, 494 nm, 536 nm and 343 nm, respectively. Emission spectra of the dyes were then filtered at 519 nm, 521 nm, 519 nm, 516 nm, 617 nm and 483 nm. These emission spectra were detected by blue (LP 425 nm), green (LP 515 nm), and red (BP 645/75 nm) filters corresponding to the emission peaks of the dyes. Images were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium).

### **Sperm and oviduct explant co-incubation**

Groups of five sperm-oviduct explants established in non-capacitating medium, were incubated for 2 h in 50  $\mu$ l droplets under oil containing (1) fluid flushed from the oviducts of mares slaughtered pre-ovulation; (2) fluid flushed from post-ovulation mare oviducts; (3) 100% FF; (4) 10% FF diluted in capacitating medium; (5) capacitating medium containing equine cumulus cells; (6) capacitating medium containing mature (MII) equine oocytes; (7) capacitating medium and (8) non-capacitating medium; all conditions were tested at both pH 7.4 and pH 7.9 except non-capacitating medium pH 7.9. Counting of the number of spermatozoa bound per mm<sup>2</sup> sperm-oviduct explant was blinded to treatment groups.

Subsequently, sperm incubated in pure fresh FF (0, 10 or 100%) at pH 7.4 and 7.9, were scored for plasma membrane and acrosome integrity and for CASA motility parameters. Additionally, the effect of fresh FF on oviduct explant viability was evaluated by examining ciliary activity and cell membrane integrity.

Next, viability parameters of suspended sperm incubated in (1) 100 kDa filtered fresh FF, (2) heat-inactivated FF, (3) charcoal-treated FF, (4) 30 kDa filtered fresh FF and (5) triple treated (heat-inactivated, charcoal-treated and 30 kDa filtered) FF were examined during a 6 h incubation.

Subsequently, the various motility parameters indicating hyperactivated motility of suspended sperm ( $10 \times 10^6$  spermatozoa / ml) incubated in 100% heat-inactivated FF, 100% charcoal-treated FF, 30 kDa filtered FF and 100% triple treated FF at both pH 7.4 and pH 7.9 were evaluated by CASA over time.

After 0.5 h of incubation in (1) heat-inactivated FF, (2) charcoal-treated FF, (3) 30 kDa filtered FF, (4) triple treated FF and (5) capacitating medium, the lateral head displacement and curvilinear velocity (indicating hyperactivated motility) of spermatozoa in suspension ( $10 \times 10^6$  spermatozoa / ml) were monitored. All FF conditions were tested at both pH 7.9 and 7.4 in 3 concentrations: (1) 100% FF, (2) 50% FF and (3) 10% FF. In addition, the  $\text{Ca}^{2+}$  dependency of hyperactivated motility was assessed in all undiluted treated FF and capacitating medium at pH 7.9 and 7.4 by adding 2 mM EDTA.

Next, the % spermatozoa that showed cytoplasmic  $\text{Ca}^{2+}$  influx was assessed by fluo-4 AM in sperm ( $10 \times 10^6$  spermatozoa / ml) incubated for 0.5 h in (1) capacitating medium, (2) 100% heat-inactivated FF, (3) 100% charcoal-treated, (4) 100% 30 kDa filtered FF and (5) 100% triple treated FF at both pH 7.4 and pH 7.9.

After 1 h incubation, membrane integrity, protein tyrosine phosphorylation and acrosome status were assessed in 9 different conditions at elevated pH 7.9: (1) 100% and (2) 10% heat-inactivated FF, (3) 100% and (4) 10% charcoal-treated FF, (5) 100% and (6) 10% 30 kDa filtered FF, (7) 100% and (8) 10% triple treated FF and (9) capacitating medium. Subsequently, the functionality of the sperm to undergo the acrosome reaction was scored after incubation in identical FF conditions (pH 7.9) followed by exposure to  $5 \mu\text{M}$   $\text{Ca}^{2+}$  ionophore A23187 for 30 min.

In addition, sperm-oviduct explants incubated in capacitating media for 2 h were washed twice and transferred to (1) 100% and (2) 10% heat-inactivated FF; (3) 100% and (4) 10% charcoal-treated FF; (5) 100% and (6) 10% 30 kDa filtered FF, (7) 100% and (8) 10% triple treated FF, (9) 5 mM procaine hydrochloride in capacitating medium and (10) capacitating medium, to determine whether induction of hyperactivation in spermatozoa bound to oviduct explants induced sperm release. Two pH's (7.4 and 7.9) were tested for each condition. The number of released spermatozoa per oviduct explant and the change in the number of spermatozoa bound per  $\text{mm}^2$  sperm-oviduct explant were counted, blind to the treatment.

Ultimately, released spermatozoa in all the hyperactivating conditions (100% heat-inactivated FF pH 7.9, 100% charcoal-treated FF pH 7.9, 100% 30 kDa filtered FF pH 7.9, 100% triple treated FF pH 7.9, 5 mM procaine in capacitating medium pH 7.4 and 5 mM procaine in capacitating medium pH 7.9) were assessed after 2 h for membrane and acrosome integrity and tail-associated protein tyrosine phosphorylation.

### Statistical analysis

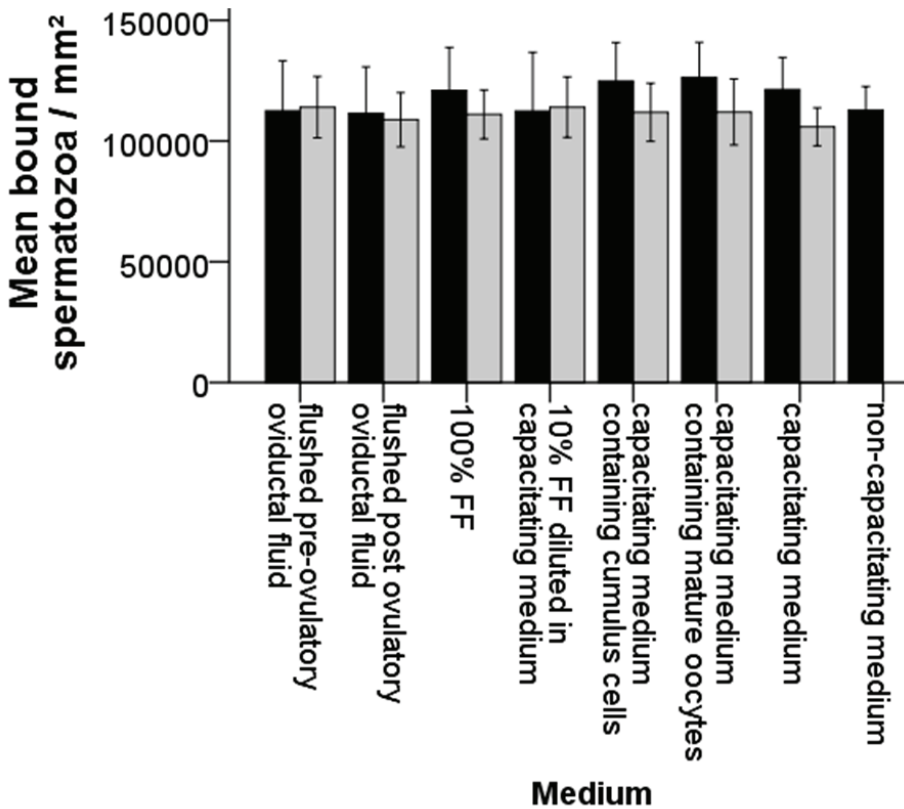
The effects of treatments on sperm parameters were assessed by analysis of variance (ANOVA). Significant differences in the number of spermatozoa released from oviduct binding, displaying motility parameters indicating hyperactivated motility (TM, PM, ALH, VCL, STR and LIN) and the percentages of viable spermatozoa were determined using repeated measures ANOVA with Greenhouse-Geisser and Bonferroni corrections, as implemented in the general linear model. The same statistical test was used to assess changes in the percentage of tail-associated protein tyrosine phosphorylated (PY+), acrosome reacted spermatozoa or spermatozoa with an increased cytoplasmic  $Ca^{2+}$  concentration over time. Scheffé post-hoc tests were performed for pairwise comparisons. Statistical analysis and graph plotting were performed using SPSS version 20 for Windows (SPSS IBM, Brussels, Belgium). Differences were considered significant if  $P < 0.05$ .

## RESULTS

### **Oviduct epithelium-bound sperm cells are not released by exposure to fluids and cells that they may encounter during sperm-oviduct binding, at either pH 7.4 or pH 7.9**

At the time of ovulation *in vivo*, the equine oviduct is thought to have an alkaline micro-environment [9]. At this time, a population of oviduct-bound sperm is released from the sperm reservoir to proceed to the ampulla of the mare's oviduct and accomplish fertilization, after induction of hyperactivated motility. In the first experiment, oviduct explants with bound stallion spermatozoa (approximately 120,000 spermatozoa bound per  $mm^2$ ; Figure 1) were incubated at both pH 7.4 and 7.9 and exposed to the various fluids and cells that they should encounter *in vivo*. After 2 h of sperm-oviduct explant incubation at both pH 7.4 and 7.9, no

significant sperm release was observed following exposure to either: (1) fluid flushed from the oviducts of mares slaughtered pre-ovulation; (2) fluid flushed from post-ovulation mare oviducts; (3) 100% FF (4); 10% FF diluted in capacitating medium; (5) capacitating medium containing equine cumulus cells, (6) capacitating medium containing mature (MII) equine oocytes, (7) capacitating medium and (8) non-capacitating medium pH 7.4 ( $P > 0.33$  for all comparisons; Figure 1). The few spermatozoa released in each treatment were nearly all membrane-damaged and immotile (data not shown).

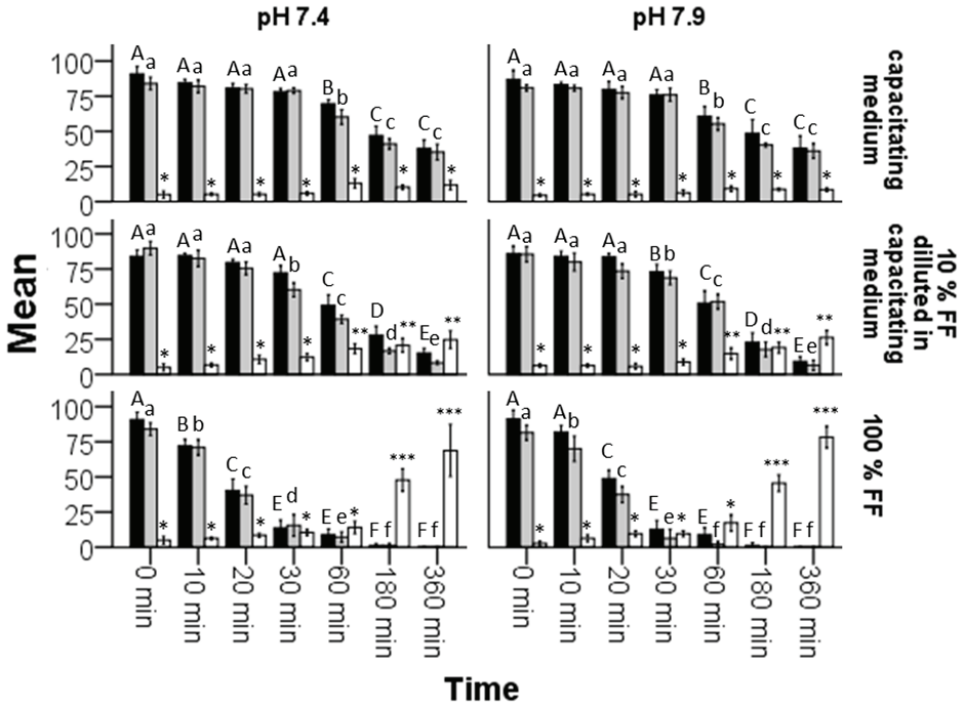


**Figure 1.** Mean ( $\pm$  s.d.) number of stallion spermatozoa bound per  $\text{mm}^2$  equine oviduct epithelium, assessed after 2 h incubation at both pH 7.4 (black bars) and 7.9 (grey bars) with; (1) oviductal flush fluid from mares slaughtered pre-ovulation; (2) oviductal flush fluid from mares slaughtered post-ovulation; (3) 100% fresh follicular fluid (FF); (4) 10% fresh FF in capacitating medium; (5) equine cumulus cells in capacitating medium; (6) mature (MII) equine oocytes in capacitating medium; (7) capacitating medium; or (8) non-capacitating medium (control) ( $P > 0.05$ ). Data represent four replicates ( $n = 40$  per group) analyzed by one-way ANOVA with post hoc Bonferroni tests for pairwise comparisons.

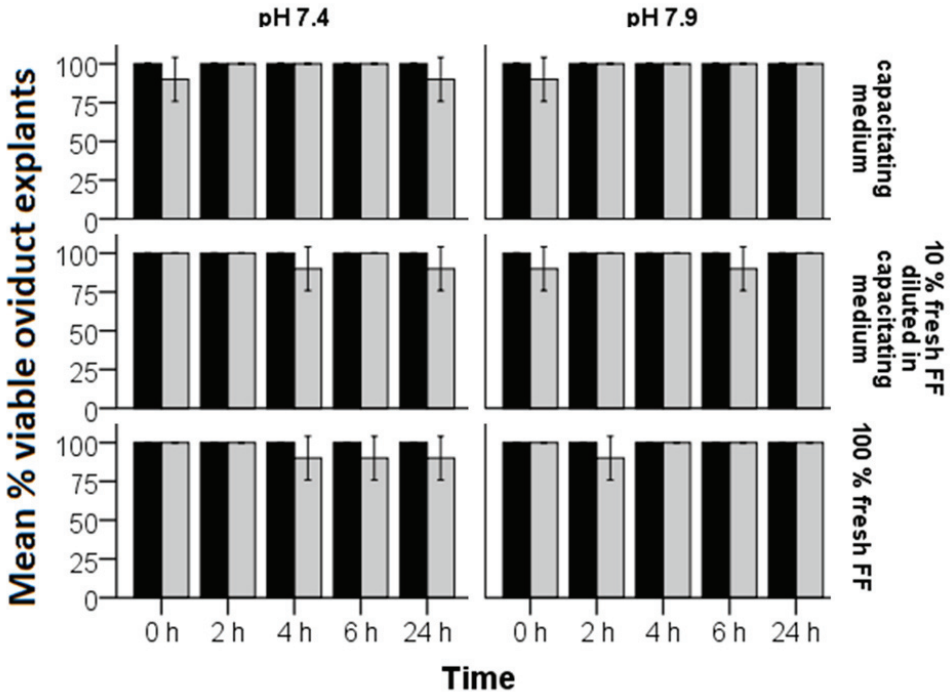
**Fresh FF has a detrimental effect on sperm membrane integrity and motility at both pH 7.4 and 7.9**

Fresh FF had a detrimental effect on sperm at both pH 7.4 and 7.9, regardless of whether the sperm were bound to oviduct explants or in suspension. The detrimental effects on sperm membrane and acrosome integrity were apparent in undiluted and in 10-fold diluted FF, albeit that at the 10-fold dilution the deteriorating effects of FF were ameliorated (see Figure 2). In each of the three incubation conditions at pH 7.4, a significant decrease in the percentage of sperm with intact membranes was observed over time, such that after 6 h in capacitating (0% FF) conditions significantly more spermatozoa remained membrane-intact ( $38 \pm 6\%$ ) than after incubation in 10% fresh FF ( $15 \pm 3\%$ ) ( $P < 0.001$ ). When sperm suspensions were incubated in 100% fresh FF, the deterioration was even more rapid ( $P < 0.001$  for all comparisons between the 3 different FF conditions except time point 0 h). After 30 min incubation, only  $14 \pm 6\%$  of the sperm population in 100% FF was still membrane-intact, and after 1 h less than 10% of the sperm were still viable. The percentage of motile sperm showed a similar deterioration to membrane integrity over time for the three incubation conditions. The destructive effect of FF on plasma membrane integrity and motility did not immediately cause deterioration of the acrosome (Figure 2). Only after more than 1 h of incubation was acrosome deterioration observed in 10% ( $21 \pm 5\%$  after 6 h;  $P = 0.02$ ) and 100% ( $48 \pm 8\%$  after 3 h;  $P < 0.001$ ) FF conditions, although this was more marked than in capacitation medium where no significant induction of acrosome deterioration was seen ( $8 \pm 4\%$  after 6 h; Figure 2). Very similar changes were observed using the same conditions at pH 7.9 (Figure 2). Thus, regardless of pH (7.4. vs 7.9), fresh FF caused concentration dependent sperm deterioration. In contrast, fresh FF did not detrimentally affect the viability of the oviduct epithelial cells lining the explants. The oviduct explants remained viable for at least 24 h when incubated in 10% or 100% fresh FF at pH 7.4, and the epithelial cells displayed similar ciliary activity between media and over time ( $100 \pm 0\%$ ); almost all cells ( $>99\%$ ) of nearly all explants ( $95 \pm 9\%$ ;  $P = 0.84$ ) remained membrane-intact. Similar findings applied for incubation at pH 7.9 (Figure 3).





**Figure 2.** Stallion sperm viability during 6 h incubation at both pH 7.4 and 7.9 in capacitating medium, 10% fresh follicular fluid (FF) diluted in capacitating medium and 100% fresh FF. The graphs depict mean ( $\pm$  s.d.) percentages of membrane-intact (black bars), motile (dark grey) and acrosome-reacted spermatozoa (light grey) ( $n=200$  spermatozoa per group) for three replicates. For membrane-intact spermatozoa, values that differ significantly are indicated by different capitals. For motile spermatozoa, values that differ significantly are indicated by different small letters. For percentage acrosome-detriorated spermatozoa, values that differ significantly are indicated by different numbers of asterisks. Analysis was performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons.

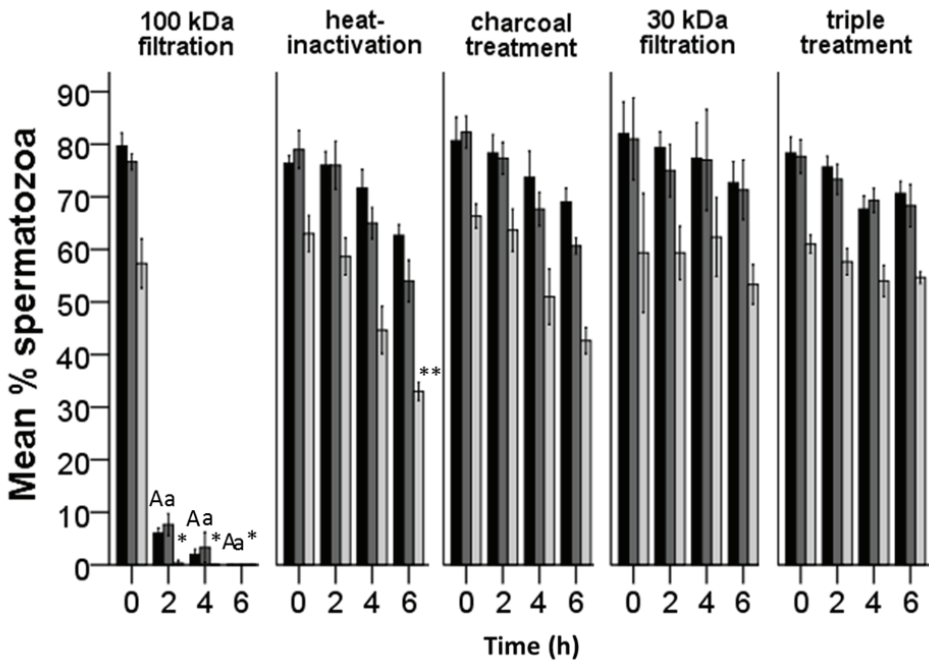


**Figure 3.** Viability of equine oviduct explants during 24 h incubation at both pH 7.4 and 7.9 in capacitating medium, 10% fresh follicular fluid (FF) and 100% fresh FF. Data represent mean ( $\pm$  SD) percent of oviduct explants ( $n=30$  per group) showing ciliary activity (black bars) or intact cell membranes (light grey bars) over three replicates. Statistical analysis was performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons.

### A 30-100 kDa heat-instable lipophilic FF factor is responsible for sperm deterioration

Fresh FF was treated in 5 different ways to elucidate properties of the sperm deteriorating substance(s): (1) centrifugation through a 100 kDa filter (to remove large proteins), (2) heat inactivation (to neutralize tertiary and quaternary folded proteins, including complement factors, by protein denaturation), (3) charcoal treatment (to remove lipids / lipoproteins and lipophilic molecules, including steroids), (4) centrifugation through a 30 kDa filter (filtered FF containing  $<30$  kDa components) and (5) triple treatment (combination of heat inactivation, charcoal treatment and 30 kDa filtration). The fresh FF fraction subjected to 100 kDa filtration, retained the membrane damaging and motility reducing effects on sperm in suspension (Figure 4). By contrast, heat inactivation, charcoal treatment, 30 kDa filtration and

triple treatment all resulted in a complete abolishment of the detrimental effects of FF on sperm in suspension (Figure 4;  $P < 0.001$ ). All FF fractions which received the former treatments are further referred to as “treated FF”. In this respect, after 6 h incubation in treated FF (heat-inactivated, charcoal-treated, 30 kDa filtration or triple treated) percentages of motile and progressively motile sperm were  $> 55\%$  and  $> 35\%$ , respectively. For spermatozoa incubated in 100 kDa filtered FF, both total and progressive motility dropped to  $< 1\%$  ( $P < 0.001$ ; Figure 4). Thus, the FF component detrimental to sperm integrity is apparently a heat instable, 30-100 kDa lipophilic entity.

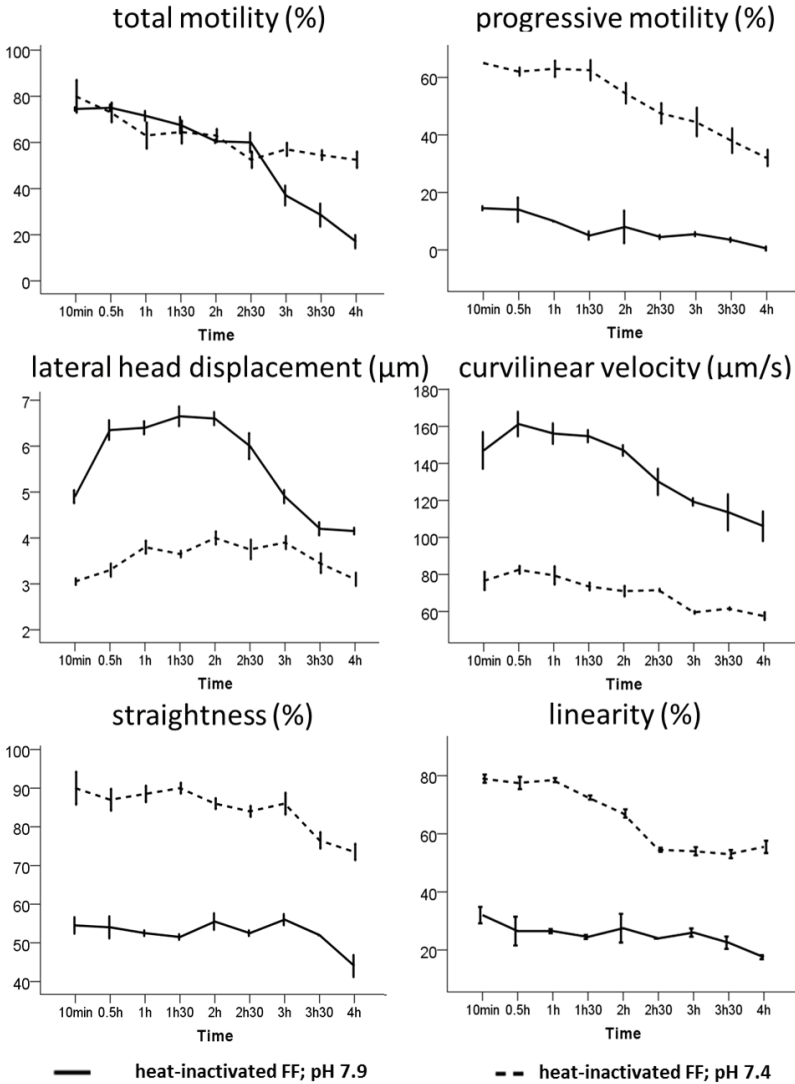


**Figure 4.** Viability of stallion sperm during a 6 h incubation in 100% FF treated in five different ways: 100 kDa filtration, heat-inactivation, charcoal treatment, 30 kDa filtration and triple treatment (heat inactivation, charcoal treatment and 30 kDa filtration). Sperm maintained viability during incubation when fresh FF was heat-inactivated, charcoal-treated, filtration through a 30 kDa filter or triple treated. Data represent mean ( $\pm$  s.d.) percent membrane-intact (black bars), motile (dark grey) or progressively motile (light grey) spermatozoa ( $n = 200$  spermatozoa per group) for three replicates. For membrane-intact spermatozoa, values that differ significantly are indicated by capitals. For total sperm motility, values that differ significantly are indicated by small letters. For progressive sperm motility, values that differ significantly are indicated by asterisks. Statistical analysis was performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons.

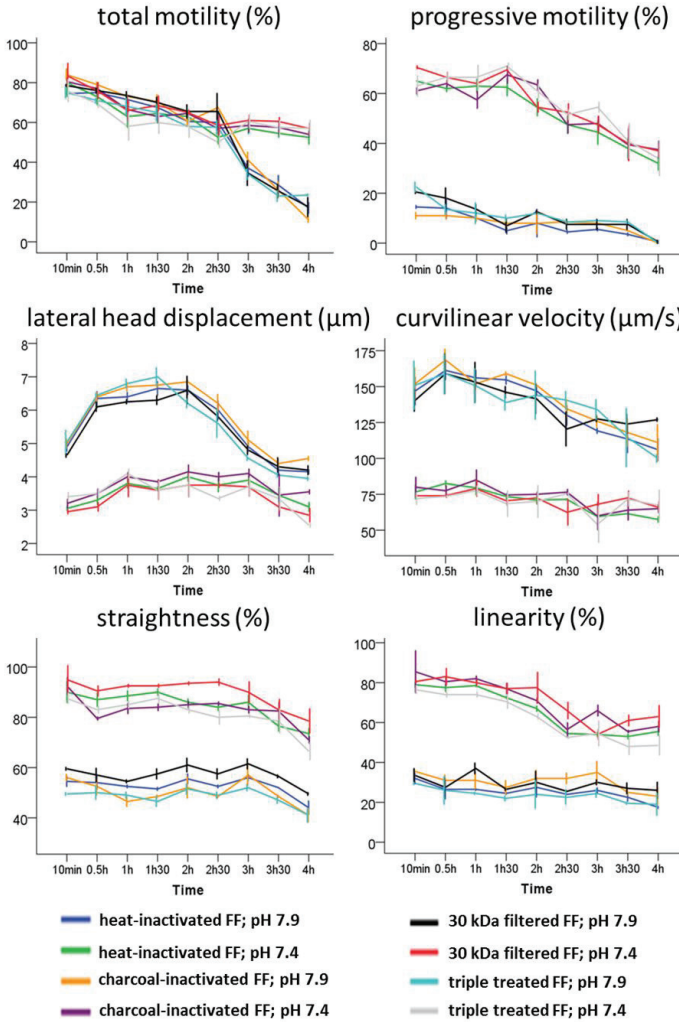
## Treated FF induces hyperactive motility responses in suspended sperm at pH 7.9 but not pH 7.4

As described previously [9], oviduct-bound spermatozoa undergo intracellular alkalinization and subsequent protein tyrosine phosphorylation in association with a locally elevated pH. While fresh pre-ovulatory FF samples had a standard physiological pH ( $7.2 \pm 0.4$ ;  $n=5$  samples;  $p=0.26$ ), we found that increasing medium pH to 7.9 in the presence of treated FF was sufficient to induce hyperactivation in stallion sperm in suspension that had not had any contact with oviduct tissues (Figure 5 and 6, and supplementary figure).

At different incubation time points (10 min, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours), six different CASA parameters associated with hyperactive motility (total motility, progressive motility, lateral head displacement, curvilinear velocity, straightness and linearity) were significantly different between spermatozoa incubated in treated FF at physiological pH (7.4) and spermatozoa incubated at slightly alkaline pH (7.9) ( $p<0.001$ ). With respect to time, sperm suspensions in pH 7.9 treated FF showed a significant rise in ALH and VCL (Loux *et al.* 2013, McPartlin *et al.* 2009) within 0.5 h of incubation, values were respectively: heat-inactivated FF,  $6.3 \pm 0.2 \mu\text{m}$  and  $161 \pm 7 \mu\text{m} / \text{s}$ ; charcoal-inactivated FF,  $6.4 \pm 0.1 \mu\text{m}$  and  $169 \pm 8 \mu\text{m} / \text{s}$ ; 30 kDa filtered FF,  $6.1 \pm 0.1 \mu\text{m}$  and  $159 \pm 14 \mu\text{m} / \text{s}$ ; triple treated FF,  $6.5 \pm 0.1 \mu\text{m}$  and  $159 \pm 13 \mu\text{m} / \text{s}$ . These ALH and VCL values remained unchanged until 2.5 h (Figure 5 and supplementary figure). After 2.5 h of incubation, ALH and VCL decreased significantly and a marked drop in total motility was observed that indicated a loss of viability (Figure 5 and supplementary figure). In contrast, sperm exposed to similarly treated FF at pH 7.4 remained progressively motile with only a slight decrease during the 4 h incubation (at 0.5 h; ALH and VCL: heat-inactivated FF,  $3.3 \pm 0.1 \mu\text{m}$  and  $83 \pm 3 \mu\text{m} / \text{s}$ ; charcoal-inactivated FF,  $3.5 \pm 0.1 \mu\text{m}$  and  $78 \pm 4 \mu\text{m} / \text{s}$ ; 30 kDa filtered FF,  $3.1 \pm 0.2 \mu\text{m}$  and  $74 \pm 2 \mu\text{m} / \text{s}$ ; triple treated FF,  $3.5 \pm 0.3 \mu\text{m}$  and  $74 \pm 1 \mu\text{m} / \text{s}$ ). In summary, during incubation in all four types of treated FF at pH 7.9, hyperactivation peaked during 0.5-1 h and had begun to decrease by 2.5 h of incubation, whereas similar conditions at pH 7.4 did not induce a similar change in motility (Figure 5 and supplementary figure).



**Figure 5.** Evaluation of different motility parameters (% total motile, % progressively motile, lateral head displacement, curvilinear velocity, straightness and linearity) during 4 h incubation of stallion sperm suspended in (1) 100% heat-inactivated follicular fluid (FF) at pH 7.9 (full black line) and (2) 100% heat-inactivated FF at pH 7.4 (dotted black line). Heat-inactivated FF stimulated sperm hypermotility at pH 7.9 but not at pH 7.4. Very similar observations were obtained for charcoal-treated, 30 kDa filtered and triple-treated FF preparations (see supplementary figure). Data represent mean ( $\pm$  s.d) for total (%) and progressively motile (%) spermatozoa, amplitude of lateral head displacement ( $\mu\text{m}$ ), curvilinear velocity ( $\mu\text{m} / \text{s}$ ), straightness (%) and linearity (%) ( $n=5$  samples in each group) for three replicates. Analysis was performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons.



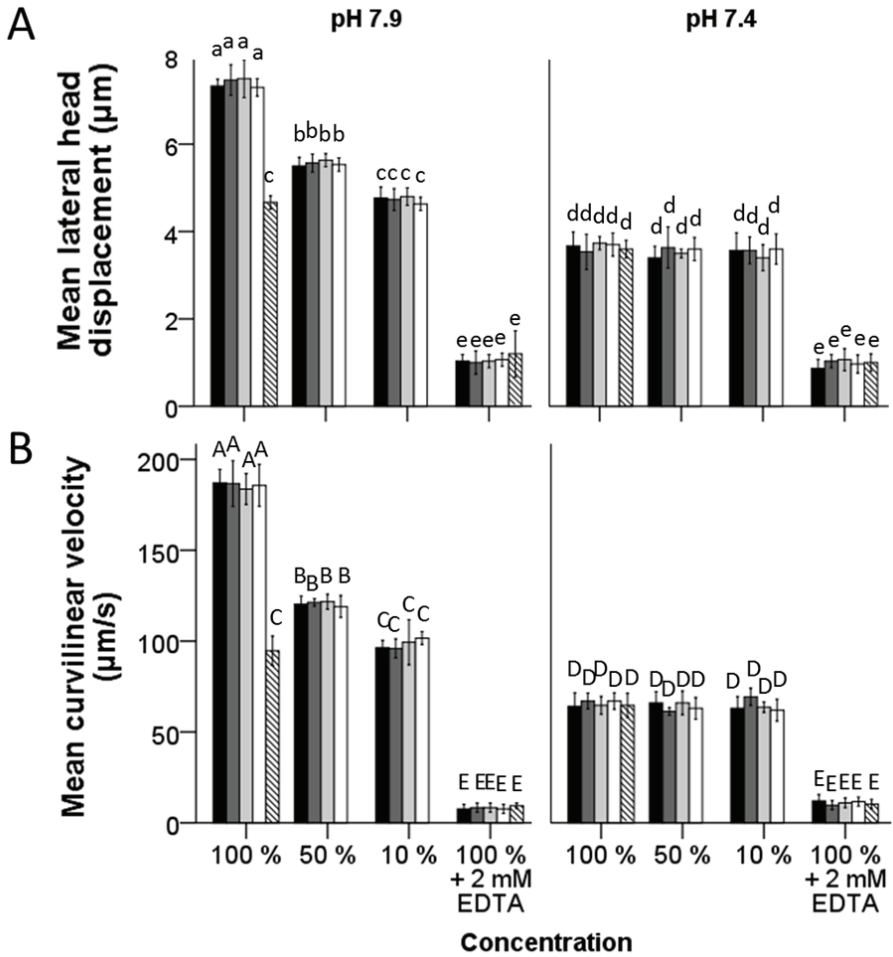
**Supplementary figure.** Evaluation of various motility parameters (% total motile, % progressive motile, lateral head displacement, curvilinear velocity, straightness and linearity) during 4 h incubation of stallion sperm in (1) 100% heat-inactivated FF at pH 7.9 (dark blue line), (2) 100% heat-inactivated FF at pH 7.4 (green line), (3) 100% charcoal-treated FF at pH 7.9 (orange line), (4) 100% charcoal-treated FF at pH 7.4 (purple line), (5) 100% 30 kDa filtered FF at pH 7.9 (black line), (6) 100% 30 kDa filtered FF at pH 7.4 (red line), (7) 100% triple treated FF at pH 7.9 (light blue line) and (8) 100% triple treated FF at pH 7.4 (grey line). At pH 7.9, all of heat-inactivated, charcoal-treated, 30 kDa filtered and triple treated FF supported sperm hypermotility whereas none did so at pH 7.4. Data represent mean ( $\pm$  s.d) of total motile (%), progressively motility (%), amplitude of lateral head displacement ( $\mu\text{m}$ ), curvilinear velocity ( $\mu\text{m} / \text{s}$ ), straightness (%) and linearity (%) ( $n=5$  samples in each group) for three replicates. Analysis was performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons.

### **Treated FF induced concentration dependent hypermotility responses**

Alkaline pH (7.9), but not pH 7.4, induced hyperactivated stallion sperm motility in the different undiluted treated FFs, as described above. The dose dependency of the hyperactivating effect was tested further by assessing ALH and VCL in sperm suspensions incubated for 0.5 h in (1) 100%, (2) 50%, (3) 10% and (4) 0% treated FF diluted in capacitating medium (pH 7.9 and 7.4).

After 0.5 h incubation in all 50% and 10% treated FF media and capacitating medium (100% or 0% FF) at pH 7.9, stallion spermatozoa showed significantly lower hypermotility parameters (ALH and VCL) compared to those exposed to undiluted, treated FF ( $P < 0.001$ ; Figure 6). Moreover, a significant effect of treated FF concentration was observed; spermatozoa incubated in 50% treated FF showed significantly higher ALH and VCL than sperm in 10% treated FF or capacitating medium (0%;  $P < 0.001$ , Figure 6). Indeed, the ALH and VCL for sperm incubated in 10% treated FF were very similar to capacitating conditions ( $P = 0.11$ ; Figure 6).

Similar to the observations for undiluted treated FF (Figures 5 and 6), sperm suspensions incubated in capacitating medium (0%) at pH 7.9 showed significantly higher ALH and VCL values than sperm in capacitating medium at pH 7.4 ( $P < 0.001$ ); however, the switch from pH 7.4 to 7.9 elicited a much less pronounced effect than in treated FF (Figure 6). Thus pH 7.9 capacitation medium was insufficient to achieve maximal hyperactivated motility. In general at pH 7.9, treated FF induced hyperactive motility of equine sperm in a concentration dependent manner.



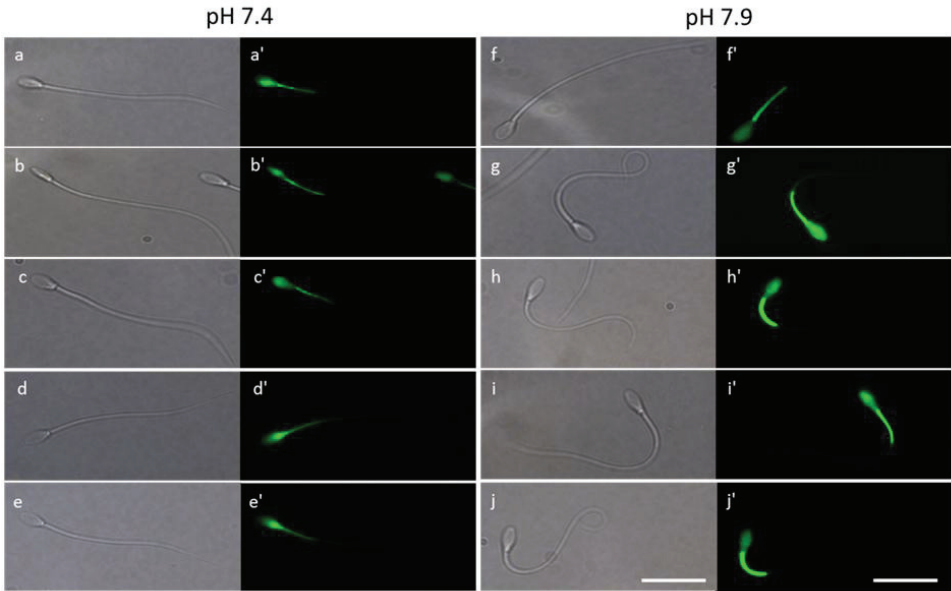
**Figure 6.** Evaluation of (a) lateral head displacement (ALH) and (b) curvilinear velocity (VCL) as indicators of hyperactivated motility in stallion sperm after 0.5 h incubation at both pH 7.9 and 7.4 in (1) heat-inactivated follicular fluid (FF: black bars), (2) charcoal-treated FF (dark grey bars), (3) 30 kDa filtered FF (light grey bars), (4) triple treated FF (white bars) and (5) capacitating medium (0% FF = 100% capacitating medium; striped bars). For all FF preparations, 100, 50 and 10% FF diluted in capacitating medium were assessed; the effect of  $\text{Ca}^{2+}$  depletion by 2 mM EDTA was tested for all FF treatments (100%) ( $n=5$  samples in each group; 3 replicates). Induction of hyperactivated motility in suspended sperm by the various undiluted FF preparations clearly required external  $\text{Ca}^{2+}$  and an elevated medium pH 7.9; a much lower hyperactivation response was observed in capacitating medium even in the presence of  $\text{Ca}^{2+}$  and at pH 7.9. For lateral head displacement, values that differ significantly are indicated by different small letters; for curvilinear velocity, values that differ significantly are indicated by different capitals. Statistical analysis was performed by repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons.



### **Hyperactivated motility induced by undiluted treated FF depends on external $\text{Ca}^{2+}$ and coincides with an increase in cytoplasmic $\text{Ca}^{2+}$ in the sperm head and mid-piece**

Hyperactivated motility in mouse sperm [21, 46] is dependent on the presence and function of the pH-gated CatSper channel, which allows a cytoplasmic  $\text{Ca}^{2+}$  influx from the sperm environment. CatSper activation has also been shown to play a role in hyperactivated motility of human sperm [47-49]. On the other hand, in stallion sperm hyperactivation induced by procaine did not seem to be dependent on external  $\text{Ca}^{2+}$ , although CatSper1 proteins were shown to be present along the principle piece [42]. We investigated whether the induction of hyperactivation in stallion sperm by treated FF at pH 7.9 was dependent on external  $\text{Ca}^{2+}$ . Addition of 2 mM EDTA to undiluted treated FF at pH 7.9 led to a marked drop in lateral head displacement (ALH) and curvilinear velocity (VCL), indicating an overall loss in sperm (hyper)motility (Figure 6). Thus, hyperactivated motility in stallion spermatozoa in undiluted treated FF was clearly dependent on an external  $\text{Ca}^{2+}$  source.

Considering that in murine and human sperm, hyperactivated motility is induced by external  $\text{Ca}^{2+}$  influx through CatSper channels, we investigated whether hyperactivated motility in stallion sperm coincided with an increased cytoplasmic  $\text{Ca}^{2+}$  concentration (monitored by fluo-4 AM) after 0.5 h exposure to undiluted treated FF. At pH 7.4, we observed that spermatozoa incubated in treated FF had a similar cytoplasmic  $\text{Ca}^{2+}$  signal to control spermatozoa incubated in capacitating medium at pH 7.4 (control;  $0 \pm 0\%$ ; heat-inactivated FF:  $3 \pm 1\%$ , charcoal-treated FF:  $2 \pm 1\%$ , 30 kDa filtered FF:  $3 \pm 2\%$  and triple treated FF:  $3 \pm 1\%$ ). By contrast, at pH 7.9 the percentage of spermatozoa with increased cytoplasmic  $\text{Ca}^{2+}$  signals was significantly higher in sperm suspensions incubated in treated FF (heat-inactivated FF,  $63 \pm 9\%$ ; charcoal-treated FF,  $71 \pm 9\%$ ; 30 kDa filtered FF,  $66 \pm 11\%$ ; triple treated FF,  $59 \pm 7\%$ ) and capacitating medium (pH 7.9; control,  $18 \pm 5\%$ ), although capacitating medium was much less supportive than treated FF ( $P < 0.001$ ). At pH 7.9 conditions, increased cytoplasmic  $\text{Ca}^{2+}$  was mainly observed in the sperm head and midpiece region and co-occurred with hypermotility (Figure 7).

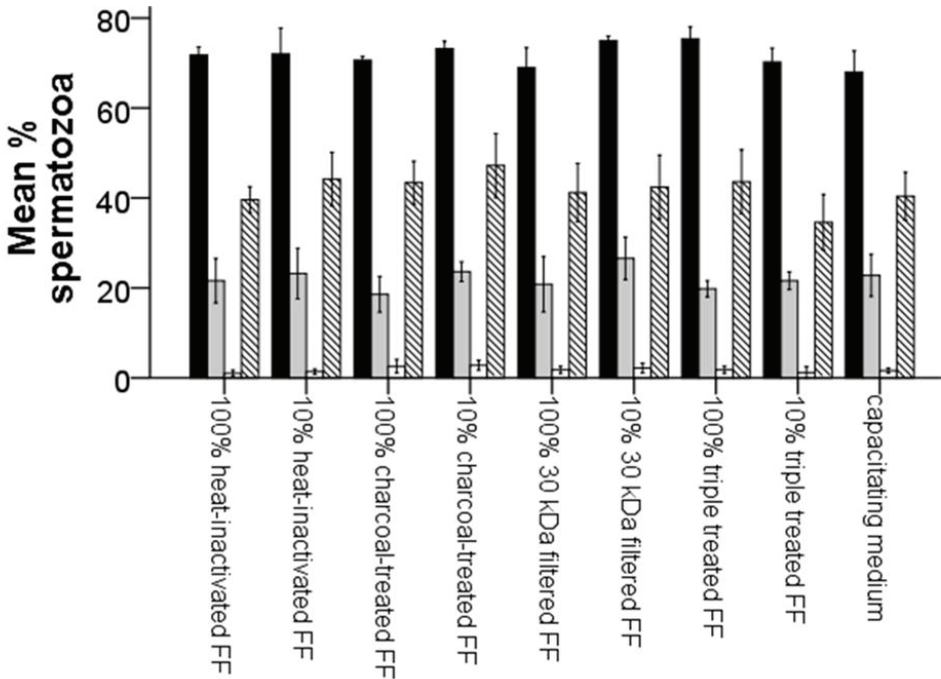


**Figure 7.** Representative DIC images to illustrate motility patterns of stallion spermatozoa after 1 h incubation in (a, f) capacitating medium (control: normal cytoplasmic  $\text{Ca}^{2+}$  concentration), (b, g) undiluted heat-inactivated follicular fluid (FF), (c, h) undiluted charcoal-treated FF, (d, i) undiluted 30 kDa filtered FF and (e, j) undiluted triple treated FF. All treatments were performed at both pH 7.4 (a, b, c, d, e) and pH 7.9 (f, g, h, i, j). In all FF preparations at pH 7.9, stallion spermatozoa showed a hyperactive motility pattern, whereas spermatozoa incubated at pH 7.4 or in capacitating medium at pH 7.9 generally did not. Representative images of fluo-4 AM labelled stallion spermatozoa (a', b', c', d', e', f', g', h', i' and j') incubated under identical conditions, showed that induction of hyperactive motility by FF preparations coincided with an intensified cytoplasmic  $\text{Ca}^{2+}$  signal (original magnification, 1000x, Bar = 25  $\mu\text{m}$ ).

**Stallion sperm in pH 7.9 treated FF suspensions undergo tail-associated protein tyrosine phosphorylation but, despite the sperm retaining acrosome responsiveness, the acrosome reaction is not induced**

When sperm suspensions were incubated in treated FF with an elevated pH (7.9), sperm membrane integrity was well preserved after 1 h of incubation (Figure 8) with at least 70% of the sperm population membrane-intact in all groups. More than 18% of the sperm showed tail-associated protein tyrosine phosphorylation, but the cells did not undergo acrosomal exocytosis in any of the treatment groups. However, sperm suspensions incubated

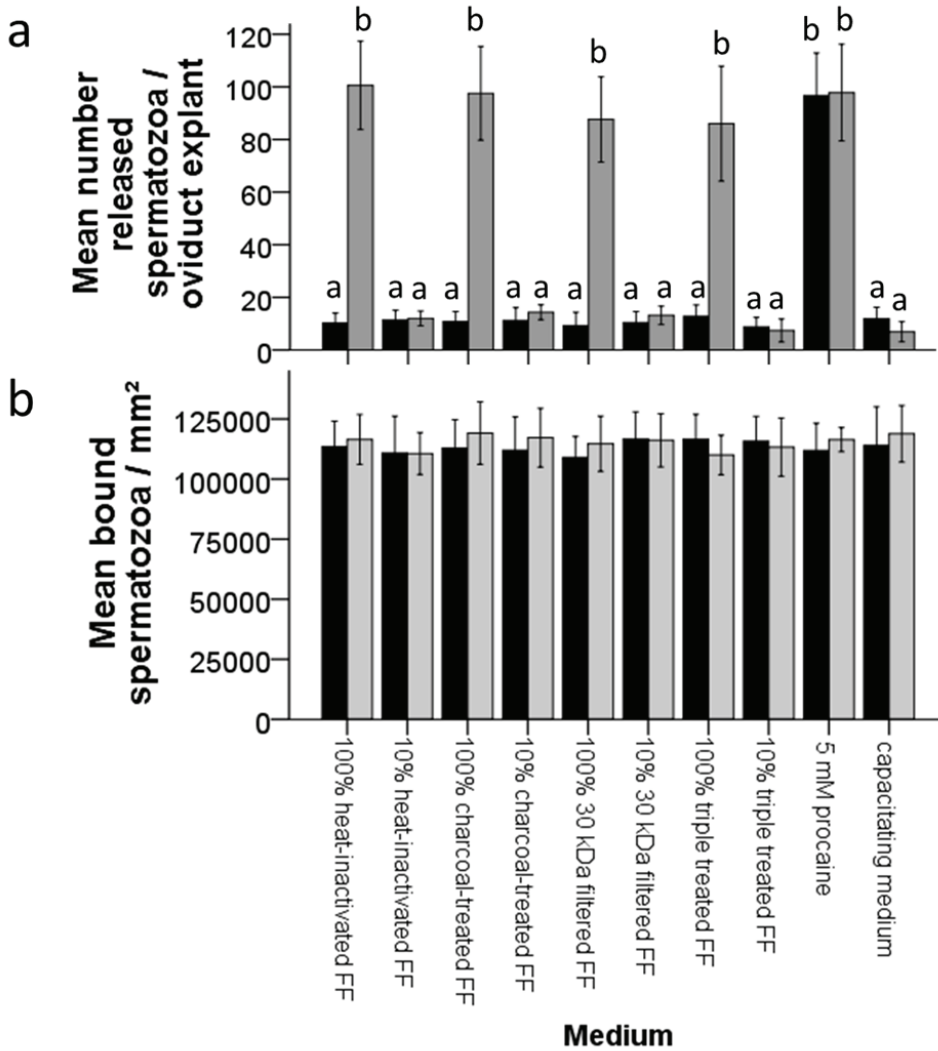
in treated FF were still able to undergo an acrosome reaction following exposure to  $\text{Ca}^{2+}$  ionophore A23187; indeed, acrosomal exocytosis was induced in more than 40% of the spermatozoa incubated in treated FF (Figure 8). In general at pH 7.9, the percentages of membrane-intact, protein tyrosine phosphorylated and acrosome intact sperm were independent of the FF concentration (100 or 10%) ( $P > 0.18$  for all comparisons; Figure 8).



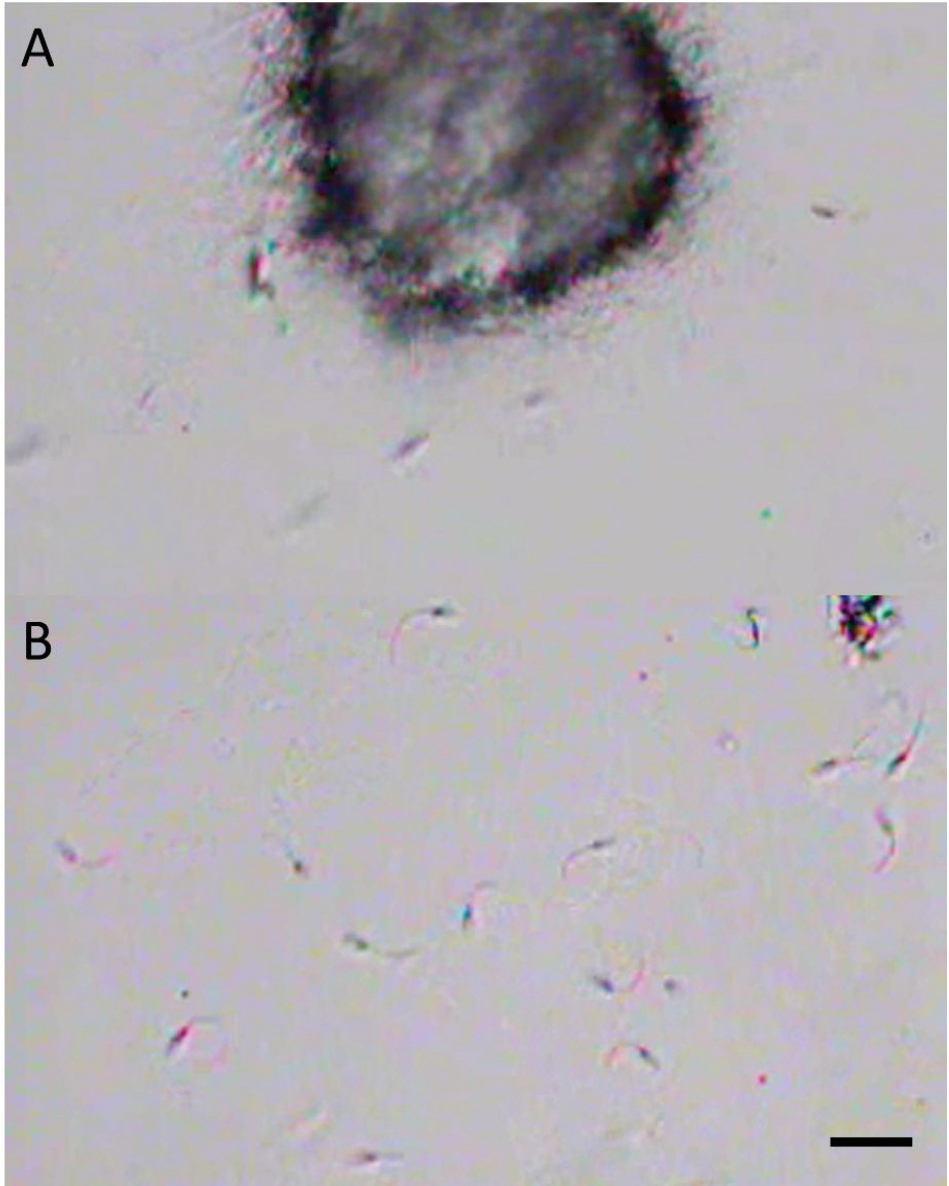
**Figure 8.** Percentages of membrane-intact, protein tyrosine phosphorylated and acrosome-reacted stallion spermatozoa after 1 h incubation in 9 different conditions at elevated pH (7.9): (1) 100% heat-inactivated follicular fluid (FF), (2) 10% heat-inactivated FF in capacitating medium, (3) 100% charcoal-treated FF, (4) 10% charcoal-treated FF in capacitating medium, (5) 100% 30 kDa filtered FF, (6) 10% 30 kDa filtered FF in capacitating medium, (7) 100% triple treated FF, (8) 10% triple treated FF in capacitating medium and (9) capacitating medium. Hyperactivating treatments also induced a pH-dependent tail-associated protein tyrosine phosphorylation, although the acrosome reaction was not stimulated. The percentage of acrosome reacted stallion spermatozoa after 1 h incubation under identical treated-FF conditions at pH 7.9 and followed by 30 min exposure to  $\text{Ca}^{2+}$  ionophore A23187 was also examined. While treated FF did not induce the acrosome reaction, the sperm were still able to undergo acrosomal exocytosis in response to  $\text{Ca}^{2+}$  ionophore. Data represent mean ( $\pm$  s.d) percent membrane-intact (black bars), protein tyrosine phosphorylated (dark grey bars) and acrosome-reacted spermatozoa before (white bars) and after (hatched bars)  $\text{Ca}^{2+}$  ionophore A23187 exposure ( $n=5$  sperm suspensions in each group) for three replicates. Analysis was performed using one-way ANOVA, with post hoc Scheffé tests for pairwise comparisons.

### **Hyperactivation induced the release of a very limited number of spermatozoa from oviduct explants**

In cattle, during the process of *in vitro* capacitation oviduct-bound spermatozoa will release from the epithelium in a hyperactivated state [50]. Since we found that treated FF can trigger sperm hyperactivation we wanted to determine whether it would induce oviduct-bound sperm to release. To this end, sperm-oviduct explant binding was allowed under non-capacitating conditions (approximately 120,000 bound spermatozoa / mm<sup>2</sup>) and, subsequently, these sperm-oviduct explants were transferred to capacitating / hyperactivating media. Alternatively, sperm-oviduct explants were incubated in the different treated FFs (heat inactivated, charcoal treated, 30 kDa filtered and triple treatment). In addition, capacitation media containing 5 mM procaine and / or at pH 7.9 were compared to pH 7.4 capacitation conditions. After 2 h of incubation, none of the conditions resulted in significant sperm release considering the total number of sperm bound per mm<sup>2</sup> (Figure 9b). However, in all conditions inducing hyperactivated sperm motility in previous experiments, a small number of hyperactivated spermatozoa (70-120 spermatozoa per oviduct explant; Figure 9a) were released from the oviduct explants (Figure 10a and Figure 10b). We conclude that the induction of sperm hyperactivated motility did not induce a massive release of capacitated spermatozoa from the oviduct epithelium. However, these findings may reflect earlier suggestions that only a very small population of spermatozoa is able to release from their binding and approach the oocyte at the time of fertilization, to reduce the risk of polyspermy [51-53].



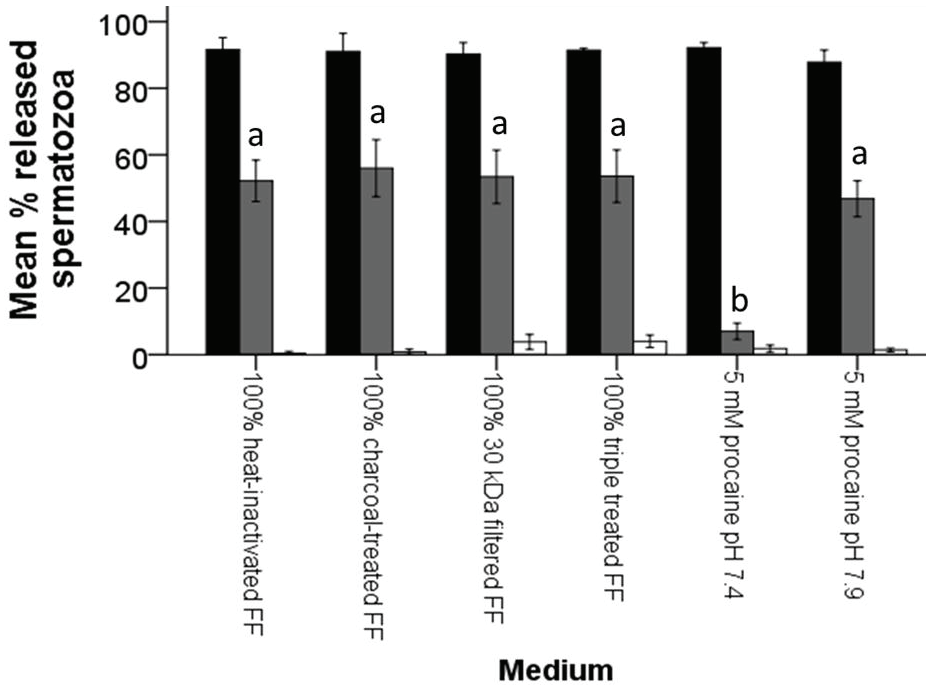
**Figure 9.** (a) Mean number of stallion spermatozoa released per oviduct explant and (b) mean spermatozoa bound per mm<sup>2</sup> epithelium, assessed after 2 h incubation in (1) 100% heat-inactivated follicular fluid (FF) (2) 10% heat-inactivated FF in capacitating medium (3) 100% charcoal treated FF (4) 10% charcoal treated FF in capacitating medium (5) 100% 30 kDa filtered FF (6) 10% 30 kDa filtered FF in capacitating medium, (7) 100% triple treated FF, (8) 10% triple treated FF in capacitating medium, (9) 5 mM procaine in capacitating medium and (10) capacitating medium. Each incubation was performed at pH 7.4 (black bars) and pH 7.9 (light grey). Only a modest release of oviduct bound spermatozoa was observed in the various hyperactivating conditions. Data represent mean ( $\pm$  s.d.) bound spermatozoa per mm<sup>2</sup> ( $n=20$  per group) for three replicates. Analysis was performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, with Scheffé post hoc tests for pairwise comparisons.



**Figure 10.** (A) Representative image from a supplementary video clip of an oviduct explant during a 2 h incubation in 100% heat-inactivated FF. Bound spermatozoa showed hyperactivated motility, as indicated by curvature of the sperm tails. (B) Representative images from a supplementary video clip of spermatozoa released from oviduct explants during incubation in 100% heat-inactivated FF. After 2 h in FF at pH 7.9, a modest release of hyperactivated spermatozoa was observed (a, b, c, d: original magnification, 400x; scale bar = 25  $\mu$ m).

**Properties of sperm released from the oviduct**

After release of hyperactivated spermatozoa from the oviduct epithelium triggered by pH 7.9 treated FF, at least 85% of the released spermatozoa were membrane-intact, > 45% showed tail-associated protein tyrosine phosphorylation, whereas hardly any had undergone acrosomal exocytosis. Similar results were achieved in the presence of procaine (at pH 7.4 and 7.9) except that tail-associated protein tyrosine phosphorylation rates were much lower (8%) in the pH 7.4 procaine condition (Figure 11).



**Figure 11.** Percentages of membrane-intact, protein tyrosine phosphorylated and acrosome-reacted stallion spermatozoa following release from oviduct explant binding during 2 h incubation in (1) 100% heat-inactivated FF, (2) 100% charcoal-treated FF, (3) 100% 30 kDa filtered FF, (4) 100% triple treated FF, (5) 5 mM procaine diluted in capacitating medium pH 7.4 and (7) 5 mM procaine diluted in capacitating medium pH 7.9 capacitating medium. Data represent mean ( $\pm$  s.d) % membrane-intact (black bars), % protein tyrosine phosphorylated (dark grey bars) and % acrosome-reacted spermatozoa (white bars). Data represent mean ( $\pm$  s.d) percentage of hyperactivated (black bars), membrane-intact (dark grey bars), protein tyrosine phosphorylated (light grey bars) and acrosome-reacted (white bars) spermatozoa (n=20 oviduct explants in each group) for three replicates. For protein tyrosine phosphorylated spermatozoa, values that differ significantly are indicated by different small letters. Analysis was performed using one-way ANOVA; Scheffé post hoc tests were performed for pairwise comparisons.

**DISCUSSION**

It is generally believed that mammalian sperm, including stallion spermatozoa, have to follow a regulated sequence of events during capacitation *in vivo* in order to achieve fertilizing capacity [25]. Ejaculated spermatozoa travel through the mare's reproductive tract to the oviduct where a sperm reservoir is formed by viable, non-capacitated spermatozoa [54, 55]. In a previous study, we reported that equine oviduct epithelial cells harvested close to the time of ovulation contain large secretory vesicles with an elevated pH. Moreover, protein tyrosine phosphorylation in the tails of spermatozoa bound to these oviduct epithelial cells was associated with contemporaneous intracellular alkalization of the spermatozoa and vesicle discharge [9]. In anticipation of oocyte arrival, spermatozoa in which capacitation has been initiated need to be released from the oviduct epithelium, an event that may require the acquisition of hyperactivated motility [12, 22]. Released capacitated / hyperactivated spermatozoa will subsequently migrate to the isthmus-ampullary junction to fertilize the mature oocyte [17, 18]. Sperm release from the oviduct epithelium by hyperactivation, and subsequent acrosome reaction after contacting the extracellular vestments of the oocyte has, however, not yet been reliably achieved *in vitro* in the horse, but may be crucial to obtaining reliable fertilization *in vitro*. Until now, only exposure to procaine has been shown to reliably induce hyperactivated motility in stallion spermatozoa [42, 45, 56], and this was independent of CatSper-related  $\text{Ca}^{2+}$  influx [42]. In the current study, we induced hyperactivation of stallion spermatozoa using slightly alkaline (pH 7.9) undiluted treated FF (i.e. either heat-inactivated, charcoal-treated, 30 kDa filtered or all three to neutralize a spermicidal factor in FF). In contrast to procaine induced hyperactivation [42], we found that the treated FF induced hyperactivation in stallion sperm was dependent on external  $\text{Ca}^{2+}$ . Under the hyperactivating conditions at pH 7.9, a significant rise in cytoplasmic  $\text{Ca}^{2+}$  levels was observed predominantly in the sperm head and mid-piece. The hyperactivated sperm remained membrane and acrosome intact and showed protein tyrosine phosphorylation, i.e. the cardinal signs of sperm capacitation without sperm deterioration. Interestingly, sperm bound to oviduct epithelium were not released *en masse* into the lumen after such treatments. Instead, only a very small population was released although a large proportion of these cells did show a capacitation-like phenotype in terms of motility, tyrosine phosphorylation and membrane integrity. In short, a combined effect of elevated environmental pH, external  $\text{Ca}^{2+}$



and unidentified FF factor(s) appear to trigger hyperactivation of stallion spermatozoa, whereas the tail-associated protein tyrosine phosphorylation response appears to depend only on pH.

By testing a variety of FF treatments, we were able to demonstrate that sperm integrity was maintained in heat-inactivated, charcoal-treated, 30 kDa filtered or triple treated FF whereas untreated fluid or a <100 kDa filtrate had a sperm deteriorating effect. This implies that the FF component that compromises sperm viability has a size between 30 and 100 kDa, but can be neutralized by heat-inactivation or charcoal treatment. Heat inactivation causes the breakdown of tertiary and quaternary protein structures and could, for example, neutralize complement factors that might be activated under *in vitro* conditions due to exposure to air [57]. Charcoal treatment extracts the lipid fraction including steroids and lipoproteins [38]. Based on these results, we hypothesize that the sperm deteriorating factor is a steroid or lipid bound to a protein. Further research is required to elucidate which component of fresh, air-exposed FF exerts a detrimental effect on sperm *in vitro*.

Interestingly, rather than stimulating sperm deterioration, the undiluted treated FF (heat-inactivated, charcoal-treated, 30 kDa filtered or triple treated) was able to induce hyperactivation of stallion spermatozoa in suspension at pH 7.9. A similar trend was also observed for spermatozoa incubated in capacitating conditions at pH 7.9, although the hyperactivated motility response was much lower than in treated FF. However, we have not yet identified the component(s) of equine FF that triggers hyperactivated sperm motility. Possible heat resistant, hydrophilic candidates (<30 kDa) in FF include carbohydrates, small heat resistant proteins and electrolytes. Theoretically, our results suggest that (1) components of FF may be involved in sperm hyperactivation in the horse oviduct or that (2) undiluted treated FF mimics capacitation conditions that are induced *in vivo* by oviduct secretions in the peri-ovulatory period. Additionally, the fact that hyperactivated motility required a slightly alkaline pH, that was not a feature of pre-ovulatory FF (pH  $\pm$  7.2), supports our hypothesis that, in the narrow and tortuous oviduct lumen, alkalization at the sperm-oviduct interface results from oviduct secretory activity at the time of ovulation [9], which in turn triggers an intracellular pH rise in oviduct-bound stallion spermatozoa and provokes protein tyrosine phosphorylation. In the present study, we additionally showed that FF components have a significant effect on sperm hyperactivation.

Undiluted treated FF stimulated the biggest increase in ALH and VCL, whereas a more muted hyperactivation response was observed when sperm were incubated in 50% and 10% FF (pH 7.9) or in capacitating medium alone (pH 7.9). These observations demonstrate a concentration-dependent effect of the unidentified FF factor(s) on stallion sperm hyperactivation.

The results of this study highlight that there is as yet no defined capacitating medium for stallion sperm. In this respect, it is pertinent that capacitating conditions that reliably and repeatably support equine *in vitro* fertilization have not yet been reported. As concluded by McPartlin *et al.* [45], a major deficit of current capacitating media appears to be the ability to induce hyperactivated motility in stallion sperm. Besides the fact that an elevated pH seems to be critical, further research needs to focus on a systematic investigation of components in treated FF compared to capacitating medium. Comparing the sperm hyperactivation supporting conditions among various mammals, we assume that this process is not well conserved. In man, it has been reported that factors in FF, and secreted by cumulus cells, can initiate hyperactivation [26, 58, 59]. In the hamster [60] and rabbit [27, 61], contact with FF also provokes sperm hyperactivation. The key elements of these ovulation associated products on CatSper induced hypermotility are bicarbonate, which supports an alkaline intracellular pH, and progesterone [17, 18, 62, 63]. On the other hand, various studies in rabbit [64], mouse [28], and large farm animals [53, 65] with relatively long pre-ovulatory periods, have indicated that sperm hyperactivation and subsequent sperm-oviduct release starts in a controlled manner shortly before ovulation. This implies that the trigger to hyperactivation must be derived from oviductal fluid or by contact with oviduct epithelia, and not from components resulting from ovulation, such as FF or the cumulus-oocyte-complex. The active components in the oviduct which induce CatSper-induced hypermotility also appear to differ between species. In the mouse, bicarbonate is the main trigger for hyperactivation [66]. In bull sperm, Marquez and Suarez [29] concluded that an increased external pH was the primary signal for sperm hyperactivation. Bull sperm can also achieve full fertilizing capacity after contact with heparin and other sulfated glycoconjugates. These molecules are abundantly present in oviductal and FF, and induce release of sperm adhering to oviduct epithelium monolayers *in vitro* by increasing their flagellar beat [67-69]. Heparin-like molecules also induce hyperactivation of free-swimming sperm [70]. The biological hyperactivating triggers for stallion sperm have not been investigated.

The experiments to examine the induction of hyperactivation by undiluted treated FF showed clearly that hyperactivated motility triggered in this way depends on extracellular  $\text{Ca}^{2+}$ . Surprisingly, these results are in contrast to procaine-induced hyperactivated motility for which external  $\text{Ca}^{2+}$  was not required [42]. In mice, hyperactivated sperm motility during capacitation is provoked by  $\text{Ca}^{2+}$  influx through pH-gated cationic CatSper channels, located along the principal piece of the sperm flagellum [13, 20-22, 46, 71]. In man, mutations in CATSPER genes have similarly been associated with infertility and abnormal sperm motility [47-49]. Whether our undiluted inactivated FF conditions induce hyperactivated motility by  $\text{Ca}^{2+}$  influx via CatSper channels or via other  $\text{Ca}^{2+}$  permeable ion channels (voltage-gated  $\text{Ca}^{2+}$  channels (CaVs), transient receptor potential (TRP) channels, cyclic nucleic gated (CNG) channels or pKD2 cation channels [22, 72] remains to be investigated, but some important elements indicate that functional pH-gated cationic CatSper channels are likely to be involved. Firstly, CatSper1 proteins have been shown to be present along the principal piece of stallion sperm [42]. Additionally, we showed that the initiation of hyperactivated motility was a specific alkaline pH dependent event. Moreover, we showed that FF-induced hyperactivated motility of stallion sperm depends on external  $\text{Ca}^{2+}$ . These observations favour the hypothesis that CatSper channels are involved in capacitation-related hyperactivated motility in the horse. This hypothesis was further supported by the increased cytoplasmic  $\text{Ca}^{2+}$  signal in sperm incubated in treated FF at pH 7.9. After 1 h incubation under hyperactivating conditions, Fluo-4 AM labelling appeared mainly in the sperm head and midpiece, but was very weak in the principle piece. It is interesting that a marked  $\text{Ca}^{2+}$  signal was not observed in the principle piece, where the CatSper channels are meant to be located. In mice, Xia and Ren [73] showed that the  $\text{Ca}^{2+}$  signal acquired during incubation in hyperactivating conditions rapidly propagated from the principle piece to the sperm head. Moreover, it has been hypothesized that the external  $\text{Ca}^{2+}$  influx through the CatSper channels subsequently induces a  $\text{Ca}^{2+}$  release from the intracellular cytoplasmic  $\text{Ca}^{2+}$  stores or the redundant nuclear envelope, involving inositol triphosphate receptors located near the sperm neck (' $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release') [18, 74-77]. This may help explain why a significant cytoplasmic  $\text{Ca}^{2+}$  rise is seen in the sperm head and mid-piece.

In this study, the acrosome reaction was not induced during sperm incubation in the treated FF samples, even though incubated sperm were still capable of acrosomal exocytosis. Previously, progesterone was shown to be the key factor inducing the acrosome reaction in

FF-exposed stallion sperm, and did so without affecting sperm viability and motility [38, 78]. It is also known that steroid hormones, including progesterone, bind to albumin in blood serum [79] and FF [80]. We propose that removing the sperm deteriorating component in FF by various treatments (heat-inactivation, charcoal treatment and 30 kDa filtration) coincidentally inactivated or otherwise extracted this steroid-protein complex. In support of this hypothesis, Cheng *et al.* [38] demonstrated that charcoal treatment, to remove progesterone from FF, also removed the stimulus for stallion sperm to undergo the acrosome reaction.

In the first experiment, we showed that contact with reproductive fluids and cells that the sperm would expect to contact in the sperm reservoir during the peri-ovulatory period, did not induce release of stallion sperm from oviduct epithelium. In the light of our subsequent hypothesis that treated FF preparations with elevated pH mimic the physiological condition in the oviduct during the peri-ovulatory period, we need to consider the possibility that, *in vitro*, the collected pre- and post-ovulatory oviductal fluids do not represent the *in vivo* condition since the oviductal fluid was (1) flushed using capacitating medium and was thus considerably diluted whereby important molecules would have been diluted; or (2) the pH of oviductal fluid may have been decreased by this method of collection. However, given the very limited amount of free fluid present in the oviduct it was impossible to collect a usable amount in pure form. Furthermore, in the final experiment we observed that even induction of hyperactivated motility by treated FF or procaine did not induce massive sperm release. However, in terms of the absolute number of spermatozoa, hyperactivating conditions (the combined effects of FF factors and elevated pH or procaine) induced a limited release of sperm bound to oviduct explants. We can suggest two reasons why the released sperm fraction was so small: (1) just like sperm-oviduct binding, sperm-oviduct release is a sperm quality selection mechanism that ensures an optimum chance of the mature oocyte being fertilized by a suitable sperm; (2) Suarez [81, 82] hypothesized that close coordination of plasma membrane changes and the induction of hyperactivated motility is a requirement for sperm release from the oviduct epithelium. Our FF hyperactivating conditions may have lacked appropriate triggers for capacitation-related plasma membrane changes. On the other hand, Hunter [51-53] showed that *in vivo* at the time of fertilization only a very small number of capacitated, hyperactivated oviduct-bound spermatozoa are able to escape from the oviduct epithelium and reach the cumulus-oocyte complex at the isthmic-ampullary junction (low

sperm:egg ratios). The biological relevance can be viewed in the context of a reduced risk of multiple oocyte penetration or polyspermy. Moreover, Hunter [55, 83] reported that, in contrast to the tight regulation during the peri-ovulatory period, during the post-ovulatory period increasing numbers of spermatozoa were able to detach from the oviduct. This does not interfere with fertilization because a stable block to polyspermy is rapidly established in fertilized, activated oocytes. In our experiments, we did not observe this enhanced release by ovulation associated factors. Two possible reasons can explain why: (1) we only used oviducts from cyclic mares in the pre-ovulatory stage or (2) our FF conditions did not support hyperactivation for long enough, given the loss of sperm viability and motility after 2.5 h incubation. In summary, these observations indicate that the induction of hyperactivated sperm motility was not sufficient to release large numbers of sperm from oviduct binding; however, a small but significant population did release.

In conclusion, we have previously shown that stallion sperm acquire important hallmarks of capacitation (elevated intracellular pH and tail-associated protein tyrosine phosphorylation) after binding to alkaline vesicle-containing pre-ovulatory oviduct epithelial cells. In the present study, we found that contact with treated FF components induces hyperactivated sperm motility using a mechanism that depends on an elevated pH and extracellular  $\text{Ca}^{2+}$ ; however, this induction of hyperactivation triggers only a modest sperm release from the oviduct epithelium. It is therefore likely that other factors that support aspects of capacitation and hyperactivation are needed to complete the cascade that prepares the spermatozoa for the acrosome reaction and penetration of the zona pellucida.

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# CHAPTER 6

## PROCAINE INDUCES CYTOKINESIS IN HORSE OOCYTES VIA A PH DEPENDENT MECHANISM

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**Adapted from:**

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*Procaine induces cytokinesis in horse oocytes via a pH dependent mechanism. Biology of Reproduction 2015; 93(1): 1–17.*



## ABSTRACT

Co-incubating equine gametes in the presence of procaine has been reported to facilitate *in vitro* fertilization, with cleavage rates exceeding 60%. It has been suggested that procaine promotes sperm penetration by inducing hyperactivated motility to accompany tail-associated protein tyrosine phosphorylation, triggered by other medium conditions like elevated medium pH. Both capacitation characteristics were also induced in treated follicular fluid with elevated pH (7.9) (Chapter 5). In this study, we demonstrate that both capacitating conditions described above trigger hyperactivation and tail-associated protein tyrosine phosphorylation in stallion sperm but were still insufficient to facilitate *in vitro* fertilization. Moreover, we found that procaine (1-5 mM) did not facilitate stallion sperm penetration of equine oocytes, but instead induced sperm-independent oocyte cytokinesis in the absence of the second polar body extrusion. Indeed,  $56 \pm 4\%$  of oocytes cleaved within 2.5 d of exposure to 2.5 mM procaine, irrespective of sperm presence. However, the cleaved oocytes did not develop beyond 8-16 cells, and the daughter cells either lacked nuclei or contained aberrant, condensed DNA fragments. By contrast, intra-cytoplasmic sperm injection (ICSI) was followed by second polar body extrusion and formation of normal blastocysts. Moreover, neither the calcium oscillations detectable using fura-2 AM staining nor the cortical granule reaction visualized by LCA-FITC staining, after oocyte activation induced by ICSI or ionomycin treatment, were detected after exposing oocytes to 2.5 mM procaine. Instead, procaine initiated an ooplasmic alkalinization, detectable by BCECF-AM staining, that was not observed after other treatments. This alkalinization was followed, after an additional 18 h incubation, by cortical F-actin depolymerization, as demonstrated by reduced actin phalloidin-FITC staining intensity, that resembled preparation for cytokinesis in ICSI-fertilized zygotes. Overall, we conclude that the induction of hyperactivated motility in tyrosine phosphorylated spermatozoa is still insufficient to fertilize equine oocytes *in vitro*. However, procaine induces cytokinesis in equine oocytes accompanied by aberrant chromatin condensation and cytoplasmic division; this explains why embryos produced after exposing equine oocytes to procaine fail to develop beyond the 8-16 cell stage.

## INTRODUCTION

The birth of two foals produced after *in vitro* fertilization (IVF) of *in vivo* matured equine oocytes was reported in 1991 [1, 2]. Unfortunately, subsequent attempts to establish a repeatable protocol for conventional IVF in horses have not been successful [3, 4], and it has been suggested that the primary deficit is the inability to adequately induce capacitation of stallion spermatozoa *in vitro*. More specifically, the absence of hyperactivated sperm motility under standard *in vitro* capacitation conditions has been proposed as the main reason why *in vitro* fertilization fails in the horse [5]. In 2009, McPartlin *et al.* [6] reported promising equine IVF results after including procaine in the co-incubation medium. The procaine induced hyperactivated motility in stallion spermatozoa, and this was concluded to be responsible for the high cleavage rates observed in the incubated oocytes. An additional important step in the capacitation process, protein tyrosine phosphorylation, was shown to be provoked by incubating stallion spermatozoa in air, and to be independent of the exposure to procaine [6, 7]. Most importantly, approximately 60% of mature (MII) oocytes developed two pronuclei during a 24 h culture subsequent to 18 h co-incubation with procaine-activated sperm. Embryo development was reported up to the 8-cell stage (day 3). More recently, Ambruosi *et al.* (2013) similarly reported that almost 40% of horse oocytes exposed to sperm and procaine formed two pronuclei after 24 h incubation in identical hyperactivation conditions [8].

Procaine is a local anesthetic known to induce a neuromuscular block and to increase the neuromuscular responses to non-depolarizing muscle relaxants, primarily via actions on the voltage-gated sodium channel [9]. The hyperactivation of sperm motility by procaine (guinea pig, [10]; horse, [6, 11, 12]) has, in the horse, been associated with a moderate increase in intracellular pH, an obligatory step in initiating pH-gated calcium influx through CATSPER channels [13]. Surprisingly, procaine-induced hyperactivated motility was not dependent on external calcium in either stallion [12] or guinea pig [10] spermatozoa, supporting the hypothesis that the calcium is mobilized from internal calcium stores, which contrasts to what is thought to happen during physiological induction of hyperactivated sperm motility [14, 15].

The two published studies on the effects of procaine on horse IVF [6, 8] used the same approach, i.e. they exposed both oocytes and spermatozoa simultaneously to procaine.

Therefore, a concurrent effect of procaine on oocyte activation cannot be excluded. Oocytes require a rise in cytoplasmic calcium after fertilization or parthenogenetic activation to resume meiosis and embark on embryonic development. During gamete fusion, a sperm-specific phospholipase C zeta (PLC $\zeta$ ) is introduced into the oocyte, and is a key factor in inducing an inositol triphosphate (IP3) mediated calcium release from intracellular stores (calcium oscillations; mouse [16], human [17]). Various methods have been employed to induce artificial calcium oscillations in mammalian oocytes *in vitro* and thereby provoke oocyte activation and embryo development. Electrical pulses [18], Ca<sup>2+</sup> ionophore [19] and strontium [20] are typically used as parthenogenetic agents, and it has been demonstrated that electrical pulses and Ca<sup>2+</sup> ionophore generate a single calcium elevation whereas multiple calcium oscillations are induced by normal fertilization or strontium [20]. Moreover, there is some evidence that procaine is able to disregulate cytoplasmic calcium rise(s) in female gametes. In pig [21, 22] and cattle [23, 24] oocytes, the calcium rise could be inhibited by injecting procaine into the cytoplasm. Further downstream in the oocyte activation pathway, the calcium rise triggers the cortical granule reaction. In many mammalian species, including the horse [25], cortical granule accumulation at the periphery of the oocyte cytoplasm (horse, [25]; pig, [26, 27]) is indicative of cytoplasmic maturation; moreover, these granules are extruded into the perivitelline space after the onset of calcium oscillations during oocyte activation [25].

Another effect of procaine on female gametes has been described in the sea urchin, namely that procaine and other weak bases provoke an elevation in cytoplasmic pH [28, 29] that, in turn, induces cortical F-actin turnover [29] as part of the dramatic changes that the cytoskeleton of a mature oocyte needs to undergo in response to activation [28]. In particular, the total amount of F-actin first increases and subsequently decreases to allow cytokinesis [30].

Beside procaine capacitating conditions, we showed also that treated follicular fluid (FF) (heat-inactivated FF, charcoal-treated FF, <30 kDa centrifuged FF and triple treated FF) at pH 7.9 induced both sperm hypermotility and tail-associated protein tyrosine phosphorylation in stallion sperm (Chapter 5). In this study, we aimed to investigate the role of both capacitating media in equine *in vitro* fertilization by assessing: 1) sperm penetration through the zona pellucida, (2) second polar body extrusion and (3) pronucleus formation.

Since the inclusion of procaine in equine *in vitro* fertilization media has been shown to induce oocyte cleavage but did not result in blastocyst formation, we additionally aimed to investigate the direct effect of procaine on equine oocytes. Moreover, (1) the activation of the oocyte's cortical granule reaction, (2) the presence and arrangement of chromatin in the cleavage products, (3) changes in cytoplasmic calcium and pH in oocytes, (4) and F-actin distribution in oocytes, were assessed. The resulting data help explain why capacitating media still fail to support equine IVF and why procaine in equine IVF media resulted in high oocyte cleavage rates without development to the blastocyst stage.

## **MATERIALS AND METHODS**

### **Chemicals and reagents**

Alexa Fluor 488-conjugated goat anti-mouse antibody, Hoechst 33342, MitoTracker Green FM and BCECF-acetoxymethyl (AM) ester were obtained from Molecular Probes (Ghent, Belgium). Monoclonal 4G10 Platinum anti-phosphotyrosine mouse antibodies were purchased from Millipore (Overijse, Belgium). Fura-2 AM ester was obtained from Invitrogen (Life Technologies, Merelbeke, Belgium) and LCA-FITC was purchased from Labconsult SPRL (Vector Labs, Brussels, Belgium). Dimethylsulfoxide (DMSO), fatty acid-free bovine serum albumin (A9418; cell culture tested), lacmoid, phalloidin-FITC, triton X-100, tween, pronase from *streptomyces griseus* and all other chemicals not otherwise listed were obtained from Sigma-Aldrich (Bornem, Belgium).

### **Collection of follicular fluid**

For each experiment, five ovaries containing a follicle at the late preovulatory stage (diameter > 35mm), were collected at the slaughterhouse and immediately transported to the laboratory. Follicular fluid (FF) was collected by individually aspirating the contents of five follicles (diameter > 35mm) using an 18 gauge winged infusion set needle attached to a 15 ml polystyrene conical tube, under low pressure provided by a vacuum pump. The FF was centrifuged at 100g for 10 min at 22°C before the supernatant was filtered through a 0.2 µm



Acrodisc® Syringe Filter with a Supor® Membrane (Pall® Corporation Life Sciences, Ghent, Belgium).

### **Treatment of follicular fluid**

Follicular fluid samples were processed as described in Leemans *et al.* (2015; Chapter 5). In brief, freshly filtered (2 µm diameter) FF was treated in 4 different ways: (1) by heat inactivation in a warm water bath at 55°C for 0.5 h; (2) charcoal treatment; (3) centrifuged through a 30 kDa filter (30 min-3000g; Vivaspin 15R, Sartorius Biolab Products, Goettingen, Germany); (4) triple treated (successive heat-inactivation, charcoal treatment and 30 kDa centrifugation); moreover heat-inactivated, charcoal-treated, 30 kDa-filtered or triple-treated FF samples were incubated in air until the pH rose to 7.9. All treated FF samples were stored in aliquots at -80°C until further use, for a maximum of one month. To control pH changes in pH elevated treated FF during sperm-oocyte incubation, experiments were performed in closed 1 ml tubes (Greiner bio-one, Vilvoorde, Belgium) to prevent contact with air during the experiment.

### **Semen collection and preparation**

Semen was collected from three adult stallions of proven good fertility using a Colorado model artificial vagina (Animal Reproduction Systems; Chino, CA, USA). The raw ejaculate was filtered through gauze to remove the gel fraction and any debris, before visual evaluation of sperm motility by light microscopy (200x) on a heated stage at 37°C; if the motility was acceptable (>65% motile), the semen was immediately transported to the laboratory for further processing. One ml of fresh semen with a concentration of 100 to 300 x 10<sup>6</sup> spermatozoa / ml was washed using a 45 / 90% Percoll® gradient [31, 32]. Next, the sperm pellet was washed once with non-capacitating medium (100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 5.5 mM glucose, 22 mM HEPES, 2.4 mM sodium lactate and 1.0 mM pyruvic acid; pH=7.4 and 280-300 mOsm / kg; [7]). Each experiment was performed using one ejaculate from each of the three stallions. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/175 and EC2013/176).

For the sperm penetration experiments, spermatozoa were labelled with MitoTracker Green FM before further processing. Briefly, 200 nM MitoTracker Green FM dissolved in 0.025% DMSO was added to the Percoll® washed sperm suspension ( $100 \times 10^6$  spermatozoa / ml) diluted in 10 ml non-capacitating medium. After 30 min incubation at 37 °C, the sperm suspension was washed twice in 10 ml non-capacitating medium (600g; 5 min).

### **Sperm capacitation / hyperactivation**

To provide conditions supportive of sperm capacitation, non-capacitating medium was modified by replacing the sodium lactate with 2.4 mM calcium lactate and adding 25 mM NaHCO<sub>3</sub> and 7 mg / ml BSA (pH=7.4; 280-300 mOsm / kg; osmolality was adjusted by graduated addition of the NaCl); this medium was pre-equilibrated for at least 2 h in a humidified atmosphere containing 5% CO<sub>2</sub> at 38.5 °C and is further referred to as capacitating medium (adapted from McPartlin *et al.* [7]). The washed sperm pellet was diluted to a concentration of  $10 \times 10^6$  spermatozoa / ml with capacitating medium. After 6 h pre-incubation in humidified air at 38.5 °C, hyperactivated motility was induced by resuspending the spermatozoa in capacitating medium supplemented with either 0, 1, 2.5 or 5 mM procaine hydrochloride (Sigma-Aldrich, Bornem, Belgium) at a final concentration of  $1 \times 10^6$  spermatozoa / ml [6]. More precisely, a stock of 10 mM procaine hydrochloride dissolved in capacitating medium was pre-equilibrated for at least 2 h in a humidified atmosphere containing 5% CO<sub>2</sub> at 38.5 °C to restore the pH to the physiological range (7.2 - 7.4). Moreover, various procaine concentrations (0, 1, 2.5 and 5 mM) were prepared by diluting the stock solution with the required amount of equilibrated capacitating medium. In this way the pH of all incubation media was adjusted to 7.2-7.4.

Similar capacitation characteristics were alternatively induced by sperm incubation ( $1 \times 10^6$  spermatozoa / ml) in undiluted treated FF at pH 7.9 (heat-inactivated FF, charcoal-treated FF, <30 kDa centrifuged FF and triple treated FF; Chapter 5).

## Oocyte maturation

Ovaries were collected from slaughtered mares (Euro Meat Group, Moeskroen, Belgium). Within 4 h after slaughter, all follicles larger than 5 mm were aspirated using a 16 gauge needle attached to a vacuum pump (~100 mm Hg), scraped with the aspirating needle and flushed with phosphate buffered saline (DPBS) containing 25 IU / ml heparin. A maximum of 30 cumulus-oocyte complexes (COCs) were transferred to 500  $\mu$ l Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) based maturation medium [33] and placed in an incubator at 38.2°C in a humidified atmosphere of 5% CO<sub>2</sub>-in-air for 28 h. After maturation, COCs were partially or completely denuded by gentle pipetting in 0.05% bovine hyaluronidase diluted in 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffered DMEM/F12 medium. Degenerated oocytes were excluded from subsequent experiments. Only completely denuded oocytes with an extruded polar body were used for piezo drill intra-cytoplasmic sperm injection (ICSI) and parthenogenetic activation by ionomycin treatment, whereas all non-degenerated oocytes were used for IVF with the assumption that an extruded polar body was present since it could not be visualized in partially cumulus-enclosed oocytes.

## ***In vitro* fertilization / oocyte activation in the presence of procaine and treated follicular fluid with elevated pH (7.9)**

Equine IVF was performed in the presence of 0, 1, 2.5 or 5 mM procaine hydrochloride, as described by McPartlin *et al.* [6] and in undiluted treated FF at pH 7.9 (heat-inactivated, charcoal treated, 30 kDa centrifuged and triple treated FF; Chapter 5).

As previously indicated using procaine conditions, sperm was incubated at  $10 \times 10^6$  spermatozoa / ml in capacitating medium for 6 h and then diluted to  $1 \times 10^6$  spermatozoa / ml in procaine containing capacitating medium to achieve final concentrations of 0, 1, 2.5 or 5 mM procaine (medium pH=7.2-7.4; previously adjusted by incubation in an atmosphere containing 5% CO<sub>2</sub>). One hundred  $\mu$ l droplets of these sperm suspensions were pipetted into petri dishes and covered with 5% CO<sub>2</sub> equilibrated mineral oil. Five completely or partially denuded mature oocytes were then transferred to each medium droplet, and the petri dishes were incubated at 38.2 °C in 5% CO<sub>2</sub> in humidified air.

Using treated FF at pH 7.9, sperm ( $1 \times 10^6$  spermatozoa / ml) and five completely or partially denuded mature oocytes were co-incubated in a closed 1 ml tube (Greiner bio-one, Vilvoorde, Belgium) in 4 different FF treatments at pH 7.9 (heat-inactivated FF, charcoal treated FF, 30 kDa centrifuged FF and triple treated FF).

After 18 h of co-incubation in both capacitating conditions, partially denuded oocytes were fully denuded by gentle pipetting in 0.05% bovine hyaluronidase in HEPES buffered DMEM/F12. Subsequently, oocytes were checked for sperm penetration or cultured for an additional 6 h to assess oocyte nuclear configuration and second polar body formation; or 2.5 days in groups of 5 oocytes per 5  $\mu$ l droplet of DMEM/F12 with 10% fetal calf serum, at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. The presumptive embryos were fixed at different developmental stages (zygote, 2-cell, 4-8 cell, 8-16 cell) to assess nuclear configuration, second polar body formation and developmental stage. Oocytes were incubated in similar procaine-containing media in the absence of sperm.

### **Parthenogenetic activation by ionomycin**

Parthenogenetically activated equine oocytes were used as negative controls for sperm penetration and its role in calcium dependent oocyte activation, including second polar body formation. The protocol was performed as described by Heras *et al.* ([34]; based on K. Hinrichs, personal communication). Briefly, cumulus-denuded mature oocytes were incubated for 4 min in 5  $\mu$ M ionomycin diluted in non-capacitating medium on a heated stage (37°C). After washing five times in wash medium (0.5% BSA in DPBS), oocytes were incubated for 30 min in non-capacitating medium and then transferred to 2 mM 6-(dimethylamino)purine dissolved in DMEM/F12 medium. After 4 h incubation, the oocytes were washed 5 times with wash medium and once with DMEM/F12. The parthenogenetically activated oocytes were cultured in groups of 5 in 5  $\mu$ l droplets of DMEM/F12 with 10% fetal calf serum at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>.

### **ICSI**

ICSI zygotes were used as a positive control for sperm penetration, second polar body formation, embryonic development up to the blastocyst stage (7-9 days after ICSI), DNA

configuration, the cytoplasmic calcium response to fertilization and F-actin redistribution. ICSI was performed as described by Smits *et al.* [35]. In brief, in preparation for ICSI the oocytes were held in (HEPES) buffered DMEM/F12 medium, and the sperm in 9% polyvinylpyrrolidone in DPBS. All manipulations were performed on the heated stage (38.5 °C) of an inverted microscope. A progressively motile sperm was immobilized and subsequently injected into the cytoplasm of a mature oocyte using a piezo drill (Prime Tech, Ibaraki, Japan). The injected oocytes were cultured in groups of 5 in 5 µl droplets of DMEM/F12 with 10% fetal calf serum at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>.

### **Quantification of tail-associated protein tyrosine phosphorylation**

Assessment of protein tyrosine phosphorylation was performed as described by Leemans *et al.* [36]. Briefly, after 6 h of incubating pre-labelled (200 nM MitoTracker Green FM in 0.025% DMSO) or non-labelled sperm in various concentrations of procaine (0, 1, 2.5, 5 mM) and atmospheric conditions (5% CO<sub>2</sub> or air), sperm suspensions were washed twice and fixed in 4% paraformaldehyde in DPBS at room temperature for 15 min. The fixative was removed by three centrifugation steps using DPBS (600g for 5 min). The washed spermatozoa were subsequently incubated in 0.1% Triton X-100 in DPBS for 10 min at room temperature to ensure complete permeabilization of the membranes. The permeabilized spermatozoa were then incubated in blocking buffer (DPBS containing 1% BSA) for 10 min at room temperature. Next, the spermatozoa were incubated overnight at 4°C in buffer containing 0.1% BSA and the mouse monoclonal 4G10@Platinum IgG<sub>2b</sub> protein anti-phosphotyrosine antibody (diluted 1:500). After incubation, unbound antibody was removed by washing the spermatozoa twice with 1 ml of DPBS containing 0.1% BSA (600g for 5 min). The spermatozoa were then stained with a monoclonal goat anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen, Molecular Probes, Ghent, Belgium) for 1h at room temperature. After immunolabelling, the non-bound antibody conjugates were removed by washing three times with DPBS containing 0.1% BSA, and once using DPBS (600g for 5 min). The immunolabelled spermatozoa were mounted on glass slides under a cover slip and sealed with nail polish. The proportion of spermatozoa with green fluorescent tails among the total sperm population (with Hoechst 33342 fluorescent heads) was determined by randomly scoring 200

spermatozoa. Samples were examined using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400x.

### **Sperm penetration, DNA configuration and embryonic development**

Oocytes and zygotes were fixed at different developmental stages in 4% paraformaldehyde in DPBS at room temperature for 1 h. The fixative was removed by washing the oocytes twice in wash medium. Next, the fixed oocytes were incubated in a 3.2  $\mu$ M Hoechst 33342 solution in wash medium for 10 min at room temperature. The oocytes were then washed 4 times in wash medium and mounted on siliconized glass slides (Marienfeld, Germany) using 1,4-Diazabicyclo[2,2,2]octane (DABCO) as antifade, and sealed with nail polish. Excessive pressure from the cover slip was prevented by placing a few droplets of vaseline on the microscope slides prior to mounting. Starting from the incubation in Hoechst 33342, oocytes were shielded from the light to prevent premature fading. Mounted slides were kept at 4°C in the dark until evaluation. The presence of a MitoTracker Green FM positive sperm tail in the oocyte cytoplasm indicated sperm penetration through the zona pellucida (fertilization) while the Hoechst stain visualized the DNA of both the oocyte and the spermatozoa. The presence of a second polar body containing condensed DNA was also determined using both Hoechst staining and a fluorescent microscope and light microscopy, because it is a hall mark of oocyte activation during fertilization and signals the completion of the second meiotic division of the maternal chromatin. After 2.5 d in culture, the ability of equine oocytes / zygotes to undergo nuclear duplication and cell cleavage was assessed. Alternatively, oocyte degeneration was apparent if the oocytes showed an irregular oolemma or shape.

To confirm the validity of the MitoTracker Green FM labelled sperm-oocyte penetration experiments, *in vitro* fertilization was alternatively performed using unlabelled spermatozoa with sperm-penetration assessed by post-fixation lacmoid staining, as described by Martinez *et al.* [37]. Briefly, after IVF incubation and removal of the cumulus cells, denuded oocytes were mounted on a glass slide and fixed with acetic alcohol (acetic acid to ethanol, 1:3 v:v) for at least 24 h. The fixed oocytes were stained with 1% (w:v) lacmoid in acetic acid. The stained oocytes were immediately evaluated under a phase contrast microscope at x400 magnification. Oocytes were considered to have been penetrated

(fertilized) when at least one sperm tail was visible within the oocyte cytoplasm and the DNA of both the oocyte and the spermatozoa could be visualized. The presence of a second polar body was also examined.

### **Calcium oscillation pattern analysis**

The possibility that 2.5 mM procaine induced oocyte activation by triggering calcium influx was investigated by imaging calcium oscillations. The calcium oscillation patterns were compared to those for oocytes activated parthenogenetically using ionomycin, and for oocytes fertilized by ICSI. The protocol was based on that described by Nikiforaki *et al.* [38]. Briefly, morphologically normal MII stage equine oocytes were loaded with 7.5  $\mu\text{M}$  of the ratiometric calcium sensitive dye fura-2 acetoxymethyl (AM) ester in non-capacitating medium at 38.5 °C in air for 20 min, and then washed repeatedly in non-capacitating medium. The oocytes were then transferred to a 20  $\mu\text{l}$  droplet of capacitating medium containing 2.5 mM procaine or 5  $\mu\text{M}$  ionomycin, or subjected to ICSI in the absence of either activator, and incubated under equilibrated mineral oil on a 35 mm diameter glass bottom dish (MatTek Corp., cat.no. P35G-0P 14-C, Ashland, USA).

Calcium imaging was performed on the pre-heated stage (38.5 °C) of an inverted epifluorescence microscope (TH4-200, Olympus Soft Imaging Solutions GmbH, Belgium) equipped with a 10 $\times$  objective (100 $\times$  magnification) and UV light provided by a 75W Xenon arc lamp and modulated by neutral density filters. Recording cytoplasmic calcium began immediately after the exposure of oocytes to procaine or ionomycin, and within 40 min after ICSI. In the procaine and ionomycin groups, fluorescence measurements were made every 10 s for 6 h with a filter switch that provided excitation alternating between 340 and 380 nm; no illumination was applied between measurements. Similar measurements were performed every 30 s for 16 h in oocytes fertilized by ICSI. The concentration of free intracellular calcium was assumed to be proportional to the ratio of fluorescence at 340/380 (expressed in arbitrary units, AU). Baseline fluorescence was then set to ratio = 1. The microscope was equipped with an Okolabs stage micro-environment chamber enclosed in a CO<sub>2</sub> microscope cage incubator so that all measurements were conducted at 37 °C in the presence of 6% CO<sub>2</sub>. Oocytes that did not show any calcium oscillations in the ionomycin group were considered non-activated while, in the ICSI group, non-reactive oocytes were considered non-fertilized.

In these two groups, only oocytes that showed a calcium signal were included in subsequent analysis of calcium oscillation patterns.

### **Distribution of the cortical granules**

The protocol for assessing cortical granule distribution was adapted from Carneiro *et al.* [25]. Briefly, the zona pellucida (ZP) was first removed by incubating the denuded oocytes for 2-5 min at 38.5°C in 0.3% pronase from streptomyces griseus in HEPES-buffered TCM-199 with Hank's salts. The oocytes were then fixed in 4% paraformaldehyde in DPBS for 30 min at room temperature. Next, the oocytes were washed twice in wash medium before being incubated for 2 h in blocking solution (0.1% BSA, 0.75% glycine and 0.2% NaN<sub>3</sub> in DPBS) at room temperature. After this step, oocytes were incubated for 1 h at room temperature in permeabilization solution (0.5% Triton X-100 and 0.05% Tween in blocking solution). Permeabilized oocytes were washed twice in wash medium before labelling the cortical granules by incubation for 15 min at room temperature in 10 µg / ml fluorescein isothiocyanate-labelled *Lens culinaris* agglutinin (FITC-LCA) in blocking solution. To verify the nuclear status, the chromatin was counterstained with 3.2 mM Hoechst 33342 in wash medium for 10 min at room temperature. Subsequently, the oocytes were mounted on glass microscope slides as described above. LCA labelled oocytes were further divided into two categories based on the distribution of the cortical granules: (1) clearly visible cortical granules at the periphery of the ooplasm, indicating oocyte cytoplasmic maturation and (2) absence of cortical granules in the oocyte cytoplasm, indicating cortical granule exocytosis.

### **Assessing cytoplasmic pH of procaine exposed oocytes**

Mature oocytes were washed twice using non-capacitating medium and stained with 5 µM of the pH-sensitive dye BCECF-AM in non-capacitating medium by incubation at 38.5 °C for 30 min. The extracellular dye was then removed by washing the oocytes twice in non-capacitating medium, and the washed oocytes were transferred to capacitating medium in which they were incubated for a further 20 min to allow de-esterification of the BCECF-AM. Subsequently, the BCECF signal was measured in oocytes mounted on glass slides after 0, 1, 3 and 6 h incubation in capacitating medium containing 0, 1, 2.5, 5 and 10 mM procaine.



Imaging of oocyte cytoplasmic pH was performed on the pre-heated stage (38.5°C) of a fluorescence microscope (Leica DM 5500 B microscope; Leica Microsystems GmbH; Wetzlar, Germany) equipped with a 10× objective (100x magnification) and with UV light provided by a 120 W Hg lamp and modulated by neutral density filters. Recording of cytoplasmic pH was performed using a filter switch that provided excitation alternating between 440 and 490 nm. The cytoplasmic pH was proportional to the ratio of fluorescence at 440/490 (expressed in arbitrary units, AU). Baseline fluorescence was then set to ratio = 1.

To make representative images, the fluorescent signal for BCECF-AM labelled oocytes was acquired after 1 h incubation in various procaine concentrations and loaded into the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium). We set the baseline pH as that observed in 0 mM procaine under capacitating conditions at 1 h. We then assigned this fluorescence intensity a value of 0 by adjusting the settings such that no fluorescence was observed in BCECF-AM labelled oocytes in 0 mM procaine medium. All other oocytes incubated under 1, 2.5 and 5 mM procaine conditions were imaged with identical settings.

### **F-actin Distribution**

The distribution of F-actin in horse oocytes was assessed as described previously by Van den Broeke *et al.* [39]. Oocytes were fixed for 30 min in 4% paraformaldehyde in DPBS at room temperature. After being washed twice in wash medium, the oocytes were permeabilized using 0.1% Triton X-100 in DPBS for 1 h at room temperature. Subsequently, oocytes were washed twice in wash medium and incubated with 40 µM FITC-labelled phalloidin in DPBS for 1 h at room temperature. Next, the oocytes were washed in DPBS and mounted on glass slides as described previously. The intensity of the FITC signal correlates with the amount of F-actin. In this study we considered the amount of F-actin in mature oocytes as the reference intensity. Increased or similar FITC intensity indicated the beginning of the cell cycle whereas a decreased FITC signal indicated the end of cell cycle associated with the preparation for cytokinesis.

### **Sperm motility assessment**

To assess the combined effects of MitoTracker Green FM and DMSO on sperm motility, parameters of MitoTracker Green FM labelled sperm in suspension were evaluated using a computer-assisted sperm analyzer (CASA: Hamilton-Thorne motility analyzer Ceros version 12.3d; Hamilton-Thorne Research, Beverly, MA, USA). Under defined capacitating conditions, BSA was replaced with PVA to avoid the marked sperm agglutination noted for stallion sperm after centrifugation in BSA-containing medium [12]. For each analysis, 10  $\mu$ l of sperm solution diluted in non-capacitating medium was mounted on a pre-warmed glass slide (Marienfeld, Lauda-Königshofen, Germany) and maintained at 37°C using a minitherm stage warmer. Five randomly selected microscopic fields in the center of the slide were scanned 4 times each, generating 20 scans for every sample. The mean of the 5 scans for each sample was used for statistical analysis. The settings of the CASA-software HTR 12.3 for analyzing motility parameters of stallion sperm, were based on Loomis and Graham [40] and described previously by Hoogewijs *et al.* [41]. To evaluate the effect on sperm viability of 0, 200, 400, 800 and 1000 nM MitoTracker Green FM dissolved respectively in 0, 0.025, 0.05, 0.10 and 0.125% DMSO, the percentages of motile and progressively motile sperm were assessed. To assess the effect of 0, 1, 2.5 and 5 mM procaine on hypermotility parameters of sperm pre-labelled with 200 nM MitoTracker Green FM in 0.025% DMSO or non-labelled sperm, 2 motility parameters were evaluated: amplitude of lateral head displacement (ALH, in  $\mu$ m; ALH is the mean width of head oscillations), curvilinear velocity (VCL, in  $\mu$ m / s; VCL is the average velocity of a sperm head along its actual, two-dimensional curvilinear trajectory). Finally, an increase in these 2 motility parameters, ALH and VCL, was used to demonstrate hyperactivated motility in stallion sperm [6, 12].

### **Microscopic imaging**

Embryonic development was assessed using a CCD ICD-46E camera (Ikegami Tsushinki Co. Ltd., Japan) attached to an Olympus IX70 inverted microscope (Olympus Belgium N.V., Aartselaar, Belgium). Images of cleaved oocytes were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium).

Sperm labelling by various MitoTracker Green FM concentrations, sperm penetration through the zona pellucida, second polar body formation and DNA configuration of embryo development stages, the cortical granule distribution, cytoplasmic pH and F-actin distribution were determined by means of fluorescence microscopy using a Leica DM 5500 B microscope equipped with excitation filters with band pass 340/380 nm, 450/490 nm, 560/40 nm and a 120 W mercury lamp. MitoTracker Green FM, Hoechst 33342, LCA-FITC, BCECF-AM and phalloidin-FITC were excited using 490 nm, 345 nm, 495 nm, 490 nm and 495 nm wavelengths, respectively. The emission spectra were detected by Blue (BP 470/40 nm) and Green (LP 515 nm) filters corresponding to the emission peaks of the dyes at respectively 516 nm, 478 nm, 519 nm, 530 nm and 519 nm. Images were acquired using the Image Database program (Leica, Van Hopplynus N.V., Brussel, Belgium). The various fluorophores were checked for signal overlap; no bleed through of signals was detected.

Sperm penetration through the zona pellucida, DNA configuration, the cortical granule and F-actin distribution were confirmed by confocal microscopy using a Leica TCS SPE-II laser scanning spectral confocal system (Leica Microsystems GmbH; Wetzlar, Germany), equipped with an ACS APO 63X oil immersion objective (Leica) and linked to a DM 2500 upright microscope (Leica Microsystems). The fluorescent dyes were excited using a diode laser and analyzed using similar detection filters to those described for fluorescence microscopy. The images were obtained using Leica confocal software. For each wavelength, digital optical sections were collected using Z-series acquisition every 0.5  $\mu\text{m}$ . Corresponding DIC images and images of lacmoid-stained oocytes were acquired using the Leica DM 5500 B fluorescence microscope described above.

Oocyte cleavage, second polar body formation and DNA configuration were confirmed by confocal microscopy using an inverted Nikon A1R confocal microscope (Nikon Instruments, Paris, France), mounted on a Nikon Ti body, using a 60 $\times$  / 1.4 Plan Apo oil immersion lens. Hoechst was excited using a 405 nm diode laser and a 488 nm Argon laser was used for simultaneous DIC imaging. Images were acquired using Nikon Elements Software and a pinhole setting of 1 Airy unit and constant acquisition settings (laser power, gain and offset, scan speed). Digital optical sections were collected across an axial range that spanned the oocyte, at a step size of 1  $\mu\text{m}$ .

## Statistical analysis

The effect of the combination of MitoTracker Green FM and DMSO on sperm motility parameters, the effect of different concentrations of procaine on embryonic development, the relative cytoplasmic pH change induced by different concentrations of procaine and the amount of F-actin in the cortical region were assessed by analysis of variance (ANOVA). Significant differences were determined using repeated measures ANOVA with Greenhouse-Geisser and Bonferroni correction, as implemented in the general linear model. Scheffé post-hoc tests were performed for pairwise comparisons. Differences were considered significant if  $P < 0.05$ .

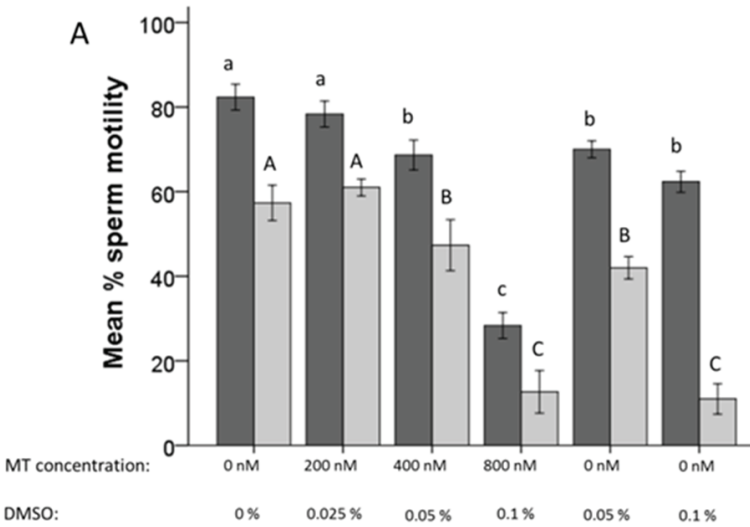
The effect of different fertilization conditions on sperm-oocyte penetration, extrusion of the second polar body, pronucleus formation and the effect of procaine on cortical granule reaction induction were analyzed by binary logistic regression for binomially distributed data. Where differences existed, further comparisons of groups were performed by chi-square analysis ( $\chi^2$  fit tests). All experiments were repeated three times. Differences were considered significant if  $P < 0.05$ . All analyses were performed using SPSS version 20 for Windows (SPSS IBM, Brussels, Belgium).

## RESULTS

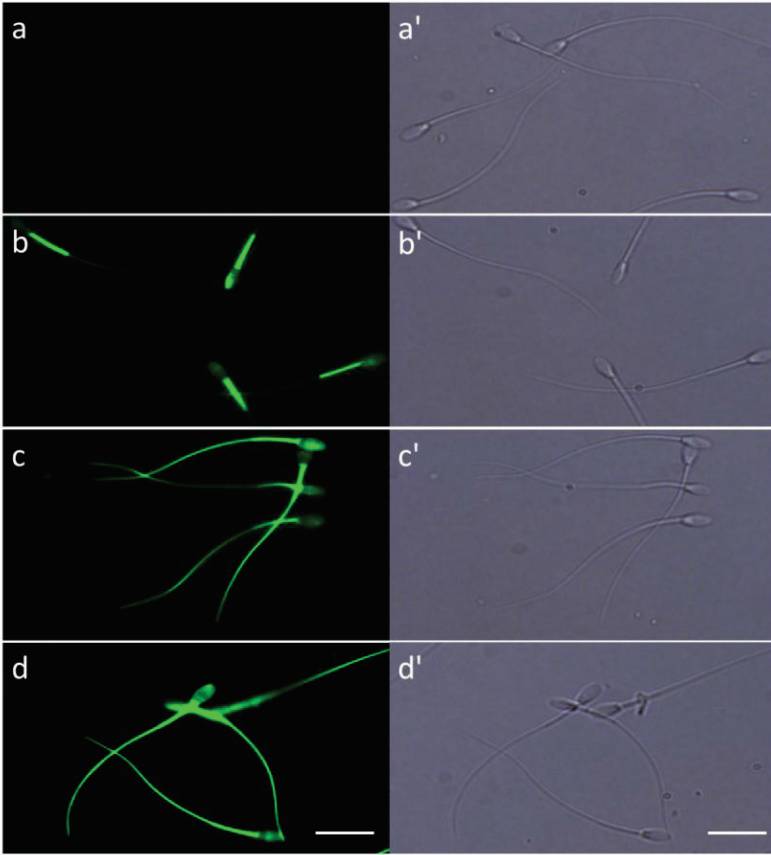
### Effect of procaine and MitoTracker Green FM on sperm motility characteristics

To assess the effect of MitoTracker Green FM and DMSO on sperm motility, 10 ml sperm suspensions ( $100 \times 10^6$  spermatozoa / ml) were incubated for 30 min with various concentrations of this mitochondrion specific stain dissolved in DMSO. In the control sperm suspension (0 nM MitoTracker Green FM, 0% DMSO) in our study,  $82 \pm 3\%$  of the spermatozoa were motile and  $57 \pm 4\%$  were progressively motile. Incubation with 200 nM MitoTracker Green FM and 0.025% DMSO did not significantly affect motility (total motility:  $78 \pm 3\%$ ;  $P = 0.32$ , progressive motility:  $61 \pm 2\%$ ;  $P = 0.21$ ). However, incubation with 0.05% DMSO, irrespective of the additional presence of 400 nM MitoTracker Green FM, depressed motility ( $P = 0.01$  and  $0.03$  for total and progressive motility, respectively). Total ( $69 \pm 4\%$ ) and progressive motility ( $47 \pm 5\%$ ) in the presence of 400 nM MitoTracker Green FM and

total ( $71 \pm 2\%$ ) and progressive motility ( $42 \pm 3\%$ ) in the presence of 0.05% DMSO without MitoTracker did not differ ( $P = 0.31$  and  $0.15$  for total and progressive motility, respectively). Higher concentrations of DMSO further disrupted both total and progressive sperm motility independent of the inclusion of MitoTracker Green FM, although the latter appeared to have an additional inhibitory effect on total but not progressive motility (Figures 1). We note that the presence of 0.1% DMSO also resulted in abnormal sperm movement. We concluded that 200 nM MitoTracker Green FM was the most suitable concentration for assessing sperm penetration through the zona pellucida, since there was no observable effect on gross sperm motility while a clear MitoTracker Green FM signal could be observed in the sperm mid-piece (Figure 2). The DMSO and MitoTracker Green FM concentrations used were relatively low compared to those used in successful *in vitro* fertilization experiments with bovine [42] and mouse [43] gametes.



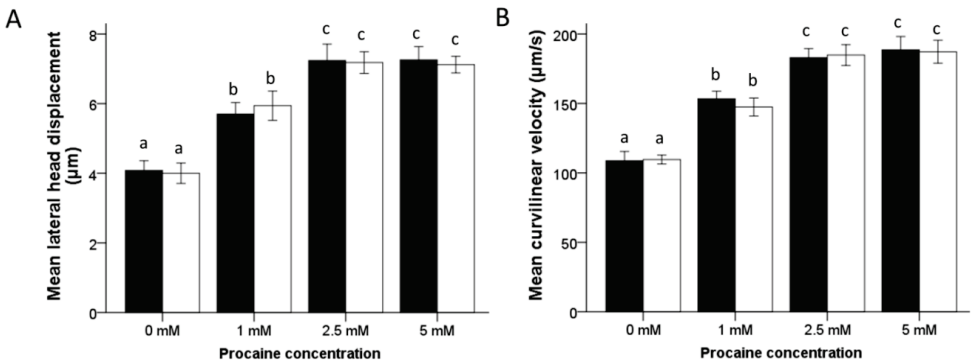
**Figure 1.** CASA motility parameters for stallion sperm suspensions after 30 min incubation with various concentrations of MitoTracker Green FM (MTG) dissolved in DMSO: 0 nM MTG, 200 nM MTG and 0.025% DMSO, 400 nM MTG and 0.05% DMSO, 800 nM MTG and 0.1% DMSO, 0 nM MTG and 0.05% DMSO and 0 nM MTG plus 0.1% DMSO and subsequently diluted in 10 ml non-capacitating medium. Bars show mean ( $\pm$  s.d.) percentages of motile (dark grey bars) and progressively motile (light grey) spermatozoa for three replicates. For the percentage of motile spermatozoa, values that differ significantly are indicated by different small letters (motile sperm) or capitals (progressively motile sperm). Comparisons were performed using repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction, followed by Scheffé post hoc tests for pairwise comparisons.



**Figure 2.** Fluorescence microscope images of spermatozoa stained with MitoTracker Green FM (MTG) dissolved in DMSO (a) 0 nM MTG, (b) 200 nM MTG and 0.025% DMSO, (c) 400 nM MTG and 0.05% DMSO and (d) 800 nM MTG and 0.1% DMSO. Corresponding light microscope images (e, f, g, h) were taken by DIC (original magnification, 1000 x; Bar = 25  $\mu$ m).

In Chapter 5 we showed that differently treated FF at pH 7.9 induced hyperactivated motility. To assess the effect of the various procaine, MitoTracker Green FM and DMSO concentrations on sperm hypermotility, non-labelled and Mitotracker Green FM labelled (200 nM MitoTracker Green FM in 0.025% DMSO) sperm preparations ( $10 \times 10^6$  spermatozoa / ml) were first incubated for 6 h in capacitating conditions with an elevated pH (7.9) and subsequently for 30 min in various procaine concentrations (0, 1, 2.5 and 5 mM). Compared to procaine-free capacitating conditions (ALH:  $4.0 \pm 0.3 \mu$ m and VCL:  $110 \pm 3 \mu$ m / s), sperm

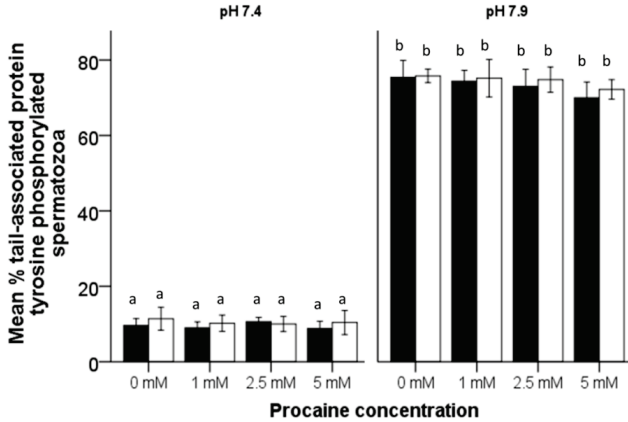
ALH and VCL measurements were significantly higher when procaine was included (1 mM: ALH  $5.9 \pm 0.4 \mu\text{m}$  and VCL  $147 \pm 6 \mu\text{m} / \text{s}$ ; 2.5 mM: ALH  $7.1 \pm 0.3 \mu\text{m}$  and VCL  $185 \pm 8 \mu\text{m} / \text{s}$  and 5 mM: ALH  $7.0 \pm 0.2 \mu\text{m}$  and VCL  $187 \pm 13 \mu\text{m} / \text{s}$ ) and became more pronounced as the procaine concentration increased. In accordance with the motility data described above, no significant effect on sperm hypermotility parameters was observed when sperm suspensions were pre-labelled with 200 nM MitoTracker Green FM dissolved in 0.025% DMSO (0 mM: ALH  $4.1 \pm 0.2 \mu\text{m}$  and VCL  $118 \pm 6 \mu\text{m} / \text{s}$ ; 1 mM: ALH,  $5.7 \pm 0.3 \mu\text{m}$  and VCL  $153 \pm 5 \mu\text{m} / \text{s}$ ; 2.5 mM: ALH  $7.2 \pm 0.5 \mu\text{m}$  and VCL  $183 \pm 6 \mu\text{m} / \text{s}$  and 5 mM: ALH  $7.2 \pm 0.3 \mu\text{m}$  and VCL  $188 \pm 11 \mu\text{m} / \text{s}$ ) (Figure 3A and 3B).



**Figure 3.** Motility patterns indicative of hyperactivated motility were assessed by CASA and included (A) lateral head displacement (ALH) and (B) curvilinear velocity (VCL) in 200 nM MitoTracker Green (MTG) + 0.025% DMSO labelled (black bars) and non-MTG labelled (white bars) stallion sperm suspensions after 6 h incubation in capacitating medium and subsequently 30 min incubation in 0, 1, 2.5 and 5 mM procaine dissolved in capacitating medium (pH=7.4) (n=5 samples in each group; 3 replicates). Hyperactivated motility in sperm suspensions was clearly triggered by 2.5 and 5 mM procaine while motility indicative of hypermotility (ALH and VCL) was not evident at lower procaine concentrations. Pre-labelling stallion spermatozoa with MTG did not have any effect on hyperactivated motility. For both lateral head displacement and curvilinear velocity, values that differ significantly are indicated by different small letters. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were performed for pairwise comparison.

## Effect of procaine and MitoTracker Green FM on sperm tail-associated protein tyrosine phosphorylation

In chapter 5 we showed that differently treated FF at pH 7.9 induced tail-associated protein tyrosine phosphorylation in stallion sperm. To assess the effect of MitoTracker Green FM dissolved in DMSO and procaine on tail-associated protein tyrosine phosphorylation, sperm suspensions ( $10 \times 10^6$  spermatozoa / ml) were incubated for 6 h in 0, 1, 2.5 and 5 mM procaine at 2 different pHs, namely pH 7.4 (5% CO<sub>2</sub> in air incubation) and pH 7.9 (air incubation). Similar to previous reports [36, 44, 45], in these capacitating conditions tail-associated protein tyrosine phosphorylation was induced by an atmospheric air-related pH increase to 7.9, whereas procaine had no significant effect on this capacitation-associated event (pH 7.4 conditions: 0 mM,  $11 \pm 3\%$ ; 1 mM,  $10 \pm 2\%$ ; 2.5 mM,  $10 \pm 2\%$  and 5 mM,  $10 \pm 3\%$  compared to pH 7.9 conditions: 0 mM,  $76 \pm 2\%$ ; 1 mM,  $75 \pm 5\%$ ; 2.5 mM,  $75 \pm 3\%$  and 5 mM,  $72 \pm 3\%$ ). Moreover, no effect of MitoTracker Green FM dissolved in DMSO was evident for rates of tail-associated protein tyrosine phosphorylation (pH 7.4 conditions: 0 mM,  $10 \pm 2\%$ ; 1 mM,  $9 \pm 2\%$ ; 2.5 mM,  $11 \pm 1\%$  and 5 mM,  $9 \pm 2\%$  compared to pH 7.9 conditions: 0 mM,  $75 \pm 5\%$ ; 1 mM,  $74 \pm 3\%$ ; 2.5 mM,  $73 \pm 5\%$  and 5 mM,  $70 \pm 4\%$ ) (Figure 4).

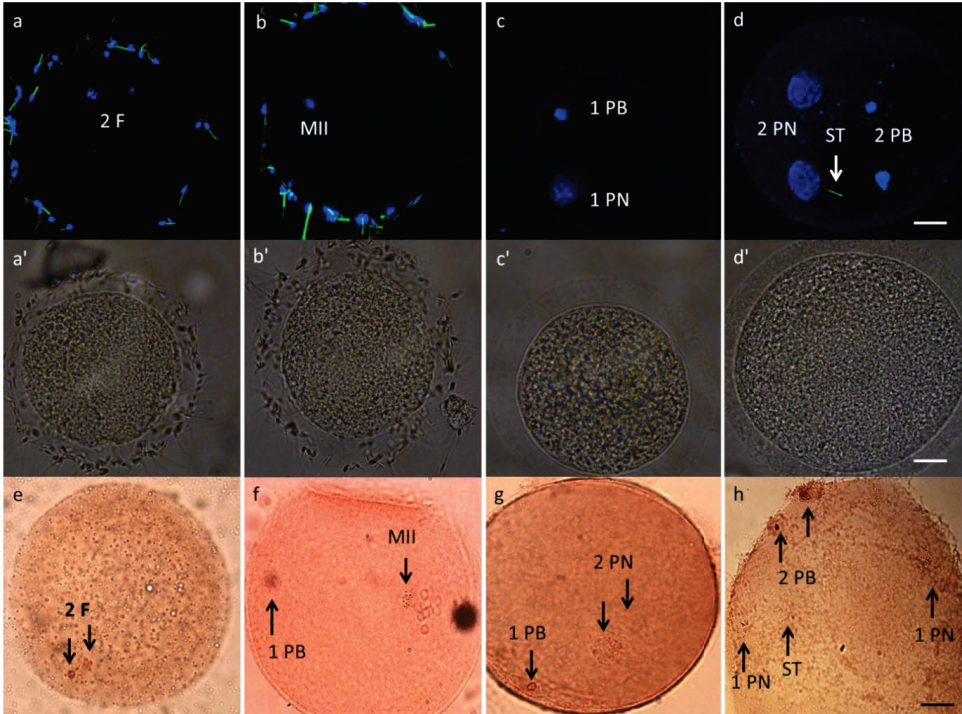


**Figure 4.** The percentage of spermatozoa pre-labelled with 200 nM MTG dissolved in 0.025% DMSO (black bars) and 0 nM MTG (white bars) and subsequently incubated for 6 h in 0, 1, 2.5 and 5 mM procaine that showed tail-associated protein tyrosine phosphorylation was assessed at pH 7.4 and pH 7.9 ( $n=5$  samples in each group; 3 replicates). Tail-associated protein tyrosine phosphorylation in stallion spermatozoa was clearly related to elevated medium pH 7.9 whereas procaine and MTG-DMSO did not have any effect. Values that differ significantly are indicated by different small letters. Repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were performed for pairwise comparison.



**Procaine and treated follicular fluid at pH 7.9 do not induce penetration of equine oocytes by sperm *in vitro***

In previous equine studies, fertilization after conventional IVF was assessed by determining the presence of at least 2 pronuclei in oocytes fixed 24 h after the *in vitro* fertilization incubation [8, 46, 47]. McPartlin *et al.* [6] assessed pronuclear formation after 18 h of gamete co-incubation and an additional 24 h culture. This would not however differentiate definitively between true fertilization following sperm penetration and parthenogenetic activation of an unfertilized oocyte. McPartlin *et al.* [6] did not report the oocyte-activation specific appearance of a second polar body in *in vitro* fertilized oocytes. We consider definitive proof of fertilization to include the presence of at least one sperm in the cytoplasm of the oocyte as assessed using; (1) labelled spermatozoa e.g. with 200 nM MitoTracker Green FM shortly before gamete co-incubation to label the mitochondria in the mid-piece [42] or; (2) post-fixation lacmoid staining of *in vitro* fertilized oocytes. In addition, the formation of the second polar body could be assessed by both Hoechst staining and light microscopic evaluation (3) and would confirm completion of the second meiotic division. Equine gamete co-incubation in the presence of 0, 1, 2.5 or 5 mM procaine (Figure 5a) and in treated FF at pH 7.9 (Figure 5b) did not result in sperm penetration through the zona pellucida in any cases ( $0 \pm 0\%$ ) as assessed by MitoTracker Green FM pre-labelling. Neither did the presence of cumulus cells around the oocytes affect sperm-oocyte penetration rates. Oocytes activated parthenogenetically using ionomycin (Figure 5c) were used as a negative control, and also showed no evidence of MitoTracker Green FM labelling in the oocyte cytoplasm. As a positive control for stability of the stain, sperm were injected into mature (MII) equine oocytes by ICSI (Figure 5d); in these oocytes the sperm tail was clearly visible inside the oocyte cytoplasm (P-value < 0.001 for all comparisons). The failure of oocyte penetration during IVF was confirmed using unlabelled sperm and post-fixation lacmoid staining (Figure 5e, 5f, 5g and 5h). Furthermore, none of the procaine or FF treated oocytes formed a second polar body as evidence of completion of the second meiotic division ( $0 \pm 0\%$ ). By contrast,  $89 \pm 7\%$  of ICSI fertilized oocytes showed a normal second polar body containing condensed DNA (see Figure 5d and 5h). Together these data support the hypothesis that procaine and treated FF (pH 7.9) does not support sperm penetration or *in vitro* fertilization.



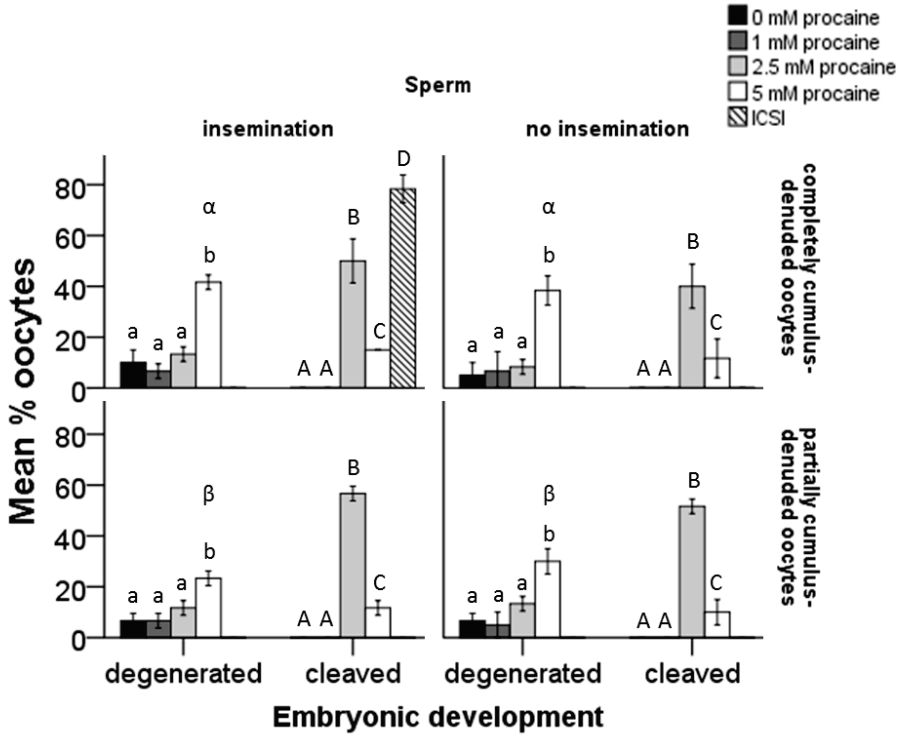
**Figure 5.** Confocal microscope images of a cumulus-free oocyte after 18 h co-incubation with spermatozoa in (a) 5 mM procaine diluted in capacitating medium and (b) heat-inactivated FF (pH 7.9), and stained with Hoechst and Mitotracker Green FM to assess sperm penetration, (c) an oocyte activated parthenogenetically using ionomycin (negative control) and (d) an oocyte fertilized by ICSI (positive control). Corresponding light microscopic images (a', b', c', d') were taken by DIC. The formation of a second polar body and the presence of a spermatozoon inside the cytoplasm of oocytes incubated with sperm, in the presence or absence of procaine, was never observed. Indeed, the formation of pronuclei was a rare observation (<3%) in oocytes incubated with sperm. Similar observations were made for gamete co-incubation in treated FF (pH 7.9). Light microscopic DIC images of an oocyte after 18 h co-incubation with unlabelled sperm in (e) 5 mM procaine or (f) heat-inactivated FF, (g) an oocyte activated parthenogenetically using ionomycin (negative control) and (h) an ICSI-fertilized oocyte (positive control) after post-fixation lacmoid staining were also examined. Both methods of evaluation indicated that IVF in the presence of procaine induced neither fertilization nor normal parthenogenesis, but instead triggered DNA fragmentation. In similarity, IVF in the presence of treated FF (pH 7.9) did not induce fertilization or parthenogenesis although oocytes were arrested in metaphase II (n=20 oocytes; three replicates; original magnification, 630 x; Bar = 25  $\mu$ m. ST; sperm tail. PN; pronucleus. PB; polar body. F; condensed DNA fragments).

**Procaine induces cell cleavage up to the 8-16 cell stage**

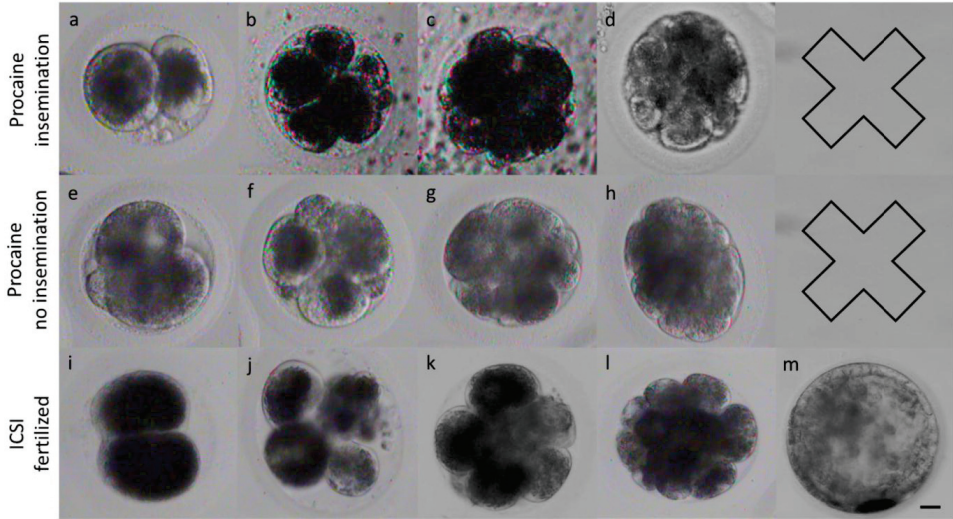
After 2.5 days of culture, we observed a degenerative effect of 5 mM procaine on the oocyte. In capacitating conditions,  $7 \pm 3\%$  of completely cumulus-denuded oocytes (CD) and  $5 \pm 5\%$  of partially cumulus-denuded oocytes (PD) degenerated; in 1 mM procaine  $8 \pm 4\%$  CD and  $7 \pm 4\%$  PD degenerated; in 2.5 mM procaine  $12 \pm 3\%$  CD and  $10 \pm 3\%$  PD; and in 5 mM procaine  $38 \pm 6\%$  CD and  $27 \pm 4\%$  PD degenerated. This suggested a concentration-dependent toxic effect of procaine on developing embryos that could be partially inhibited by the presence of cumulus cells around the oocytes during the 24 h gamete co-incubation ( $P < 0.001$  for all comparisons; Figure 6). Moreover, no significant degenerative effect on treated FF (pH 7.9) exposed oocytes could be observed (heat-inactivated FF,  $9 \pm 4\%$  CD and  $5 \pm 3\%$  PD; charcoal-inactivated FF,  $5 \pm 7\%$  CD and  $4 \pm 4\%$  PD; 30 kDa filtered FF,  $10 \pm 4\%$  CD and  $7 \pm 2\%$  PD; and triple treated FF,  $8 \pm 6\%$  CD and  $7 \pm 3\%$  PD).

Interestingly, in our hands in the presence of 2.5 mM procaine,  $44 \pm 12\%$  CD and  $56 \pm 4\%$  PD oocytes cleaved, whereas cleavage was significantly reduced in the presence of 5 mM procaine ( $12 \pm 6\%$  CD and  $8 \pm 4\%$  PD). Significantly more oocytes started to cleave when the cumulus investment was left in place during gamete co-incubation ( $P < 0.001$  for all comparisons; Figure 6). A significantly higher cleavage rate was achieved in ICSI-fertilized oocytes ( $78 \pm 8\%$  of injected oocytes;  $P < 0.001$ ). Tellingly, oocytes that cleaved in the presence of procaine never developed beyond the 8-16 cell stage even though, after exposure to 2.5 mM procaine in capacitating conditions,  $70 \pm 5\%$  CD and  $67 \pm 8\%$  PD of the cleaved oocytes reached the 8-16 cell stage ( $31 \pm 2\%$  CD and  $37 \pm 4\%$  PD of the incubated oocytes). A similar pattern was observed after exposure to 5 mM procaine, when  $63 \pm 3\%$  CD and  $65 \pm 5\%$  PD developed to the 8-16 cell stage ( $8 \pm 2\%$  of CD and  $5 \pm 1\%$  of PD oocytes). The presence of cumulus cells around the oocyte did not have a significant effect on the percentage of cleaved oocytes that reached the 8-16 cell stage ( $P > 0.14$  for all comparisons). Moreover, no effect on oocyte cleavage could be observed in 0 and 1 mM procaine conditions and the 4 treated FF conditions (pH 7.9) ( $<1\%$  in all groups). In short, cleaved embryos that formed in the presence of procaine never ( $0 \pm 0\%$ ) reached the blastocyst stage whereas  $15 \pm 6\%$  of ICSI oocytes did develop to blastocysts (Figure 7). After 5-6 days of incubation, all presumptive embryos derived from procaine treatments started to degenerate. Moreover, none of these parameters differed significantly between oocytes incubated in the presence or

absence of spermatozoa. This indicates that procaine induced oocyte activation, cleavage and embryo development were all sperm-independent (Figure 6 and 7).



**Figure 6.** Percentage of completely or partially cumulus-denuded oocytes that had degenerated or cleaved after 2.5 days in culture following fertilization incubation in capacitating medium containing 0, 1, 2.5 or 5 mM procaine with or without spermatozoa. ICSI fertilized oocytes were used as a positive control for cleavage (hatched bar). In the presence of 2.5 and 5 mM procaine, oocytes cleaved up to the 8-cell stage. Cleavage was not observed for 1 mM procaine or capacitating medium (0 mM procaine). Data represent mean ( $\pm$  s.d.) percentages of oocytes after incubation in capacitating medium (black bars), 1 mM procaine in capacitating medium (dark grey bars), 2.5 mM (light grey bars) and 5 mM procaine (white bars);  $n=20$  oocytes in each group, three replicates. Values that differ significantly ( $p<0.05$ ) are indicated by different small letters for degenerated oocytes or capitals for cleavage. Values that differ significantly between completely and partially cumulus-denuded oocytes are indicated by Greek letters ( $p<0.05$ ). The numbers of degenerated and cleaved oocytes were analyzed by binary logistic regression, with chi-square ( $\chi^2$ ) tests performed for pairwise comparison.



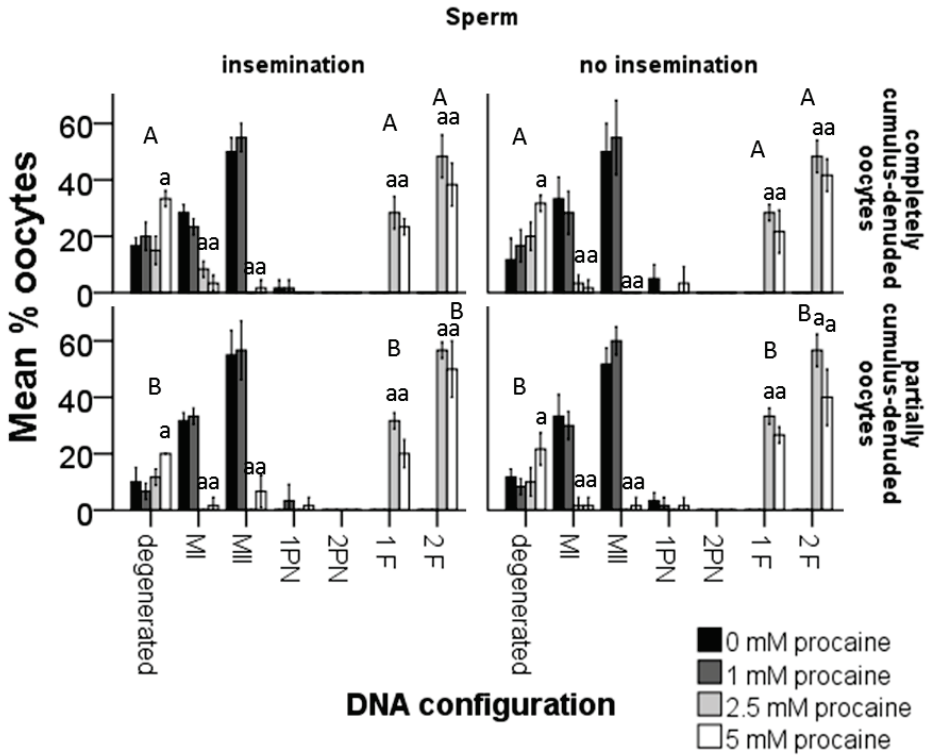
**Figure 7.** Micrographs of embryo developmental stages (a, e and i: 2-cell; b, f and j: 4-cell; c, g and k: 8-cell; d, h and l: 16-cell; m: blastocyst) after oocytes were incubated for 18 h in 2.5 mM procaine in capacitating medium with or without sperm, and subsequently cultured in a DMEM/F12 plus 10% FBS based medium. Oocytes that cleaved in the presence of procaine never developed further than the 8-16 cell stage. ICSI fertilized oocytes were used as positive control (original magnification, 400x: Bar = 20  $\mu$ m).

### Procaine-induced embryonic cleavage is associated with aberrant DNA segregation and fragmentation

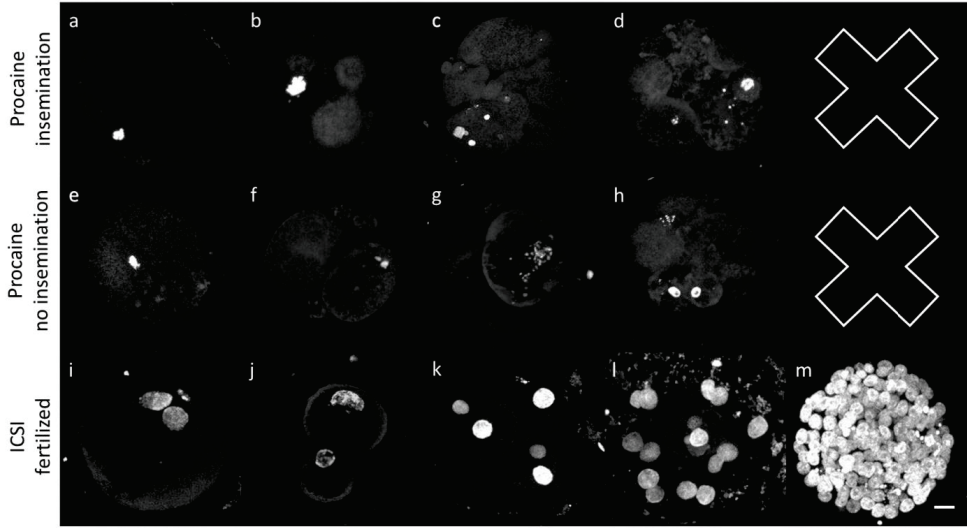
As indicated above, procaine triggered cleavage of horse oocytes while treated FF (pH 7.9) did not. We also analyzed the effect of procaine (0, 1, 2.5 and 5 mM procaine in capacitating conditions) and treated FF (pH 7.9) on nuclear configuration during embryo development. After 24 h incubation in the presence of 5 mM procaine, we observed significantly more DNA fragmentation in the oocytes than in oocytes exposed to 0, 1, or 2.5 mM procaine or treated FFs (pH 7.9). Moreover, the incidence of oocytes with fragmented DNA was higher for the completely denuded (CD) than for the partially cumulus-denuded oocytes (PD) (Figure 8). In addition, in 0 (control) and 1 mM procaine, the majority of oocytes displayed a normal metaphase spindle (MI:  $32 \pm 3$  and MII:  $55 \pm 9\%$ ) indicating that procaine up to 1 mM did not affect the DNA configuration (Figure 8). A very similar pattern was observed in oocytes incubated in treated FF with elevated pH (7.9) (heat-inactivated FF,

MI:  $27 \pm 7$  and MII:  $61 \pm 5\%$ ; charcoal-inactivated FF, MI:  $31 \pm 5$  and MII:  $54 \pm 7\%$ ; 30 kDa filtered FF, MI:  $29 \pm 3$  and MII:  $55 \pm 4\%$ ; and triple treated FF, MI:  $33 \pm 7$  and MII:  $58 \pm 6\%$ ). By contrast, after exposure to 2.5 or 5 mM procaine one (1F) or two (2F) very condensed fragments of DNA were observed instead (2.5 mM procaine:  $32 \pm 3\%$  1F and  $57 \pm 3\%$  2F; 5 mM procaine:  $20 \pm 5\%$  1F and  $50 \pm 10\%$  2F, respectively; Figure 8). The presence of cumulus cells around the oocytes during procaine exposure ( $P < 0.001$  for all comparisons) protected against DNA fragmentation. The formation of normal healthy (pro)nuclei (1 or 2 PN), indicating that oocytes had undergone fertilization or viable parthenogenesis, was a rare finding in procaine-treated oocytes (less than  $3 \pm 3\%$ ; Figure 8) and treated FF (pH 7.9) exposed oocytes (less than  $2 \pm 3\%$ ). In contrast to oocytes incubated with sperm at various procaine concentrations,  $87 \pm 5\%$  of ICSI fertilized oocytes formed two pronuclei 24 h after injection using the same maturation and culture conditions used for procaine-exposed oocytes ( $p < 0.001$  for all comparisons).

During development of embryos formed in the presence of procaine, the majority of the apparent daughter cells did not contain DNA at all, while others displayed condensed, fragmented pieces of DNA (Figure 9). In ICSI-fertilized oocytes, every cell contained a nucleus (Figure 9). None of these parameters evaluated after procaine exposure differed between oocytes incubated in the presence or absence of spermatozoa, again indicating that procaine-induced cleavage events were sperm-independent ( $P > 0.11$  for all comparisons; Figure 8 and 9).



**Figure 8.** Percentages of completely or partially cumulus-denuded oocytes that showed (1) degeneration, (2) meiosis I stage (MI), (3) meiosis II stage (MII), (4) 1 pronucleus (1PN), (5) 2 pronuclei (2PN), (6) 1 DNA fragment (1F) and (7) 2 DNA fragments (2F) after 24 h in fertilization incubation in capacitating medium containing 0, 1, 2.5 and 5 mM procaine with or without spermatozoa. In general, this experiment showed clearly that procaine-exposed oocytes rarely form pronuclei, but instead exhibit condensed DNA fragments. Data represent mean ( $\pm$  s.d.) percentages of oocytes after incubation in capacitating medium (black bars), 1 mM procaine in capacitating medium (dark grey bars), 2.5 mM (light grey bars) and 5 mM procaine (white bars); n=10 oocytes in each group, three replicates. Values that do not differ significantly ( $p < 0.05$ ) within each type of DNA configuration are indicated by the same small letter. Values that do not differ significantly between cumulus-free and cumulus-intact oocytes are indicated by the same capital letter ( $p < 0.05$ ). The numbers of degenerated and cleaved oocytes were analyzed by binary logistic regression, with chi-square ( $\chi^2$ ) tests performed for pairwise comparison.

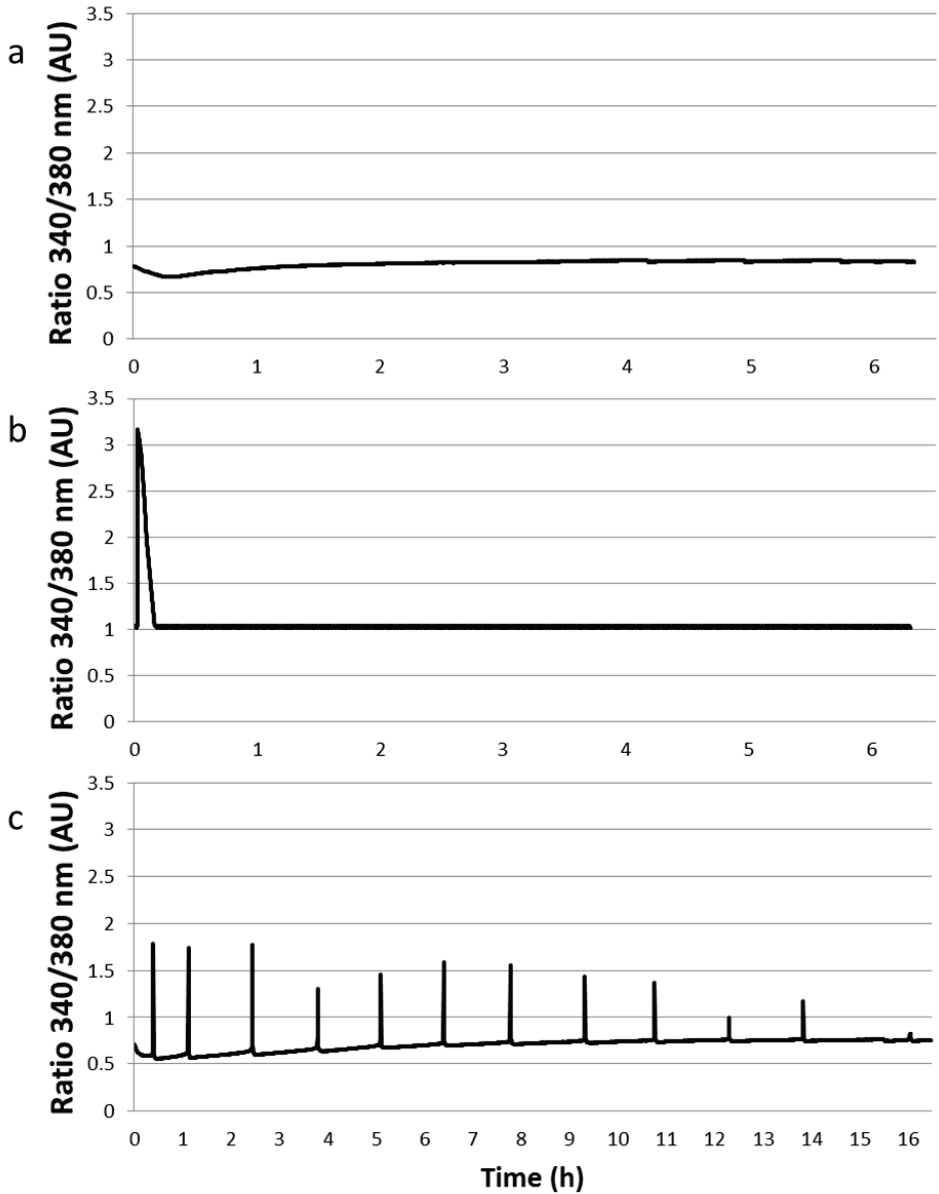


**Figure 9.** Confocal micrographs to demonstrate the DNA of different embryo developmental stages (a, e and i: uncleaved oocyte; b, f and j: 2-3-cell stage; c, g and k: 5-6-cell; d, h and l: 8-16-cell and m: blastocyst) after incubation for 18 h in 2.5 mM procaine in capacitating medium with or without sperm, followed by culture in a DMEM/F12 plus 10% FBS based medium. Many cells contained no DNA, and the visible DNA was very condensed and fragmented. ICSI fertilized oocytes were used as positive control (original magnification, 400x: Bar = 20  $\mu$ m).

### Procaine does not induce cytoplasmic calcium oscillations in horse oocytes

With respect to what has been reported for various mammalian species [38, 48, 49], we wanted to determine whether procaine-induced horse oocyte activation evoked calcium oscillations. To this end, we ratiometrically measured calcium oscillations in procaine- and ionomycin-exposed, and in ICSI-fertilized, horse oocytes. In the case of ICSI, fertilized oocytes exhibited a series of cytoplasmic calcium rises over at least 16 h (Figure 10c). This pattern was similar to that reported previously in mouse [48], human [38, 48] and equine [50] oocytes. Similar to reports for mouse and human oocytes [51, 52], parthenogenetic activation by ionomycin induced a single early rise in intracellular calcium (Figure 10b). By contrast, incubation of *in vitro* matured equine oocytes in 2.5 mM procaine did not trigger cytoplasmic calcium fluctuations during a 6 h incubation (Figure 10a).

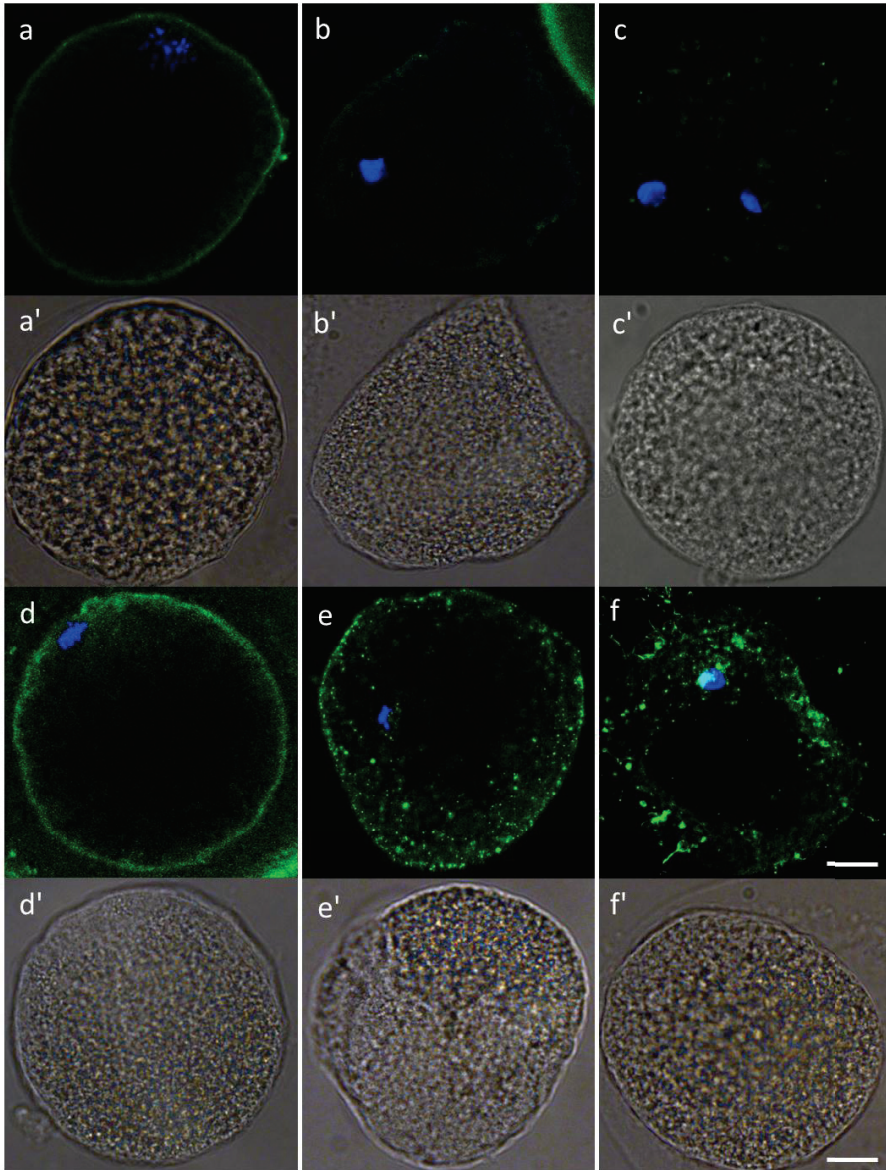




**Figure 10.** Representative cytoplasmic calcium oscillations assessed using the ratiometric dye, fura-2 AM, in equine oocytes exposed for 6 h to (a) 2.5 mM procaine and (b) 5  $\mu$ M ionomycin or for 16 h after (c) fertilization by ICSI. Procaine did not induce a cytoplasmic calcium rise in equine oocytes, whereas a single early cytoplasmic calcium rise was observed after exposure to ionomycin, and multiple calcium oscillations were evident after fertilization by ICSI (n=20 oocytes for the procaine group; n=5 oocytes for the ionomycin group; n= 15 oocytes for the ICSI group).

### **Procaine does not induce cortical granule exocytosis**

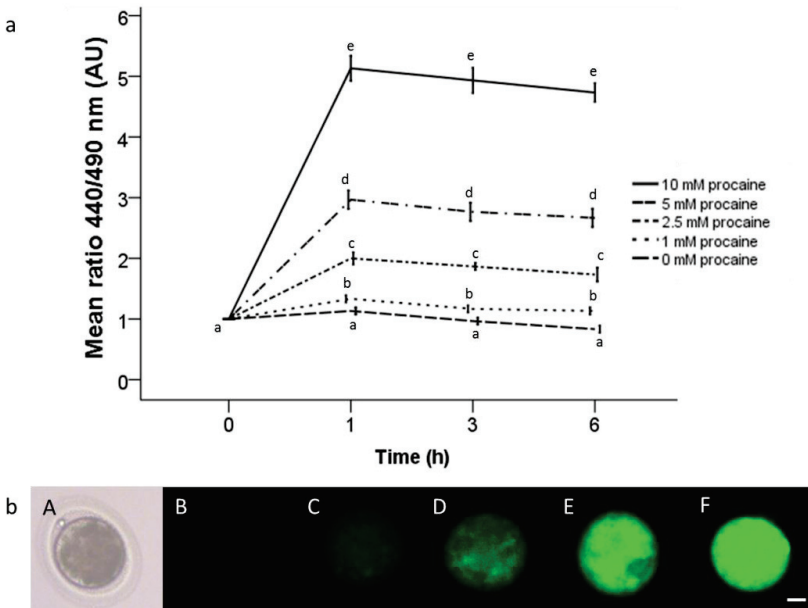
To alternatively assess calcium signaling during oocyte activation we used lectin staining (LCA-FITC) to determine whether procaine could induce the cortical reaction. After oocyte maturation, we found that the cortical granules were concentrated in the periphery of the cytoplasm of most oocytes ( $83 \pm 3\%$ ; Figure 11a). When the oocytes were subsequently incubated for 6 h in capacitating conditions (0% procaine), the same cortical granule pattern was generally maintained ( $85 \pm 5\%$ ; Figure 11d). Ionomycin-activated and ICSI fertilized oocytes lost their cortical granules, indicating that the oocytes had undergone calcium-dependent cortical granule extrusion (ionomycin:  $85 \pm 4\%$ , ICSI:  $82 \pm 7\%$ ; Figure 11b and 11c). By contrast, procaine-activated oocytes did not extrude their cortical granules during a 6 h procaine / IVF incubation; instead a proportion of the cortical granules were maintained in the periphery of the cytoplasm and even more were redistributed to more centrally in the cytoplasm (2.5 mM procaine:  $76 \pm 7\%$ ; 5 mM procaine:  $68 \pm 5\%$ ; Figure 11e and 11f).



**Figure 11.** Cortical granule exocytosis was assessed by LCA-FITC staining in (a) mature oocytes; (b) 6 h after exposure to 5  $\mu$ M ionomycin; (c) 6 h after ICSI; 6 h after exposure to (d) 0 mM, (e) 2.5 mM procaine and (f) 5 mM Procaine (n=10 oocytes in each group; three replicates). Corresponding light microscopic images (a', b', c', d', e', f') were taken by DIC. Procaine did not induce cortical granule exocytosis but ICSI fertilization and ionomycin activation did. Moreover, in the procaine-activated oocytes, the cortical granules were present not only in the periphery of the oocyte cytoplasm but also more centrally (original magnification, 630x: Bar = 25  $\mu$ m).

### Procaine induces cytoplasmic alkalinization in horse oocytes

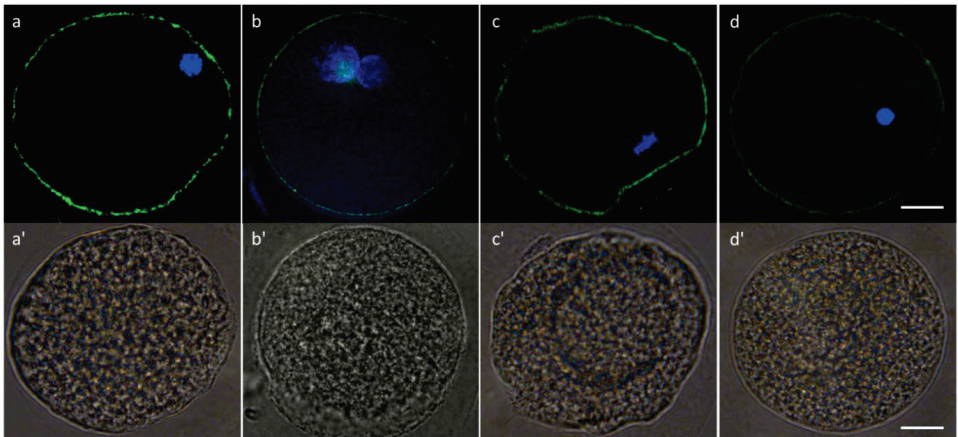
Using BCECF-AM staining, we ratiometrically assessed the effect on horse oocytes of exposure to 0, 1, 2.5, 5 and 10 mM procaine. It transpired that procaine induced a rapid, concentration-dependent increase in the BCECF-AM ratio during the first hour of incubation (after 1 h in 0 mM procaine:  $1.13 \pm 0.06$ ; in 1 mM procaine:  $1.33 \pm 0.06$ ; in 2.5 mM procaine:  $1.87 \pm 0.10$ ; in 5 mM procaine:  $2.97 \pm 0.15$ ; and in 10 mM procaine:  $5.13 \pm 0.21$ ) which remained constant during the remainder of the 6 h culture period ( $P > 0.11$  for all comparisons; Figure 12a). The procaine concentration dependent increase in BCECF-AM fluorescence in horse oocytes at 1 h of incubation is shown in Figure 12b. The BCECF-AM fluorescence in horse oocytes incubated in 0 mM procaine was assigned as the baseline to which oocytes exposed to procaine differed significantly ( $p < 0.001$ ).



**Figure 12.** (a) Intracellular pH was assessed at 0, 1, 3 and 6 h using the ratiometric dye BCECF-AM in equine oocytes exposed to 0, 1, 2.5, 5 and 10 mM procaine in capacitating medium. Increasing procaine concentration was associated with an increase in cytoplasmic pH. Values are mean ( $\pm$  s.d.) BCECF-AM ratio in oocytes exposed to 0, 1, 2.5, 5 and 10 mM procaine ( $n=5$  oocytes in each group; three replicates). Values that differ significantly are indicated by different small letters ( $p < 0.05$ ). Comparisons were performed by repeated measure ANOVA with Greenhouse-Heisser and Bonferroni correction; Scheffé post hoc tests were used for pairwise comparisons. (B) Changes in BCECF-AM fluorescence intensity in equine oocytes exposed to 0, 1, 2.5, 5 and 10 mM procaine in capacitating media for 1 h. A clear procaine concentration dependent fluorescence signal (indicating a pH rise) was observed (original magnification, 100x; Bar = 25  $\mu$ m).

### Procaine induces depolymerization of cortical F-actin in horse oocytes

To assess the effect of procaine on cortical F-actin turnover in preparation for cytokinesis, we used phalloidin-FITC staining. Two different phalloidin-FITC staining patterns were observed (Figure 13). The amount of F-actin present in the cortical region of mature oocytes was relatively high (Figure 13a). After 18 h exposure to procaine (2.5 mM), the majority of oocytes showed a clear decrease in F-actin abundance (Figure 13d; only  $24 \pm 6\%$  oocytes retained the more intense phalloidin labelling pattern), whereas in standard conditions (0 mM procaine) the F-actin pattern was similar to that seen in pre-incubation MII oocytes ( $81 \pm 5\%$ ; Figure 13c;  $P < 0.001$ ). As expected, 18 h after injection ICSI-fertilized oocytes also showed a decrease in F-actin abundance (Figure 13b) with only  $26 \pm 5\%$  retaining the abundant phalloidin labelling pattern. The similarity in actin cytoskeleton depolymerization in ICSI-fertilized and procaine-treated oocytes indicates that at least some aspects of procaine-induced cytokinesis of equine oocytes are similar to fertilization-induced cleavage.



**Figure 13.** Cortical F-actin distribution as assessed by actin phalloidin-FITC staining in (a) mature oocytes and (b) ICSI-fertilized oocytes (18 h after ICSI), compared to oocytes exposed for 18 h to (c) 0 mM (capacitating), and d) 2.5 mM procaine ( $n=10$  oocytes in each group; three replicates). Corresponding light microscopic images (a', b', c' and d') were taken by DIC. Exposure of horse oocytes to procaine was associated with depolymerization (reduced abundance) of F-actin (original magnification, 630x; Bar = 25  $\mu$ m).

## DISCUSSION

The principal aim of this study was to determine if hyperactivated, protein tyrosine phosphorylated stallion spermatozoa allows to fertilize mature equine oocytes *in vitro*. We found that both procaine capacitating conditions and treated FF at pH 7.9 did not support equine IVF. Instead, procaine had a direct effect on equine oocytes, inducing cytokinesis followed by further cleavage up to the 8-16 cell stage, which was not accompanied by sperm penetration or second polar body formation. Interestingly, we also noted that in procaine-activated oocytes the DNA in the metaphase II plate condensed without prior formation of a proper pronucleus. During cytoplasmic cleavage, these condensed DNA fragments segregated to one of the two daughter cells only, indicating that DNA duplication did not take place. Moreover, we clearly demonstrated that procaine-induced cytokinesis in equine oocytes was not stimulated by a rise in cytoplasmic calcium, in marked contrast to the situation in oocytes fertilized by ICSI or parthenogenetically-activated by ionomycin. Instead, exposure of equine oocytes to procaine induced a rapid increase in cytoplasmic pH followed by a pH-dependent reduction in F-actin, both of which are important steps in cytokinesis [29].

The first important observation was that tail-associated protein tyrosine phosphorylated, hyperactivated stallion spermatozoa were not able to penetrate equine oocytes *in vitro* despite the presence of 0, 1, 2.5 or 5 mM procaine or being exposed to treated FF at pH 7.9, as evidenced by incubating oocytes with spermatozoa labelled with MitoTracker Green FM. These results were confirmed on oocytes treated with unlabelled sperm using post-fixation lacmoid staining. That similar cleavage rates under procaine conditions were achieved irrespective of the presence of spermatozoa, further suggests that the primary effect of procaine was not to trigger sperm penetration. Moreover, oocytes that cleaved in the presence of procaine did not form a second polar body, suggesting failure of normal oocyte activation. By contrast, the injection of a single sperm cell into an oocyte by ICSI was followed by (1) normal second polar body formation and (2) normal pronucleus formation, demonstrating that the incubation conditions were adequate for oocyte maturation and embryo development. Possibly, an oviduct derived factor is essential for equine oocyte penetration by a sperm, but we do not yet know whether that factor is implicated in modifying the cumulus / zona pellucida or the sperm surface. Without that oviduct factor, it appears that sperm cannot fertilize the oocyte, regardless of the presence of cumulus cells

In a number of previous studies, the success of fertilization was determined exclusively by the presence of 2 pronuclei at 20-24 h after sperm-oocyte co-incubation [6, 8, 46]. Although we agree that the detection of two pronuclei and cell cleavage are generally indicative of fertilization in the horse, neither parthenogenetic activation followed by the formation of two maternal pronuclei nor oocyte cytokinesis can be excluded by these endpoints. That is why we decided to determine whether a sperm cell could be shown to enter the oocyte under the procaine / capacitating conditions. Labelling bull spermatozoa with MitoTracker Green FM had previously been shown not to interfere with fertilization and embryo development in a bovine IVF system [42]. Because horse-specific fertilization characteristics are not yet known, and to exclude any possible interfering effect of MitoTracker Green FM and DMSO on sperm-oocyte penetration, unlabelled sperm and postfixation lacmoid staining were used to confirm the failure of sperm penetration. Moreover, pre-labelling sperm with MitoTracker Green FM dissolved in DMSO did not affect sperm (hyper)motility or tail-associated protein tyrosine phosphorylation. Thus, MitoTracker Green FM sperm pre-labelling was not responsible for failure of sperm-oocyte penetration in procaine / capacitating conditions and pre-labelling sperm with this dye may be a valuable additional tool for assessing fertilization in the horse, as has been shown in other mammalian species [42, 43]. In our study, not a single spermatozoon was observed in the cytoplasm of an oocyte in any of the IVF conditions, and we conclude that fertilization does not occur under normal or procaine-supplemented capacitating conditions or treated FF conditions with elevated pH (7.9). We also note that, while procaine induced cleavage of the oocytes, none of the resulting 'embryos' formed a visible second polar body. Previous studies have not addressed the presence of a second polar body in the cleaved oocytes [6, 8]. The absence of the second polar body is a clear indication that the second meiotic division of the maternal DNA is not completed normally. In this respect, DNA fragmentation is also visible in both nuclei of the two-cell stage procaine-induced embryo depicted by McPartlin *et al.* [6]. We speculate that the aberrant chromatin condensation and fragmentation in procaine-treated oocytes prevents development beyond the third or fourth cytoplasmic cleavage. By contrast, sperm injected oocytes developed into normal blastocysts and showed normal second polar body formation, normal pronucleus formation, and had no signs of unequal DNA division or DNA condensation and fragmentation, demonstrating that both the oocytes and embryo culture conditions were adequate to support embryo development.

A second important observation was that procaine induced a rise in cytoplasmic pH in horse oocytes. At concentrations of  $\geq 5$  mM procaine, the resulting high pH exerted a degenerative effect on the oocytes that was not observed at  $\leq 2.5$  mM. In sea urchin eggs, procaine acts as a proton acceptor and thereby mediates cytoplasmic alkalinization [28]. At 5 mM procaine, the rise in cytoplasmic pH was presumably too high and therefore induced oocyte degeneration. This effect could be partially countered by the presence of a cumulus cell investment around the mature oocyte. At lower concentrations of procaine (2.5 mM), the rise in cytoplasmic pH did not induce degeneration but instead triggered cytoplasmic cleavage up to the 8-16 cell stage. This procaine concentration most likely induced the appropriate rise in cytoplasmic pH to activate equine oocytes, whereas a lower procaine concentration did not provoke sufficient cytoplasmic pH change to activate cytokinesis. Similar to our results, exposure of sea urchin eggs to procaine induced a cytoplasmic pH increase [53]. Procaine, and some other local anesthetics, are tertiary amines that show characteristics of weak bases. Winkler and Grainger [28] demonstrated that procaine acted as a proton acceptor within the oocyte cytoplasm. In sea urchin eggs, elevated cytoplasmic pH mediated cortical non-filamentous actin polymerization and a rapid increase in the amount of F-actin [29, 54]. Elevated pH acts directly on actin / actin binding protein complexes [55, 56]. The polymerization of cortical non-filamentous actin to F-actin, to construct a functional contractile ring to mediate cell cleavage, followed by a decrease in F-actin is required for cytoskeleton reorganization prior to cytokinesis [54, 57, 58].

It therefore appears that procaine can induce cytokinesis in equine oocytes, but that this is not accompanied by normal nuclear division and mitosis. As mentioned above, after 18 h incubation in 2.5 mM procaine, the DNA configuration changed from a metaphase II plate to one or two very condensed DNA fragments, without prior pronucleus formation. Moreover, when cytoplasmic cleavage was initiated, the DNA did not divide appropriately, such that during the subsequent oocyte divisions the majority of the 'cells' did not contain nuclear material. An additional explanation can be found in studies in other systems; in cultured myogenic cells, procaine exerts a concentration dependent toxic effect on the DNA [59] and, in fertilized sea urchin eggs, high concentrations of procaine (10 mM) were associated with the inhibition of DNA synthesis and the polymerization of tubulin, which has an important function in the construction of the meiotic spindle [60, 61]. These events appear to be related to the procaine-induced elevation in cytoplasmic pH [61] and are most probably the cause of



the DNA fragmentation that we observed. It is also unlikely that this compacted DNA could initiate embryonic genome activation, such that more advanced stages would inevitably degenerate. In conclusion, we assume that 2.5 mM procaine induced the appropriate cytoplasmic pH shift to cause cytokinesis without DNA replication.

A third important observation was that procaine did not induce oocyte activation via the normal pathway, which begins with a rise in cytoplasmic calcium. In various mammalian species, oocyte activation is induced by a cytoplasmic calcium rise soon after fertilization or after contact with the parthenogenetic agent [62]. In our study we found that, as reported for other mammalian species, ionomycin induced an early single cytoplasmic calcium rise, whereas ICSI induced calcium oscillations, in mature oocytes. A rise in oocyte cytoplasmic calcium is generally accepted to induce both 'early' and 'late' events of the fertilization process, where the early events include the cortical reaction, sodium influx and a respiratory burst, and the late events include an increase in intracellular pH, DNA and protein synthesis and chromosome replication and segregation [63, 64]. However, after 6 h exposure to 2.5 mM procaine no cytoplasmic calcium rise was observed in equine oocytes and neither did procaine induce the calcium dependent cortical reaction. Similar observations have been reported in sea urchin eggs [65, 66] in which procaine failed to trigger either calcium influx [65] or release from intracellular calcium stores [66]. Also in pig [21, 22] and cattle [23, 24], the oocyte-activation associated calcium rise could be inhibited by injecting procaine into the cytoplasm, because even low concentrations (max 200  $\mu$ M) of procaine were able to block the ryanodine receptors on the calcium channels of the cytoplasmic calcium stores. These observations reinforce the conclusion that calcium oscillations early in fertilization and the subsequent cortical reaction, as seen in horse oocytes subjected to ICSI, do not take place in the presence of procaine.

Finally, we showed that the total amount of F-actin in the equine oocyte decreased significantly during 18 h of incubation in the presence of 2.5 mM procaine in a similar fashion to that observed in sperm injected oocytes. Reduction in F-actin has to occur at the end of the cell cycle in preparation for cytokinesis [30]. Moreover, a cyclic increase and decrease in the amount of cortical F-actin has been observed during early cleavage divisions, with a peak near the beginning of the cell cycle and a trough during cytokinesis [30]. In sea urchin eggs, weak bases such as procaine can stimulate pH mediated actin turnover [67-70]. Moreover, ADF /

cofilin (AC) proteins exhibit a pH dependent role in F-actin turnover [71, 72] consisting of an alkalinity-associated increase in F-actin depolymerization activity, with a critical concentration around neutral pH [73], and a temporary concentration at the contractile ring during cytokinesis [74]. ADF / cofilin (AC) proteins have been found in all eukaryotic cells thus far examined [75-78] and are therefore likely to be involved in cytokinesis of the horse zygote or oocyte.

It may be argued that sea urchins are not representative of fertilization events in mammals. However, similarities in fertilization-mediated events, like calcium induced oocyte activation and the subsequent cortical reaction, have been reported [79]. Following the cytoplasmic calcium transient, an efflux of protons from the egg results in an elevated cytoplasmic pH in fertilized sea urchin eggs [80], which initiates cortical F-actin turnover [29, 30]. However, the physiological role of a raised cytoplasmic pH in mammalian oocyte activation is not clear [62]. Further research is required to elucidate whether such events are evolutionarily conserved between the sea urchin and mammalian species. More importantly, proof is required that a rise in cytoplasmic pH contributes to oocyte activation during normal fertilization or ionomycin treatment in horses. Theoretically, a pH rise in a horse oocyte could induce: 1) a non-physiological activation of pH-sensitive enzymes involved in actin polymerization / cytokinesis or, as in sea urchins, 2) a physiological response that results in cytokinesis.

In conclusion, we have shown that hyperactivated, protein tyrosine phosphorylated stallion spermatozoa does not facilitate penetration of horse oocytes by spermatozoa, despite the direct stimulating effect of procaine and treated FF (pH 7.9) on hyperactivated motility in stallion spermatozoa. In our hands, procaine instead induced a pH rise dependent cytokinesis in equine oocytes, without inducing an intracellular calcium increase, albeit only over a small range of procaine concentrations. Cleaved oocytes developed to the 8-16 cell stage without undergoing proper DNA duplication and without the formation of a second polar body. Moreover, the unequally divided DNA deteriorated by becoming condensed and fragmented.

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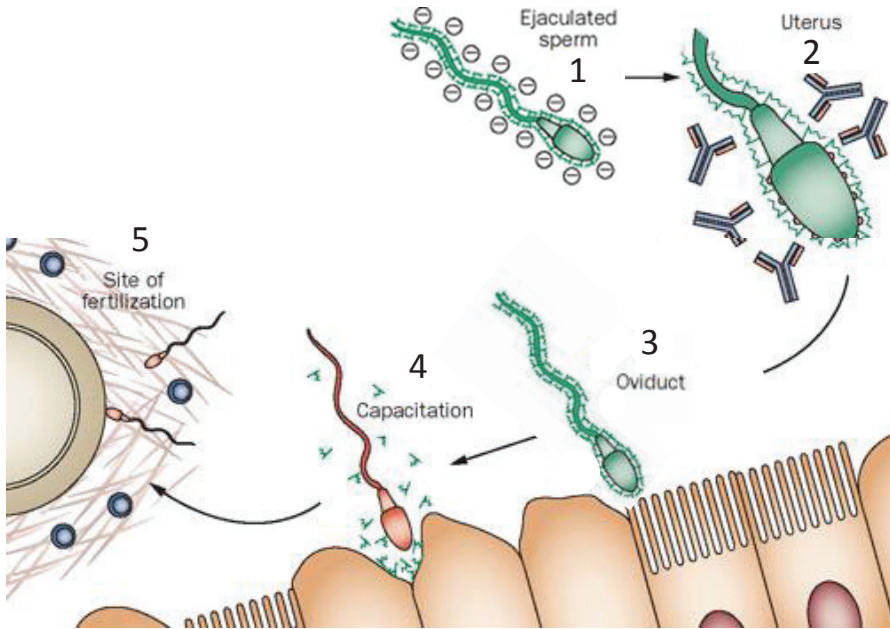
# CHAPTER 7 GENERAL DISCUSSION

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When the original concept for this research plan was first drafted in 2010, little was known about the possible triggers present in the oviduct of the mare near ovulation to force the stallion sperm through the capacitation process. Between 1990 and 2000, a few American researchers studied sperm-oviduct interaction in horses using an oviduct monolayer model [1-15]. Since then, almost no new data have been reported concerning this topic in the horse.

The general aim of this thesis was to optimize an *in vivo*-like oviduct explant model in order to improve our current understanding on how spermatozoa behave in the oviduct and eventually achieve the ability to fertilize, a process known as “capacitation” [16, 17]. Based on research in other mammalian species, Suarez [18] postulated the following hypothesis on *in vivo* capacitation. Briefly, millions of sperm cells are inseminated in the uterine body in order to fertilize only one oocyte. Of those millions spermatozoa, only thousands reach the isthmus of the oviduct. As they arrive, many are trapped and held in a sperm reservoir near the caudal site of the oviduct until close to ovulation. This sperm reservoir is established by the reversible binding of spermatozoa to the oviduct epithelium, a process which is facilitated by a  $\text{Ca}^{2+}$ -dependent lectin binding in cattle and other mammals. In the timespan prior to ovulation, the fertility of bound sperm is maintained in the sperm reservoir. In the late pre-ovulatory period, capacitation triggers force the bound sperm to undergo various capacitation-related events. Finally, sperm cells will escape from the reservoir by achieving hyperactivated motility and will move to the site of fertilization, i.e. the isthmic-ampullary junction. Capacitated spermatozoa will penetrate the oocyte’s extracellular vestments eventually resulting in fertilization. Polyspermic fertilization is also controlled by the sperm reservoir by allowing only a few sperm cells at a time to reach the oocyte in the ampulla. Additionally, the release of the cortical granules in the perivitelline space of the oocyte after the first sperm cell has penetrated and oviduct secretions like osteopontin [19], oviduct-specific glycoprotein and heparin [20] support the sperm reservoir in preventing polyspermy.



**Figure 1:** Schematic figure illustrating the hypothesis of Suarez: ‘How do mammalian sperm achieve fertilizing capability in the female genital tract’. (1) Ejaculated sperm is coated with seminal plasma de-capacitating factors over its entire surface (2) which provides protection in the uterus from surveillance and response by both the innate and adaptive immune systems of the female. Moreover, this coating also inhibits an early onset of the capacitation process and imparts a high negative charge that enables sperm to swim smoothly through the negatively charged genital tract fluid / mucus. (3) Upon arrival in the oviduct, viable, non-capacitated sperm cells bind to the cilia and microvilli of oviduct epithelium. Sperm cells are kept in a quiescent state until ovulation is imminent. A reservoir of sperm cells is formed as more sperm cells migrate into the oviduct and bind to the epithelium. (4) In the peri-ovulatory period, capacitation triggers arise in the oviduct fluid and exposed sperm cells start the capacitation process. By achieving hyperactivated motility, sperm cells are able to escape from the oviduct reservoir (5). Liberated, hyperactivated sperm will migrate to the site of fertilization. Now, sperm cells can penetrate the hyaluronan-rich cumulus matrix and bind to / penetrate through the zona pellucida of the oocyte. (Figure adapted from Tollner *et al.* [21]).

Because so few data are available on the mechanisms regulating sperm capacitation in the horse, we focused in this thesis on (1) the molecular basis of equine sperm-oviduct binding, (2) the identification of the capacitation mechanisms that induce tail-associated protein tyrosine phosphorylation and hyperactivation, (3) the possible induction of release of hyperactivated, protein tyrosine phosphorylated spermatozoa from the oviduct epithelium, and (4) the fertilizing capability of hyperactivated, protein tyrosine phosphorylated spermatozoa.

# 1. Sperm-oviduct interactions

## 1. Type of sperm-oviduct binding

In a few mammalian species like hamster, pig, cattle and horse, the molecular base of the sperm-oviduct binding consists of a  $\text{Ca}^{2+}$ -dependent lectin binding [22]. Using the oviduct monolayer model, Dobrinski *et al.* [1] showed in the horse that D-galactose carbohydrate moieties expressed on the oviduct epithelium exerted a key role as oviduct receptor in the equine sperm-oviduct interaction. Unfortunately, the  $\text{Ca}^{2+}$ -dependency of this binding was not tested. In chapter 3 of this thesis, we could not confirm the data of Dobrinski *et al.* using both the oviduct explant and oviduct apical plasma membrane model. Although carbohydrates including galactosyl moieties were highly expressed along the oviduct epithelium, we clearly demonstrated that several tested carbohydrates including D-galactose were not involved in this binding reaction. Additionally, we showed that the sperm-oviduct explant binding was not dependent on  $\text{Ca}^{2+}$  nor did a disulphide (S-S) covalent binding facilitate this interaction. As such, we could conclude that the equine sperm-oviduct epithelium interaction *in vivo* is not exclusively based on a single lectin or S-S covalent binding.

However, we were not able to elucidate the type of oviduct receptor(s) which is / are indeed responsible for the equine sperm-oviduct binding. Furthermore, the specificity of the equine sperm oviduct binding can be questioned as well both *in vitro* as *in vivo*. The carbohydrate-protein (lectin) interactions demonstrated *in vitro* between sperm and oviduct epithelial cells might represent either the totality of the pre-ovulatory sperm binding reaction or only a proportion of that reaction. If the latter is true, a strong degree of non-specific binding is present *in vivo*. This hypothesis was supported by studies [23, 24] showing a similar sperm binding capacity to tracheal epithelium, another type of ciliary epithelial cells. These observations strongly suggest the ‘non-specific’ nature of the binding of stallion spermatozoa to the oviduct instead of an exclusive binding mechanism regulated by a lectin or S-S interaction. It can be hypothesized that a secondary binding can be realized after competing with a primary carbohydrate receptor. Additionally, not only the type of carbohydrate but also the carbohydrate conformation / isomer might be important. Moreover, the presentation of the carbohydrate moiety within a larger glycoprotein determines the binding affinity too. Carbohydrates on both the oviduct as sperm cell surface might cooperate as well to facilitate sperm-oviduct binding. Cooperativity is mainly regulated by hydroxyl

groups which may participate in carbohydrate-protein interaction as both donor as acceptor (reviewed by del Carmen Fernandez-Alonso [25]). Moreover, carbohydrate-binding proteins show very shallow and solvent exposed binding sites. They make only few direct contacts with their target ligands. Thus, a key phenomenon leading to the specificity of such interactions resides in “multivalency”, arising from multiple protein-carbohydrate interactions which cooperate in a recognition event to achieve the necessary functional affinity. This leads to the need of multiple receptors arranged in such a way to bind efficiently to multiple saccharide ligands [26, 27]. Also, individual receptors can contain more than one binding site or oligomerize to form larger structures with multiple binding sites. As an example, asialoglycoprotein receptors on the surface of both hepatocytes and peritoneal macrophages bind terminal *N*-acetylgalactosamine residues and terminal galactose residues. However, the hepatic receptor binds oligosaccharides with terminal *N*-acetylgalactosamine residues more tightly than ligands with terminal galactose residues, but the macrophage receptor shows no such differential binding affinity [28].

## **2. Is *in vivo* sperm binding to oviduct epithelium in the isthmic sperm reservoir necessary for capacitation / fertilization?**

The caudal isthmus has been identified as sperm reservoir in mares prior to ovulation [14, 29, 30]. However, the necessity for sperm to interact with isthmic oviduct epithelial cells *in vivo* to achieve fertilizing capability is a point of discussion. When spermatozoa are directly inseminated in the equine oviduct via the infundibulum, similar pregnancy rates are obtained as after AI in the uterine body [31]. This suggests that the uterine body and the isthmus are both no obligatory sites for stallion spermatozoa to pass prior to capacitation / fertilization while the ampulla is a crucial environment for stallion spermatozoa to achieve the ability to capacitate / fertilize. As such, the horse is an important exception as in many species like human, cattle and pig, sperm capacitation, fertilization and blastocyst development can be achieved *in vitro* in the absence of any of these epithelia.

We hypothesize that the sperm-oviduct binding should be considered as a pre-ovulatory storage strategy associated with sperm quiescence and not a prerequisite in the capacitation process. Moreover, we hypothesize that sperm capacitation is induced by oviduct secretions containing the appropriate capacitation triggers. Indeed, we showed that exposing stallion spermatozoa to medium containing combined  $\text{HCO}_3^-$  (25 mM; pH= 7.4) and albumin decreased dramatically the density of bound sperm on the oviduct epithelia coinciding with

massively increased sperm head-to-head agglutination. Sperm head-to-head agglutination is considered in the pig as an early stage of the capacitation process *in vitro* due to removal of anti-agglutinin from the sperm surface. This suggests that capacitation under our capacitating conditions occurred along the apical part of the sperm plasma membrane (Chapter 3 and 5). As in the pig [32-34], we hypothesize that head-to-head agglutination in stallion sperm coincides with (1) the removal of sperm surface bound de-capacitation factors (seminal plasma proteins) and / or (2) early plasma membrane changes. Further research should focus in this area of the sperm capacitation pathway.

Tail-associated protein tyrosine phosphorylation is an important hallmark for capacitation. In Chapter 4, we observed that *in vitro* oviduct bound stallion sperm showed higher rates of protein tyrosine phosphorylation – in a time-dependent manner when compared to unbound spermatozoa. Initially, we hypothesized that the interaction between the sperm cells and the oviduct was important *in vitro* to switch on the intracellular capacitation processes. However, triggering capacitation in oviduct explant bound sperm is more likely due to the release of “pro-capacitating factors” from the secretory oviduct cells. This hypothesis is supported by the fact that a similar protein tyrosine phosphorylation response has been reported for stallion sperm suspensions when the pH of the capacitation medium was alkaline [35, 36]. It may therefore be significant that the secretory cells in the oviduct explants contained large alkaline vesicles (Chapter 4), although secretion of this vesicles only marginally raised the pH of the incubation medium. These observations suggested that *in vitro* oviduct secretions probably form an alkaline gradient around each oviduct explant. *In vivo* however, the alkaline secretions may be diluted to a much lesser extent by oviduct fluid and may therefore induce an alkaline local microenvironment that is sufficient to induce sperm protein tyrosine phosphorylation without the need for the sperm to bind to the oviduct. Certainly, in our *in vitro* oviduct explant system, direct contact between spermatozoa and oviduct explants was also necessary to induce intracellular alkalization consistent with capacitation induction (Chapter 4). Combining the various observations, it is hypothesized that a local increase in the  $\text{HCO}_3^-$  concentration is essential for stallion sperm to capacitate and subsequently fertilize [37].

All these data support our hypothesis that equine sperm capacitation does not depend on physical contact with oviduct epithelium but that the exposure to oviduct secretions is of

utmost importance. Considering our results, we hypothesize that the *in vivo* sperm-oviduct binding depends on the timing when non-capacitated sperm cells enter the oviduct:

- (1) If non-capacitated sperm cells arrive in the early pre-ovulatory oviduct, the majority of the sperm population will bind to the oviduct epithelium in the isthmus and form a sperm reservoir. As such, sperm cells are stored in the oviduct until the moment that capacitation triggers appear near ovulation.
  
- (2) If non-capacitated sperm cells arrive in the peri-ovulatory oviduct, the sperm cells immediately get in touch with the oviduct secretions which contain the capacitation triggers and subsequently lose their affinity to bind to the oviduct. Sperm cells should not be stored as fertilization should occur as soon as possible.

## 2. Sperm-oviduct release: The number of released spermatozoa

In Chapter 5, we demonstrated that only treated follicular fluid (pH 7.9) was able to induce hyperactivated motility if sperm cells were previously bound to oviduct explants in both non-capacitating and capacitating conditions. Although an important proportion of bound spermatozoa showed hyperactivated motility combined with tail-associated protein tyrosine phosphorylation, only a small number of spermatozoa was released from the oviduct epithelium. Moreover, capacitating conditions including  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and albumin did not facilitate hyperactivated motility nor was sperm release induced (Chapter 3). An interesting question that still needs to be answered is: “What is the physiological number of sperm cells that should be released from the oviduct reservoir to fertilize the oocyte at the moment of ovulation?”. In cattle, it has been demonstrated *in vitro* that heparin [38, 39] and S-S reductants like penicillamine [39-41] are both capacitation triggers which are able to extensively release sperm from oviduct monolayers. Both triggers are also present *in vivo* in oviduct and follicular fluid. In contrast, a low sperm:egg ratio is initially *in vivo* essential for monospermic fertilization [42]. To this end, the oviduct strongly regulates the number of spermatozoa detaching from the epithelium and progressing to the fertilization site in the peri-ovulatory period. Near ovulation, when at least one oocyte will soon be shedded into the oviduct, a small number of sperm cells is capacitated and released from the oviduct



epithelium [43-47]. Such a controlled release of a few capacitated spermatozoa leads to a low sperm:egg ratio at the isthmic-ampullary junction and reduces the risk of polyspermic fertilization [44, 45, 47]. These observations strongly support our *in vitro* findings of discrete sperm release in hyperactivating capacitating conditions. The stringent regulation *in vivo* around the time of ovulation is in sharp contrast with the progressively increasing post-ovulatory release of sperm cells. This release however occurs too late in time to influence fertilization [47-51]. The endocrine activity of the ipsilateral gonad overall controls these pre- and post-ovulatory events within the oviduct [44, 48, 52, 53]. A local counter-current transfer of hormones between the ovarian vein and oviduct branch of the ovarian artery [44, 47, 48, 52] regulates the cycle-dependent changes in the oviduct, specifically the microenvironment created by the oviduct secretions, which is essential for the onset of sperm capacitation, the timing of sperm release and fertilization [43]. The lack of the extensive release of sperm cells in our *in vitro* capacitating / release conditions can be explained as follows: (1) hypermotility *in vitro* was not maintained for a sufficient timespan (max 2.5 h) and / or (2) our capacitating / hyperactivating conditions still lack one or more capacitating trigger(s) which induce(s) other capacitating events. It has previously been hypothesized that sperm plasma membrane changes are also important to release spermatozoa from the oviduct epithelium [22]. In a follow-up study, these capacitation-related membrane changes should be studied more in detail.

### 3. Elevated pH is a key component in equine capacitating conditions

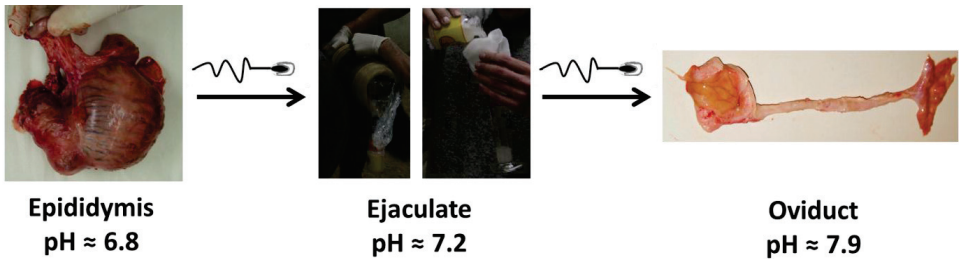
The acid-base balance in the male and female reproductive tract is finely modulated [54, 55]. The male reproductive tract and more specific the epididymis contains acidic luminal fluid (pH $\approx$ 6.5; 3-4 mM HCO<sub>3</sub><sup>-</sup>) [56-59] which renders mature sperm quiescent. Due to the secretion of HCO<sub>3</sub><sup>-</sup> in the cauda of the epididymis, the pH slightly increases and the sperm gains progressive motility upon arrival. During ejaculation, spermatozoa are mixed with seminal plasma resulting in a further increase of the pH to 7.2 (25-30 mM HCO<sub>3</sub><sup>-</sup>) [59], but at the same time seminal plasma contains also de-capacitation factors preventing capacitation. In contrast, luminal fluids of the female reproductive tract of various mammalian species, i.e. the uterus and especially the oviduct, tend to be alkaline [60-62]. In the uterus, spermatozoa are

still mixed with seminal plasma whereas upon entering the oviduct the sperm is separated from these de-capacitation factors (reviewed by Liu *et al.* [37]). It has been suggested that an increased, slightly alkaline pH is present in the oviduct lumen in the peri-ovulatory period [35]. The concentration of  $\text{HCO}_3^-$  during the complete pre-ovulatory period probably varies though the pH in the oviduct will never be lower than 7.4 (Figure 2).

We showed for the first time that the number of alkaline secretory vesicles located in the epithelium of oviduct explants decreased significantly during sperm binding (Chapter 4). As such, an alkaline local microenvironment is created *in vitro* during capacitation.

In response to elevated pH 7.9 capacitating conditions, stallion sperm showed:

- (1) a clearly decreased density of sperm binding to the oviduct but instead induced head-to-head agglutination. This event is already induced by capacitating conditions at pH 7.4 but a further elevated pH 7.9 supports this property as well (Chapter 3).
- (2) a gradual increasing intracellular pH associated with an increased protein tyrosine phosphorylation of the sperm tail (Chapter 4 and [35, 36]).
- (3) induced hyperactivated motility which facilitates sperm release in a discrete fashion (Chapter 5).

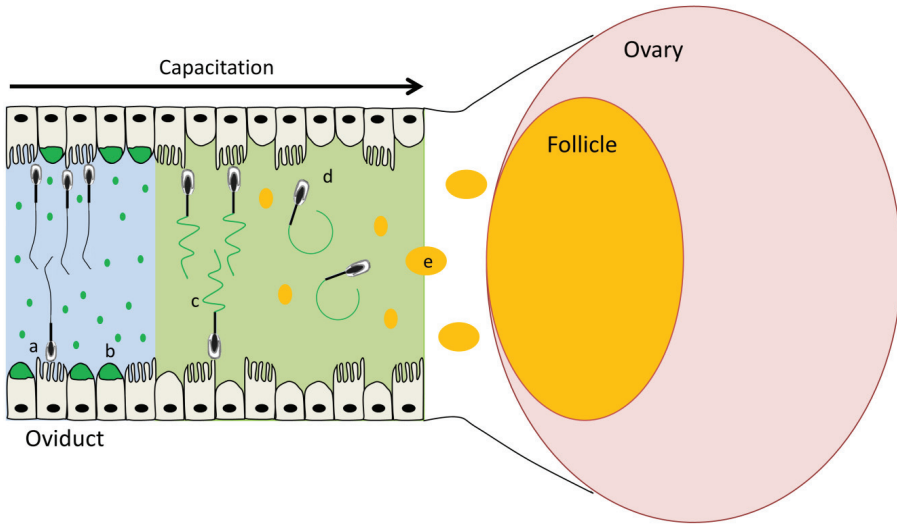


**Figure 2:** Evolvement of the pH of luminal genital tract fluids that stallion sperm encounter before achieving fertilizing capability. The epididymis contains acidic luminal fluid which renders mature sperm quiescent. During ejaculation, spermatozoa are mixed with seminal plasma which prevents capacitation. In the peri-ovulatory period, inseminated spermatozoa arrive in the slightly alkaline environment of the oviduct. This condition is essential to force stallion sperm through the capacitation process.

The effect of increased environmental pH on capacitation strongly suggests that equine sperm capacitation / fertilization media require a standard higher pH compared to other mammalian species. However, our capacitating conditions do not facilitate the acrosome reaction (Chapter 4 and 5) and equine IVF (Chapter 6) yet. Considering our observations in the horse, alkaline secretory vesicles in the oviduct epithelium are not demonstrated yet in other mammalian species. Very likely in the horse, the oviduct secretions contain additional pH-sensitive factors. The effect of these unidentified factors should be tested in the future by using native oviduct fluid to perform equine IVF. In this way a redefined capacitating medium including all important capacitating / fertilization triggers might be established after identifying the active capacitation triggers. So far, the plasma membrane changes necessary to induce the acrosome reaction at the final stage of capacitation are not supported.

## 4. Capacitation-related changes of the sperm cells in the equine oviduct

In Chapter 4 and 5 we showed that the combined effect of the elevated environmental pH, the external  $\text{Ca}^{2+}$  and the unidentified follicular fluid factor(s) induced hyperactivation, tail-associated protein tyrosine phosphorylation and sperm release from the oviduct epithelium. The question remains whether or not follicular fluid enters the tiny oviduct in the horse *in vivo* after ovulation and whether it mixes with the oviduct secretions to induce stallion sperm capacitation. In Chapter 4, we showed that secretory epithelial cells contained large, alkaline vesicles which were gradually secreted in the capacitating medium. Subsequently, only tail-associated protein tyrosine phosphorylation was observed in oviduct-bound spermatozoa but no hyperactivation or sperm-oviduct release. In contrast, these three capacitation events were successfully induced during incubation in undiluted follicular fluid with elevated pH (7.9) (Chapter 5). Therefore, we initially hypothesized that a mix of oviduct secretions and follicular fluid components induced sperm capacitation in the peri-ovulatory oviduct (Hypothesis 1) (Figure 3).



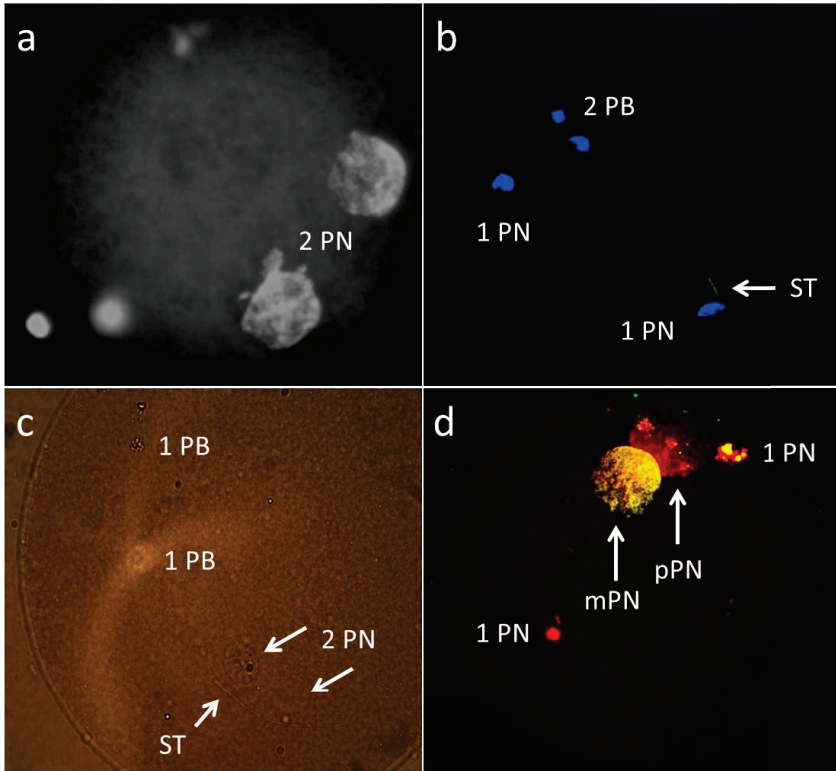
**Figure 3:** A schematic overview of hypothesis 1 whereby capacitation of stallion sperm *in vivo* is induced by a mix of oviduct secretions and follicular fluid (a: sperm-oviduct binding, b: secretion of the alkaline vesicles of the secretory epithelial cells, c: induction of hypermotility and tail-associated protein tyrosine phosphorylation, d: release of sperm from oviduct epithelium, (e) a small amount of follicular fluid enters the oviduct after ovulation).

However, we subsequently showed that an elevated pH (7.9) is also essential for stallion spermatozoa capacitation. Since pre-ovulatory follicular fluid is not alkaline (pH  $\pm$  7.2), the physiological relevance of hypothesis 1 can be questioned. Unfortunately, the pH of equine oviduct fluid is not measured yet during the complete secretory phase of the oviduct epithelium in the peri-ovulatory period. It might be that capacitation in the narrow and tortuous oviduct lumen might be induced by oviduct secretions only (Hypothesis 2). Hypothetically, the unidentified follicular fluid factor(s) might be secreted by the oviduct epithelium as well. In the latter point of view, the fact that we observed only protein tyrosine phosphorylation in sperm bound to oviduct explants (Chapter 4) might be due to a dilution gradient of pro-capacitating factors whereby the secretory epithelial cells supported the alkaline local microenvironment but the concentration of the unidentified follicular fluid factor(s) was too low to induce the other capacitation events.

## 5. Fertilization, parthenogenesis or cytokinesis?

We showed in Chapter 6 that equine oocytes *in vitro* can be parthenogenetically activated using ionomycin by facilitating the  $\text{Ca}^{2+}$  release from the oocyte's intracellular  $\text{Ca}^{2+}$  store [63, 64]. As such, the oocyte activation after gamete fusion is perfectly mimicked by indirectly increasing the cytoplasmic  $\text{Ca}^{2+}$  concentration. Procaine acts more downstream in the oocyte activation process and induces only cytokinesis events via a pH-dependent depolymerization of F-actin while nuclear activation is not triggered (Chapter 6). However, the concentration of procaine used to induce cytokinesis activation appeared to be DNA toxic. Besides the fact that procaine does not facilitate fertilization in the horse, it is also not of use to study oocyte activation events. McPartlin and co-authors [65] claimed in 2009 that procaine facilitated equine IVF by inducing sperm hypermotility. In their study, oocyte cleavage rates of 61% were reported but cleaved oocytes never developed further than the 8-16 cell stage (at this stage genome activation is taking place in horse embryos). On the other hand, the direct effect of procaine on equine oocytes was never considered in this study. We showed that procaine not only induced cytokinesis instead of fertilization or normal parthenogenesis but also severe DNA fragmentation in equine oocytes (Chapter 6). Therefore, more stringent evaluation methods are needed to assess fertilization in the horse as the reported fertilization rates in the study of McPartlin *et al.* [65] are likely a result of induced cytokinesis (Chapter 6). Due to the low incidence of parthenogenesis (around 5%) in currently used IVF systems in farm animals, the presence of two pronuclei after 20-24 h gamete co-incubation visualized by nuclear chromatin stainings such as Hoechst or propidium iodide, is generally considered as a valid method to assess fertilization [66-68]. Considering the poor equine IVF rates (see introduction: Table 1), the presence of 2 pronuclei as confirmation of fertilization seems however not conclusive to distinguish normal fertilization from parthenogenesis. Indeed, using techniques as pre-labelling sperm with MitoTracker Green FM / Hoechst and lacmoid post-fixation, we were able to differentiate fertilization (two PB within the perivitelline space, two PN and a sperm tail within the ooplasm) from parthenogenesis (one PB, one or two PN and no sperm tail) (Figure 4). Alternatively, we recently established a staining technique to differentiate the paternal from the maternal pronucleus in equine zygotes produced by ICSI [67]. We found that the paternal and maternal pronucleus in equine zygotes displayed different histone 3 methylation (H3K9me3) patterns. This staining technique could

not be applied to the procaine experiments as the exposed oocytes did not form normal pronuclei but instead produced condensed DNA fragments (Figure 4).



**Figure 4:** Methods to distinguish fertilization from parthenogenesis. (a) Previously the presence of 2 pronuclei (2 PN), evaluated by nuclear stainings as Hoechst, was considered conclusive to assess fertilization of equine oocytes (image from McPartlin *et al.* 2009). However, this method cannot exclude parthenogenesis. Therefore, the additional presence of the sperm tail (ST) and the second extruded polar body (PB) showed by (b) MitoTracker Green FM pre-labelling combined with Hoechst postfixation or (c) lacmoid postfixation, should be demonstrated; or (d) paternal (pPN) and maternal (mPN) pronuclei in equine zygotes should be distinguished by different histone 3 methylation (H3K9me3) patterns (image kindly provided by drs Sonia Heras-RBU-Ugent).

## 6. Optimization of the currently used equine capacitating / IVF medium

Direct gamete co-incubation in procaine capacitating conditions is not an adequate way to perform equine IVF regarding its direct toxic effect on the DNA of equine oocytes

(Chapter 6). However, future research might focus on evaluating the effect on embryo development of ICSI fertilized oocytes after exposure to procaine for 18 h or verifying if stallion spermatozoa are still hypermotile after washing the procaine-exposed sperm. If so, procaine can still be applied in equine IVF protocols. Moreover, we showed that tail-associated protein tyrosine phosphorylated and hyperactivated spermatozoa still failed to penetrate the oocyte's extracellular vestments (Chapter 6). So, a working equine IVF system is still not realized which might be explained by the fact that we were unable to induce the membrane changes leading to the acrosome reaction with our improved, elevated pH capacitating conditions (pg 221-222). Further research might focus on the biological triggers which induce these capacitation-related membrane changes. In conclusion, the exact trigger for full capacitation resulting in a repeatable, working equine IVF system is still unknown. The composition of capacitating key factors in oviduct fluid is usually mimicked *in vitro* by establishing an efficient capacitation / fertilization medium. Based on our data and many other reports, we hypothesize that essential capacitating factors are currently still lacking. Determining these missing factors in oviduct secretions (Chapter 5) and / or follicular fluid will be an enormous challenge in the future.

## 7. Further considerations

The main aim of this thesis was to elucidate the *in vitro* capacitation triggers of equine spermatozoa in order to establish an efficient and repeatable equine IVF system. Unfortunately, we succeeded only partially in our goals. We identified the biological capacitation triggers which facilitate the tail-associated protein tyrosine phosphorylation and hyperactivated motility in stallion sperm. As the acrosome reaction could not be induced, further research should focus on capacitation-related membrane changes which were not studied in this thesis. However, it should be verified first if our improved capacitation conditions also support: (1) the increased lipid fluidity of the sperm plasma membrane (using merocyanine 540 staining), (2) the cholesterol extraction from the sperm plasma membrane (by filipine staining) and (3) the lateral redistribution of the lipid rafts in the apical region (by caveolin and flotilin isolation from the apical sperm plasma membrane). If not, further research should focus as well on the biological triggers inducing these capacitation-related membrane events. We are convinced that inducing the sperm plasma membrane changes eventually will support the acrosome reaction. When combined with the capacitation triggers

that induce tail-associated protein tyrosine phosphorylation and hyperactivated sperm motility, stallion sperm will be able to penetrate the cumulus and ZP barriers. Subsequently, the penetrated sperm cell will fuse with the oocyte to accomplish fertilization.

Nanotechnology as microfluidic automation - the automated routing, dispensing, mixing, and / or separation of fluids through microchannels - by 3D prints, inserts and microfluidics has been introduced recently in reproductive biology to mimic more closely the physiological function of the oviduct *in vivo*. For example in pig, IVF of porcine oocytes in microchannels resulted in a higher incidence of monospermic fertilization compared to oocytes fertilized in the traditional microdrop system with comparable penetration and male pronucleus formation rates [69]. In the horse, further research should focus on cultivating oviduct cell cultures in microfluidic channels. The switch from a static to a more dynamic model will generate a more robust oviduct epithelium cell model in terms of morphological, ultrastructural and physiological features. The development of such a model will improve the knowledge on gamete-oviduct interaction including sperm capacitation and fertilization.

Developing an efficient conventional IVF system is imperative since the only method so far to produce IVF foals, intracytoplasmic sperm injection (ICSI), has some disadvantages. Besides the fact that ICSI in the horse requires very expensive equipment and highly trained technicians, the oocyte is traumatized which results in a low average success rate. Only a few labs worldwide are able to achieve satisfying ICSI results (blastocyst rates of 20%) and can implement this technique in a clinical setting to produce foals from subfertile horses. If a repeatable conventional IVF system could be established in the future, a cheaper and more ergonomic system would be available. However, stallions with capacitation-related fertility problems are not eligible for this method. Once complete capacitating conditions can be established, ejaculates can be scored *in vitro* before performing AI or IVF. Testing the *in vitro* capability of the sperm to undergo the different steps of capacitation will give more information about the *in vivo* fertility. In addition to the general sperm fertility assessment in practice, this approach will save a lot of time, money and labor because infertile stallions with capacitation-related problems can immediately be excluded for AI and IVF. ICSI is the only method to obtain offspring from these stallions.



## 8. General conclusions

Based on the results obtained in this thesis, following conclusions can be drawn:

1. Carbohydrate moieties expressed along the oviduct epithelium do not facilitate sperm-oviduct binding. Using our oviduct explant and apical plasma membrane model,  $\text{Ca}^{2+}$  - dependent lectin or disulphide interaction did not facilitate this binding exclusively. Unfortunately, the exact nature of the sperm oviduct binding in horses could not be determined.
2. A reduced density of sperm bound to stallion oviduct epithelia was observed when sperm was exposed to capacitating conditions, due to the combined effect of  $\text{HCO}_3^-$  and albumin. This is probably related to the coinciding sperm head-to-head agglutination, an event associated with early capacitation.
3. An essential role of elevated pH (7.9) in stallion capacitation events was clearly demonstrated. Tail-associated protein tyrosine phosphorylation and increased cytoplasmic pH in stallion sperm was induced by releasing alkaline secretory granules towards the oviduct bound sperm. Additionally, switching from progressive to hyperactivated sperm motility also depended on an elevated pH. Further research should focus on the anticipated role of CATSPER on hyperactivated motility induced by elevated pH conditions.
4. Generally, it has been accepted that tail-associated protein tyrosine phosphorylated, hyperactivated spermatozoa are able to support equine IVF. However, capacitation triggers supporting these events still failed to allow the *in vitro* fertilization of equine oocytes. It is clear that equine capacitating media still lack some essential components.

Procaine has a direct effect on equine oocytes by inducing cytokinesis which unfortunately coincides with DNA fragmentation. The latter explains why oocytes exposed to procaine never develop further than the 8-16 cell stage. In conclusion, procaine is not able to support equine IVF.

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# SUMMARY

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Horse breeding has evolved immensely the past century as equine reproduction is no longer limited to natural breeding. Based on the successes achieved in human infertility treatments, various assisted reproductive techniques have also been introduced in equine practice to produce foals from subfertile horses. Applying these techniques also accelerates the genetic progress in the horse breeding industry and supports the conservation of endangered horse breeds and wild equids. Techniques like artificial insemination and embryo transfer (ET) are nowadays already very successful while the *in vitro* production of equine embryos (IVP) gradually gains popularity as horse infertility treatment. Currently, IVP can only be commercially provided using intracytoplasmic sperm injection (ICSI). Unfortunately, ICSI is very expensive and labor intense, requires a lot of expertise and most importantly, the blastocyst rate is very low.

Conventional *in vitro* fertilization (IVF) might be an alternative for ICSI to produce equine embryos. Although two foals were born by IVF in the early nineties, this initial success could not be repeated later on. Limited successes claiming *in vitro* fertilization were not repeatable between labs and even not within the same lab. Apparently, stallion sperm is *in vitro* not able to penetrate the zona pellucida (ZP), a glycoprotein layer surrounding the oocyte. It is generally accepted that equine IVF does not succeed due to insufficient sperm capacitation *in vitro* and not because of oocyte maturation abnormalities. Indeed, *in vivo* matured oocytes could not be fertilized *in vitro* while transfer of *in vitro* matured oocytes to the oviduct of an inseminated mare yielded similar pregnancy rates as normal AI. Additionally, penetrating the ZP mechanically by ICSI using *in vitro* matured oocytes also results in normal fertilization.

In general, this thesis mainly focused on fundamental capacitation-related changes of stallion sperm, a process which sperm cells undergo in order to achieve the ability to fertilize. First, we aimed to study the interaction between stallion spermatozoa and oviduct epithelium receptors (Chapter 3). Next, the physiological triggers to induce tail-associated protein tyrosine phosphorylation (Chapter 4) and hyperactivated motility (Chapter 5) were identified. As sperm capacitation events *in vivo* are facilitated in the oviduct near ovulation, these events were *in vitro* studied in a more *in vivo*-like model by developing two bioassays, i.e. an oviduct explant and an oviduct apical plasma membrane model (Chapter 3, 4 and 5).

*In vivo*, the sperm binding to oviduct epithelium at the caudal part of the oviduct is probably an essential step to recruit and store viable and potentially fertile non-capacitated spermatozoa prior to fertilization, the so called sperm reservoir. In several mammalian species like cattle, hamster and pig, this molecular interaction between sperm and oviduct epithelium is based on a  $\text{Ca}^{2+}$ -dependent carbohydrate-lectin recognition. In cattle, glycosaminoglycans, S-S reductants and capacitation triggers like albumin,  $\text{Ca}^{2+}$  or  $\text{HCO}_3^-$  change the affinity of the sperm cells for the oviduct epithelium and induce the release of the bound sperm. In the horse, D-galactose has previously been identified as a key-molecule facilitating the sperm-oviduct binding using the oviduct monolayer model. In Chapter 3, we evaluated the role of various carbohydrates, glycosaminoglycans, lectins, S-S reductants and capacitation factors like albumin,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  in the sperm-oviduct interaction in the horse using an oviduct explant and oviduct apical plasma membrane model. Despite the high expression of *N*-acetylgalactosamine, *N*-acetylneuraminic acid (sialic acid) and D-mannose or D-glucose in the oviduct epithelium, D-galactose moieties were not detected. Using a competitive binding assay and pretreatment of this assay with *N*-glycosidase F, we were able to demonstrate that the equine sperm-oviduct binding was not exclusively regulated by a  $\text{Ca}^{2+}$ -dependent lectin or disulphide (S-S) binding. Moreover, the combined sperm capacitating factors albumin and  $\text{HCO}_3^-$  even severely reduced (>10 fold) the sperm affinity for the oviduct epithelium. Instead, the affinity between stallion sperm cells increased considerably resulting in  $\text{Ca}^{2+}$ -independent head-to-head agglutination.

*In vivo*, the sperm-oviduct binding near ovulation is an essential step as well in the capacitation process preparing the sperm for fertilization. One of the most important hallmarks of sperm capacitation is the tail-associated protein tyrosine phosphorylation. In many species, tail-associated protein tyrosine phosphorylation can *in vitro* be induced by exposing spermatozoa to  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  and albumin. These conditions, however, are deficient in cattle and equine. In cattle, capacitation can be induced by adding heparin-like glycosaminoglycans to the capacitating medium. Exposure to these capacitation triggers *in vitro* induces a considerable increase in tail-associated protein tyrosine phosphorylated spermatozoa. Unfortunately, heparin-like glycosaminoglycans have no capacitation-related effects on stallion sperm (Chapter 3). Using the oviduct explant model, *in vitro* binding to oviduct epithelium appears essential to induce capacitation in stallion spermatozoa (Chapter 4). Oviduct-bound spermatozoa show a time-dependent protein tyrosine phosphorylation

response which is not observed in unbound spermatozoa or spermatozoa incubated in oviduct explant conditioned medium. Interestingly, both oviduct-bound and unbound sperm remains motile with intact plasma membrane and acrosome. As protein tyrosine phosphorylation is induced in equine spermatozoa using media with high pH, the intracellular pH of oviduct explant cells and bound spermatozoa was subsequently monitored. Apparently, the sperm-oviduct interaction facilitated the tail-associated protein tyrosine phosphorylation in bound sperm by creating an alkaline microenvironment surrounding each oviduct explant. The secretory epithelial cells contained large, alkaline vesicles which disappeared during sperm-oviduct binding. Moreover, a time-dependent gradual increase in intracellular pH was observed in oviduct-bound spermatozoa, while unbound spermatozoa do not show intracellular pH changes.

Inducing hyperactivated motility near ovulation is considered as a next essential step in capacitation to release the bound spermatozoa from the oviduct epithelium. Defined capacitating medium alone or enriched with glycosaminoglycans or S-S reductants appeared insufficient to induce sperm release from oviduct epithelium, most likely due to the inability of stallion spermatozoa to induce hyperactivated motility when bound to the oviduct (Chapter 3 and 5). In Chapter 5, possible *in vivo* hyperactivating / release conditions were tested by adding female genital tract cells and fluids to the sperm-oviduct explant model. Surprisingly, flushed pre-ovulatory and post-ovulatory oviduct fluid, 100% and 10% follicular fluid, cumulus cells and mature equine oocytes did not trigger sperm release or hyperactivated motility. As native follicular fluid even was detrimental to sperm viability, the sperm deteriorative component (heat sensible, lipophilic, 30-100 kDa factor) was eliminated by heat inactivation, charcoal treatment or 30 kDa filtration, respectively or in combination. Sperm suspensions exposed to treated follicular fluid induced clearly hyperactivated motility in stallion sperm at pH 7.9 but not at pH 7.4. Moreover, the elevated pH combined with the extracellular  $\text{Ca}^{2+}$  and a heat resistant, hydrophilic, <30 kDa component of follicular fluid apparently triggered tail-associated protein tyrosine phosphorylation, elevated cytoplasmic  $\text{Ca}^{2+}$  concentration and hyperactivated motility in stallion spermatozoa. Interestingly, incubation in these hyperactivating conditions induced only a limited release of oviduct pre-bound sperm.

One study from 2009 suggested a central role for tail-associated protein tyrosine phosphorylated and hyperactivated stallion spermatozoa in successful equine IVF. Co-incubating equine gametes in the presence of 5 mM procaine facilitated equine IVF, with cleavage rates higher than 60%. Unfortunately, embryos did not develop further than the 8-16 cell stage. In the latter study, it was hypothesized that procaine promoted sperm penetration by inducing hyperactivated motility to accompany the tail-associated protein tyrosine phosphorylation, triggered by elevated pH medium conditions. In our study, both tail-associated protein tyrosine phosphorylation and hyperactivated motility of stallion spermatozoa were achieved using treated follicular fluid with elevated pH (Chapter 5). However, the fertilizing ability of tail-associated protein tyrosine phosphorylated, hyperactivated spermatozoa was tested by performing IVF using both procaine and treated follicular fluid (pH 7.9) capacitating conditions (Chapter 6). When fertilization was assessed by sperm penetration, extrusion of the second polar body and pronuclei formation, it appeared that fertilization failed under both *in vitro* conditions. Moreover, equine oocytes exposed to 2.5 and 5 mM procaine showed cleavage, independently from the co-incubated sperm. The cleaved oocytes though did not develop beyond 8-16 cells, and the blastomeres either lacked nuclei or contained aberrant, condensed DNA fragments. Instead of increasing the cytoplasmic  $Ca^{2+}$  level of exposed oocytes, procaine initiates an ooplasmic alkalinization followed by a cortical F-actin depolymerization resulting in cytokinesis and aberrant chromatin condensation in equine oocytes.

In conclusion, more fundamental insights on capacitation-related changes of stallion spermatozoa in the oviduct during the peri-ovulatory period are provided in this thesis. Equine *in vitro* capacitating media should standard be adjusted to pH 7.9 when considering its effect on sperm capacitation including cytoplasmic  $Ca^{2+}$  and pH rise, tail-associated protein tyrosine phosphorylation and hyperactivated motility. Unfortunately, a repeatable and standardized equine IVF protocol is not established yet as the current capacitating conditions still lack the capacitation triggers responsible for the acrosome reaction. The missing capacitation triggers in follicular and / or oviduct fluid should be identified and added to the defined equine capacitating medium in order to establish IVF in the horse.

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# SAMENVATTING

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De laatste eeuw heeft de paardenfokkerij een enorme vooruitgang gekend waarbij, naast natuurlijke dekking, ook gebruik wordt gemaakt van geassisteerde voortplantingstechnieken. Vruchtbaarheidsbehandelingen die ontwikkeld zijn bij de mens, worden nu gebruikt bij het paard om veulens te produceren van subfertiele paarden. Daarnaast wordt de genetische vooruitgang van bepaalde bloedlijnen met behulp van deze technieken versneld en zijn deze belangrijk voor het in standhouden van bedreigde paardenrassen en wilde paardachtigen. Kunstmatige inseminatie en embryotransplantatie worden momenteel routinematig uitgevoerd bij het paard. Ook de *in-vitro* embryoproductie wint aan populariteit als alternatieve vruchtbaarheidsbehandeling. Op dit moment is de *in vitro* productie van paardenembryo's enkel mogelijk met behulp van intracytoplasmatische sperma-injectie (ICSI). Uiteindelijk worden de *in vitro* geproduceerde blastocysten individueel overgezet in de baarmoeder van een draagmerrie via embryotransplantatie. De blastocystpercentages die bereikt worden met ICSI - een dure, arbeidsintensieve techniek die veel expertise vergt - zijn momenteel echter nog behoorlijk laag.

Een alternatief voor de *in vitro* productie van paardenembryo's is klassieke *in-vitro*fertilisatie. In de vroege jaren negentig zijn via deze techniek twee veulens geboren maar dit initieel succes kon later niet meer herhaald worden. Sindsdien zijn vele pogingen ondernomen, waarbij gebruik gemaakt werd van verschillende *in-vitro*fertilisatie procedures, met echter geen of lage, niet herhaalbare bevruchtingsresultaten. Uiteindelijk blijkt hengstensperma niet in staat om de zona pellucida, een glycoproteïenlaag rond de eicel, te penetreren. Algemeen wordt aanvaard dat *in-vitro*fertilisatie bij het paard niet werkt omdat de spermacellen bij de hengst *in vitro* onvoldoende capaciteren aangezien de *in vitro* maturatie van paardeneicellen voldoende goed verloopt. Deze hypothese wordt enerzijds ondersteund door het feit dat *in vivo* gematureerde eicellen *in vitro* niet kunnen bevrucht worden terwijl anderzijds, de transfer van *in vitro* gerijpte eicellen naar de eileider van een geïnsemineerde merrie gelijkaardige drachtresultaten oplevert als kunstmatige inseminatie. Bovendien leidt de mechanische penetratie van de zona pellucida bij ICSI ook tot normale bevruchting.

In deze thesis hebben we grotendeels gefocust op de fundamentele aspecten van capacitatiegerelateerde veranderingen die hengstenspermacellen moeten ondergaan alvorens bevruchtingskrachtig te zijn. Het doel van deze thesis was om (1) de interactie tussen hengstenspermacellen en eileiderepitheelcellen te bestuderen (Hoofdstuk 3) en (2) de fysiologische triggers voor de inductie van tyrosinefosforylatie in de spermastaart (Hoofdstuk

4) en hyperactieve motiliteit (Hoofdstuk 5) te identificeren. *In vivo* vindt capacitatie van spermacellen plaats tijdens de peri-ovulatoire periode. Om deze capacitatiegerelateerde veranderingen *in vitro* te bestuderen, werden twee modellen ontwikkeld, nl. een oviduct explant- en een oviduct apicale plasmamembraanmodel.

De binding van spermacellen *in vivo* aan eileiderepitheel ter hoogte van het caudale deel van de eileider is een essentiële stap om fertiele, niet-gecapaciteerde spermacellen te selecteren en te bewaren tot het moment van de bevruchting. Deze gebonden spermacellen worden het spermareservoir genoemd. Bij verschillende diersoorten zoals het rund, de hamster en het varken bestaat de moleculaire interactie tussen het sperma en de eileiderepitheelcellen uit een  $\text{Ca}^{2+}$ -afhankelijke koolhydraat-lectine verbinding. Bij het rund veranderen glycosaminoglycanen, S-S reducerende stoffen en capacitatietriggers zoals albumine,  $\text{Ca}^{2+}$  of  $\text{HCO}_3^-$  de affiniteit van de spermacellen voor het eileiderepitheel zodat de spermacellen het eileiderepitheel loslaten. Met behulp van een oviduct monolaagmodel is bij het paard D-galactose eerder geïdentificeerd als de sleutelmolecule die de binding tussen sperma- en eileidercel mogelijk maakt. In Hoofdstuk 3 hebben we de rol van verschillende koolhydraten, glycosaminoglycanen, lectines, S-S reducerende stoffen en capacitatie triggers zoals albumine,  $\text{Ca}^{2+}$  en  $\text{HCO}_3^-$  onderzocht in de interactie tussen sperma- en eileiderepitheelcellen bij het paard met behulp van een oviduct explant- en oviduct apicale plasmamembraanmodel. *N*-acetylgalactosamine, *N*-acetylneuraminiczuur (sialzuur) en D-mannose of D-glucose kwamen tot expressie op het eileiderepitheel terwijl de expressie van D-galactose niet kon worden aangetoond. Bovendien werd door middel van een competitieve bindingstest aangetoond dat de verbinding tussen sperma en eileidercellen niet alleen verloopt via een  $\text{Ca}^{2+}$ -afhankelijke koolhydraat-lectine of disulfide (S-S) verbinding. De incubatie van spermacellen in medium met capacitatiefactoren albumine en  $\text{HCO}_3^-$  verminderde aanzienlijk de affiniteit van de spermacellen voor het eileiderepitheel ( $>10\times$ ). Tegelijkertijd steeg de onderlinge affiniteit van hengstensperma enorm met een  $\text{Ca}^{2+}$ -onafhankelijke agglutinatie van de spermakoppen tot gevolg.

De *in vivo* interactie tussen sperma en eileiderepitheelcellen in de peri-ovulatoire periode is eveneens een essentiële stap in het capacitatieproces van spermacellen bij verschillende zoogdieren. Eén van de belangrijkste kenmerken van spermacapacitatie is tyrosinefosforylatie van de eiwitten in de spermastaart. Bij vele diersoorten kan spermacapacitatie, en dus ook tyrosinefosforylatie van eiwitten in de spermastaart,



geïnduceerd worden door de spermacellen *in vitro* bloot te stellen aan  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$  en albumine in het incubatiemedium. Deze condities blijken echter onvoldoende voor capacitatie van runder- en hengstensperma. Bij het rund wordt volledige capacitatie met een uitgebreide tyrosinefosforylatie van de eiwitten in de spermastaart geïnduceerd door heparine-achtige glycosaminoglycanen toe te voegen aan het capacitiemedium. Deze moleculen hebben echter geen effect op capacitatie van hengstensperma (Hoofdstuk 3). Met behulp van het oviduct explantmodel hebben we aangetoond dat de binding tussen sperma en eileiderepitheelcellen *in vitro* een essentiële vereiste is voor de capacitatie van hengstenspermacellen (Hoofdstuk 4). Spermacellen gebonden aan eileiderepitheel vertoonden een tijdsafhankelijke tyrosinefosforylatie van de eiwitten in de spermastaart terwijl ongebonden spermacellen of spermacellen geïncubeerd in oviduct explant-geconditioneerd medium, deze eigenschap niet vertoonden. Zowel de gebonden als de ongebonden spermacellen bleven motiel en vertoonden een intacte plasmamembraan en acrosoom. Vroeger werd reeds aangetoond dat tyrosinefosforylatie van eiwitten in de spermastaart *in vitro* geïnduceerd kon worden door hengstenspermacellen te incuberen in medium met een verhoogde pH. Als we de intracellulaire pH van de oviductexplantcellen, de oviduct-gebonden en ongebonden spermacellen evalueerden, bleek duidelijk dat tyrosinefosforylatie van eiwitten in de spermastaart bij de gebonden spermacellen veroorzaakt werd door het creëren van een alkalisch micromilieu rond iedere oviduct explant. Het secretorisch epitheel van de eileider bevat grote alkalische vesikels die vrijgesteld werden gedurende de sperma-oviduct binding. Op hetzelfde moment werd een graduele toename van de intracellulaire pH van de gebonden spermacellen waargenomen terwijl de ongebonden spermacellen geen stijging vertoonden van de intracellulaire pH.

Vervolgens moeten de spermacellen in de peri-ovulatoire periode hyperactieve motiliteit vertonen opdat ze het eileiderepitheel zouden kunnen loslaten. Gedefinieerd capacitiemedium of capacitiemedium verrijkt met glycosaminoglycanen of S-S reducerende stoffen waren als dusdanig *in vitro* onvoldoende in staat om deze loslating van hengstenspermacellen te verwezenlijken (Hoofdstuk 3 en 5). In hoofdstuk 5 werden mogelijke *in vivo* hyperactivatie- / loslatingstriggers getest door cellen en vochten van het geslachtsapparaat van de merrie toe te voegen aan het sperma-oviduct explantmodel. Gespoeld eileidervocht afkomstig van pre- en post-ovulatoire merries, 100% en 10% vers follikelvocht, cumuluscellen en rijpe eicellen bleken helemaal geen effect te hebben op

hyperactieve motiliteit en loslating van gebonden spermacellen aan het eileiderepitheel. Vers follikelvocht bleek zelfs een schadelijk effect te hebben op de leefbaarheid van hengstenspermacellen. Daarom werd de spermatoxische component (een hittegevoelige, vetoplosbare factor met een grootte tussen de 30-100 kDa) eerst geneutraliseerd door respectievelijk hitte-inactivatie, actieve kool behandeling of 30 kDa centrifugatie, of door een combinatie van deze drie behandelingen. Na deze verschillende follikelvochtbehandelingen vertoonden de spermiasuspensies die geïncubeerd werden bij pH 7.9 duidelijke hyperactieve motiliteit in tegenstelling tot de spermacellen die geïncubeerd werden bij pH 7.4. Bovendien bleken de verhoogde pH, het extracellulaire  $Ca^{2+}$  en de ongedefinieerde, hittegevoelige, wateroplosbare follikelvochtfactor kleiner dan 30 kDa, ook tyrosinefosforylatie van eiwitten in de spermastaart, een verhoogde intracellulaire  $Ca^{2+}$  concentratie en hyperactieve motiliteit te veroorzaken. Daarnaast werd ook een beperkte loslating van de gebonden hengstenspermacellen waargenomen.

In 2009 werd een studie gepubliceerd waarin men beweerde dat tyrosinegefosforyleerde, hyperactieve hengstenspermacellen in staat waren om *in-vitro*fertilisatie bij het paard te realiseren. In de aanwezigheid van procaïne zou na coïncubatie van sperma en eicellen meer dan 60% van de paardeneicellen beginnen delen. De embryos ontwikkelden echter niet verder dan het 8-16 cellig stadium. Men besloot dat spermacellen onder invloed van procaïne in staat waren om de zona pellucida te penetreren door het induceren van hyperactieve motiliteit in de tyrosinegefosforyleerde spermacellen. Hiertoe werden de spermacellen voorafgaand geïncubeerd in capacitatiemedium met een verhoogde pH. Beide capacitatiekarakteristieken werden echter ook geïnduceerd na de incubatie van hengstenspermacellen in de verschillende follikelvochtbehandelingen bij pH 7.9 (Hoofdstuk 5). Wanneer we echter het bevruchtend vermogen van de tyrosinegefosforyleerde, hyperactieve hengstenspermacellen testen door *in-vitro*fertilisatie uit te voeren in capacitatiemedium met procaïne of na de verschillende follikelvochtbehandelingen bij pH 7.9, bleken beide condities onvoldoende om *in-vitro* bevruchting van paardeneicellen mogelijk te maken (Hoofdstuk 6). De bevruchting werd beoordeeld door het evalueren van de aanwezigheid van een spermastaart in het cytoplasma van de eicel, het uitstoten van een tweede poollichaampje en de vorming van twee pronuclei. Bovendien stelden we vast dat 2.5 en 5 mM procaïne deling van paardeneicellen induceerde onafhankelijk van de aanwezigheid van spermacellen. Deze gedeelde eicellen ontwikkelden echter ook niet verder dan het 8-16

cellig stadium en bovendien vertoonden de dochtercellen ofwel geen celkern ofwel zeer gecondenseerde, afwijkende DNA fragmenten. Procaïne bleek uiteindelijk ook geen toename van de intracellulaire  $\text{Ca}^{2+}$  concentratie in de eicellen te veroorzaken maar wel een toename van de intracellulaire pH. Deze pH stijging veroorzaakt vervolgens een depolymerisatie van F-actine in de corticale regio van de eicel wat verder leidt tot de inductie van celdeling van paardeneicellen gecombineerd met DNA fragmentatie.

Deze thesis heeft bijgedragen tot het verwerven van een beter fundamenteel inzicht in de interacties tussen hengstensperma en de eileider tijdens de peri-ovulatoire periode bij het paard. Door het feit dat spermacapacitatie bij de hengst duidelijk verbetert bij een licht alkalische pH, dient de pH van *in vitro* capacitiemedia voor hengstensperma standaard aangepast te worden naar 7.9. Jammer genoeg is deze aanpassing onvoldoende om *in vitro*fertilisatie bij het paard werkzaam te maken omdat blijkbaar belangrijke capacitiefactoren nog steeds ontbreken in gedefinieerde capacitiemedia die de acrosoomreactie induceren. Het is dus belangrijk om in de toekomst de resterende capacitiefactoren in follikel- en eileidervocht te identificeren om uiteindelijk een werkzaam capacitiemedium voor paarden te kunnen ontwikkelen.



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# DANKWOORD

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Bart



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# CURRICULUM VITAE

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Bart Leemans werd geboren op 18 september 1985 te Lier, België. Na het behalen van het diploma hoger secundair onderwijs aan het Provinciaal Instituut voor Tuinbouwonderwijs te Mechelen, richting Biotechnische Wetenschappen, startte hij in 2004 met de studies Diergeneeskunde aan de Universiteit Gent. In 2010 behaalde hij het diploma Master in de Diergeneeskunde optie paard met onderscheiding.

In oktober 2010 startte hij zijn doctoraatsonderzoek – ondersteund door een IWT mandaat – aan de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Het onderzoek dat plaatsvond in het IVF laboratorium ‘Reproductive Biology Unit (RBU)’ was gericht naar de identificatie van fysiologische capacitatietriggers van hengstensperma in het oviduct van de merrie. In het kader van zijn doctoraatsonderzoek heeft hij gedurende vier maanden laboratoriumwerk verricht in het laboratorium ‘Biology of Reproductive Cells’ te Utrecht (Nederland) gesteund door een COST – Gemini – Short – Term Scientific Mission (STSM) grant. Naast zijn doctoraatsonderzoek was Bart Leemans ook werkzaam in de kliniek verloskunde, waar hij participeerde in de nacht- en weekenddiensten. In 2014 voltooide hij eveneens de doctoraatsopleiding aan de Doctoral Schools of Life Science and Medicine.

Bart Leemans is auteur of mede-auteur van verschillende publicaties in internationale en nationale wetenschappelijke tijdschriften en presenteerde zijn onderzoek op diverse internationale en nationale congressen.





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I hear, I know.  
I see, I remember.  
I do, I understand.

*Confucius*