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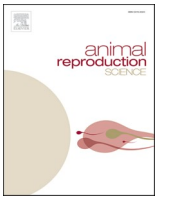

A stallion spermatozoon's journey through the mare's genital tract: *In vivo* and *in vitro* aspects of sperm capacitation

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ABSTRACT

Conventional in vitro fertilization is not efficacious when working with equine gametes. Although stallion spermatozoa bind to the zona pellucida in vitro, these gametes fail to initiate the acrosome reaction in the vicinity of the oocyte and cannot, therefore, penetrate into the perivitelline space. Failure of sperm penetration most likely relates to the absence of optimized in vitro fertilization media containing molecules essential to support stallion sperm capacitation. *In vivo*, the female reproductive tract, especially the oviductal lumen, provides an environmental milieu that appropriately regulates interactions between the gametes and promotes fertilization. Identifying these 'fertilization supporting factors' would be a great contribution for development of equine in vitro fertilization media. In this review, a description of the current understanding of the interactions stallion spermatozoa undergo during passage through the female genital tract, and related specific molecular changes that occur at the sperm plasma membrane is provided. Understanding these molecular changes may hold essential clues to achieving successful in vitro fertilization with equine gametes.

1. Introduction

Fertilization in mammalian species relies on the activation, often referred to as 'capacitation', of spermatozoa in the female genital tract (Visconti et al., 2009). After deposition of the ejaculate in either the vagina (e.g., in dogs), cervix (e.g., in pigs) or uterus (e.g., in horses), a viable sperm population will be transported through the uterus to the site of fertilization in the oviduct. A sperm reservoir, that promotes physical interaction between spermatozoa and the oviduct epithelial surface, will initially be established in the caudal isthmus. When ovulation is imminent, spermatozoa bound to the oviduct epithelium will be activated or capacitated and subsequently released from the oviduct epithelium to meet the mature oocyte (Yanagimachi, 1969; Suarez, 2002). During capacitation, various signaling pathways at the sperm membrane and cytoplasm have to be induced in the spermatozoa, leading to physiological and biochemical modifications. Only capacitated spermatozoa have the capacity to fertilize the mature oocyte (Gervasi and Visconti, 2016). The process of capacitation has been mimicked in various species in in vitro conditions, yielding standard procedures for in

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vitro fertilization and embryo production using human, cattle, pig and mice gametes (Steptoe and Edwards, 1992; Galli et al., 2003; Betteridge, 2006; Perry, 2014). In general, capacitation is induced in vitro by HCO_3^-, Ca^{2+} and a cholesterol acceptor such as albumin (review: Leemans et al., 2019a). In some species, there is need for additional specific factors in the surrounding medium to induce capacitation, e.g., glycosaminoglycans in cattle (Parrish et al., 1988) and caffeine in pigs (Funahashi et al., 2001). These observations are indicative that the capacitation process in in vitro conditions has species-specific features (Bailey at al, 2010). Unfortunately, it has not yet been possible to develop a repeatable, conventional in vitro fertilization (IVF) protocol for horses. The birth of two healthy IVF-produced foals was reported in the early 1990 s, using calcium ionophore for capacitation induction (Palmer et al., 1991; Bézard et al., 1992). Unfortunately, other laboratories have not achieved similar success with this protocol.

Different protocols used to induce capacitation in other species have been evaluated with the aim of developing a repeatable equine IVF protocol. Fertilization rates, however, remain very low, and protocols are not repeatable between different laboratories (Choi et al., 1994; Dell'Aquila et al., 1997a, 1997b; Alm et al., 2001; Hinrichs et al., 2002; Mugnier et al., 2009, reviewed by Leemans et al., 2016a). For example, the use of Ca^{2+} ionophore A23187 and heparin did not significantly improve fertilization rates (Alm et al., 2001; Li et al., 1995) with horse gametes. The use of zona pellucida (ZP) proteins, caffeine and lysophospholipids enhanced sperm capacitation but there was not a concomitant increase in fertilization rates observed (Graham et al., 1996). Overall, reported in vitro fertilization rates in horses range from 0% to 31% (for an overview see (Leemans et al., 2016a). In 2009, it was reported that procaine treatment of stallion spermatozoa induced fertilization with rates varying from 0% to 60%, presumably by inducing hyperactivated sperm motility (McPartlin et al., 2009). In 2015, however, a direct effect of procaine was found on equine oocytes, inducing oocyte cytokinesis and embryonic development to the 8–16 cell stage independent of whether spermatozoa were in the medium (Leemans et al., 2015a). There, however, was not proper division of the DNA and viable embryo development has not been reported yet. In contrast, ZP drilling and partial removal of the ZP does improve fertilization rates in horses (Choi et al., 1994; Li et al., 1995; Dell'Aquila et al., 1996), although the utility of these protocols is complicated by a large rate of polyspermy.

A relevant question is, which of the two equine gametes contribute to the failure of IVF? The lack of capacity of stallion spermatozoa to penetrate the zona pellucida (ZP) has been reported to hamper development of an efficacious equine IVF protocol (Tremoleda et al., 2004). Theoretically, both inefficient sperm capacitation and/or inadequate oocyte maturation could contribute to this failure. IVF performed with in vivo matured oocytes, however, has not been successfully performed (Palmer et al., 1991), while in vitro matured oocytes transferred to the oviduct of an inseminated mare achieve similar pregnancy rates to conventional artificial insemination (Hinrichs et al., 2002). This finding indicates that in vitro matured oocytes have the capacity of being fertilized normally. Furthermore, irreversible ZP hardening (e.g., due to premature release of cortical granules, or other post-ovulatory ZP modifications of mature oocytes) is considered unlikely to contribute to the failure of equine IVF (Hinrichs et al., 2002; Dell'Aquila et al., 1999). Stallion spermatozoa have the capacity for penetrating the ZP of (heterologous) bovine oocytes in vitro with subsequent pronuclei formation and cell cleavage (Consuegra et al., 2020; Sinowatz et al., 2003; Sessions-Bresnahan et al., 2014). These observations are indicative that stallion sperm capacitation occurs in vitro under the latter conditions and that post-ovulatory ZP modifications cannot be excluded as a barrier for efficacious IVF in horses. Overall, the argumentation above indicates that it is difficult to verify which of the two gametes is primarily responsible for the failure of IVF. Generally, however, it is assumed that the relatively higher rates of fertilization achieved in vivo are due to specific properties of the oviductal environmental milieu. Indeed, pregnancy rates are similar after artificial insemination into the uterine body and direct oviductal insemination (McCue et al., 2000). This suggests that the passage of sperm through the mare's uterus is not obligatory for capacitation/fertilization to occur.

In general, capacitation is the activation process that sperm undergo after ejaculation, during passage through the female genital tract (Suarez, 2002). A capacitating environment in most eutherian mammals will generate an influx of HCO_3^-, which in turn leads to the activation of soluble adenylyl cyclase (sAC) (pig: Harrison and Gadella, 2005; mouse: Krapf et al., 2010). As a consequence, the quantity of cytoplasmic cAMP will increase and as a result there will be activation of the protein kinase A (PKA) cascade. Activation of these latter pathways will subsequently induce and increase in membrane fluidity and change in intracellular pH (mouse: Zeng et al., 1996). At a later stage, intracellular Ca^{2+} increases and the asymmetry of the plasma membrane bilayer will collapse, resulting in exposure of phosphatidylserine and phosphatidylethanolamine on the outer leaflet of the plasma membrane (Flesch and Gadella, 2000). Next, in vitro cholesterol acceptors such as fatty acid free albumin will facilitate changes such as cholesterol depletion. As a result, the aggregation of microdomains or lipid rafts occurs that contain protein complexes with affinity for the zona pellucida (pig: van Gestel et al., 2007) and factors such as N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). SNARE proteins play an important role in the fusion of the sperm plasma membrane with the outer acrosomal membrane (pig: Tsai et al., 2007; Tsai et al., 2012). The outcome is the onset of the acrosome reaction, an exocytotic process that leads to release of lytic enzymes to locally digest the zona pellucida (pig: Tsai et al., 2010). More recently, there has been reported in mice that contact with the intercellular matrix of the cumulus cell complex rather than with the zona pellucida was sufficient to induce the acrosome reaction and disaggregate the cumulus cell complex. This allows for acrosome-reacted spermatozoa of mice to pass through the zona pellucida (Inoue et al., 2011; Jin et al., 2011). Besides these processes, the activation of PKA also supports phosphorylation of protein tyrosine residues that, especially in the sperm tail, leads to the onset of a Ca^{2+}-dependent hyperactivated motility, necessary to generate the force to cross the zona pellucida of the oocyte (man: Nassar et al., 1999). In addition, tyrosine phosphorylation increases the affinity for the zona pellucida (cat: Pukazhenthi et al., 1998) and has a proven role in the acrosome reaction (Benoff, 1998). Only fully capacitated spermatozoa bind to and penetrate the zona pellucida (Yanagimachi, 1994; pig: Harayama et al., 2012).

The aim of this review is to provide an overview of the different sites and barriers stallion spermatozoa need to overcome in vivo in order to yield a population of fully capacitated spermatozoa in the oviduct, the site of fertilization. In addition, important capacitation-related molecular interactions in stallion spermatozoa will be discussed. Because there has been published a recent review on the regulation of hyperactivated motility, tail-associated protein tyrosine phosphorylation and the acrosome reaction as these relate to

stallion sperm capacitation (Leemans et al., 2019a), the current review will focus on the capacitation-related processes that occur at the sperm membrane. This is still an area that is poorly explored in stallion sperm capacitation both in vivo and in vitro.

2. Transport of a spermatozoon through the mare's genital tract in vivo, and its role in ensuring viable populations of capacitated spermatozoa

Sperm capacitation is a strictly ordered sequence of processes in all mammals, including horses (Yanagimachi, 1994): (1) spermatozoa are ejaculated into the uterine body and transported to the utero-tubal junction (UTJ); (2) a reservoir of non-capacitated spermatozoa is established, by binding to oviduct epithelial cells at the UTJ and the caudal isthmus; (3) spermatozoa within the reservoir become capacitated just before the time when ovulation occurs; (4) the capacitated spermatozoa become hyperactively motile 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 (mouse: Jin et al., 2011; mouse: Inoue et al., 2011), (6) the acrosome reaction is induced, resulting in the capacity of 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 processes are initiated after the spermatozoa have made contact with the epithelial cells and the peri-ovulatory stage-stimulated oviduct secretions.

2.1. Transport through the uterine lumen

After ejaculation or insemination, stallion spermatozoa are transported from the mare's uterine body towards the oviduct mainly as a result of uterine contractions (passive sperm transport), with a relatively minor contribution of active sperm motility (Katila, 2001). The first spermatozoa are observed in the oviduct within 2 h post-insemination while the largest number of spermatozoa are transported into the oviduct 4 h post-insemination (Bader, 1982; Brinsko et al., 1991). Only a small number of spermatozoa are transported into the oviduct (Scott and Overstreet, 1998). There are several mechanisms that help to clear the uterus and eliminate the redundant spermatozoa. Firstly, myometrial contractions will mechanically expel the sperm through the cervix (Troedsson et al., 1998; Katila et al., 2000). Secondly, starting about 0.5 h post-insemination, an influx of polymorphonuclear neutrophils (PMNs) into the uterine lumen plays an important role in sperm phagocytosis (Kotilainen et al., 1994; Katila, 1995; Troedsson et al., 1995). This inflammatory response is harmful for all the spermatozoa being transported through the uterine lumen. Only dead spermatozoa, however, are highly susceptible to this elimination cascade, whereas viable spermatozoa are protected from binding to the PMNs and thereby from phagocytosis. Seminal plasma factors have important functions in this protective mechanism. For example, lactoferrin enhances the cell-to-cell interaction between PMNs and spermatozoa (viable and dead) in the uterus (Troedsson et al., 2014). Interestingly, CRISP-3 (cysteine rich secretory protein 3), a protein secreted by the accessory sex glands into the seminal plasma during ejaculation, induces a marked reduction in the binding between viable spermatozoa and PMNs (Doty et al., 2011). As such, the transport of viable spermatozoa to the oviduct occurs, while PMNs phagocytose the dead spermatozoa in the uterus.

During sperm transport towards the oviduct, the general hypothesis is that the onset of capacitation in viable spermatozoa needs to be inhibited. To prevent a premature capacitation response, spermatozoa are coated with decapacitation factors derived mainly from the seminal plasma during and after ejaculation. Furthermore, mammalian seminal plasma contains several extracellular vesicle populations secreted by epithelial cells at different sites of the male reproductive tract, including the accessory sex glands. For example, prostasomes, secreted by the prostatic epithelium, probably function as decapacitation factors in horses and humans because prostasomes have a lipid content consisting of mainly saturated fatty acids and large concentrations of cholesterol and sphingomyelin (horse: Arienti et al., 1998a; man: Arienti et al., 1998b; man: Carlini et al., 1997). The large cholesterol content may result in the inhibition of capacitation-related plasma membrane changes and the acrosome reaction by stabilizing the sperm plasma membrane (humans: Cross and Mahasreshti, 1997; horse: Sostaric et al., 2008a; humans: Pons-Rejraji et al., 2011), thereby preventing premature sperm capacitation. In some studies with pigs, humans and mice results have indicated that prostasomes actually promote capacitation-related processes like the acrosome reaction (Palmerini et al., 2003; Siciliano et al., 2008) and the Ca^{2+}-induced onset of hyperactivated motility, a requirement for ZP penetration (Park et al., 2011).

In horses, when spermatozoa are directly deposited in the oviduct via the infundibulum, pregnancy rates are not different from those obtained after artificial insemination into the uterine body (McCue et al., 2000). This suggests that neither the uterine body nor the oviductal isthmus is an obligatory site through which stallion spermatozoa need to be transported before capacitation/fertilization, whereas the lumen of the ampulla contains an essential environmental milieu for stallion spermatozoa to attain the ability to capacitate/fertilize.

2.2. Transport through the utero-tubal junction (UTJ)

In mares, the oviduct reservoir selects morphologically normal spermatozoa with progressive motility (Scott and Overstreet, 1998; Boyle et al., 1987; Scott et al., 2000). After transport through the uterus, stallion spermatozoa need to be transported through the UTJ, a closed muscular 'sphincter', to establish a sperm-oviduct reservoir. It is unknown if there has to be an opening of the UTJ during the pre-ovulatory period for spermatozoa to enter the oviduct. Estrous cycle-dependent effects of estrogens or seminal plasma factors such as prostaglandins may play a role in evoking the relaxation of the UTJ. It can be hypothesized that during the follicular phase the UTJ might open as a result of estrogenic actions while this structure remains closed during the progesterone-dominated luteal 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-dominated postovulatory phase of the estrous cycle, can provide additional insights into the regulation of UTJ opening or

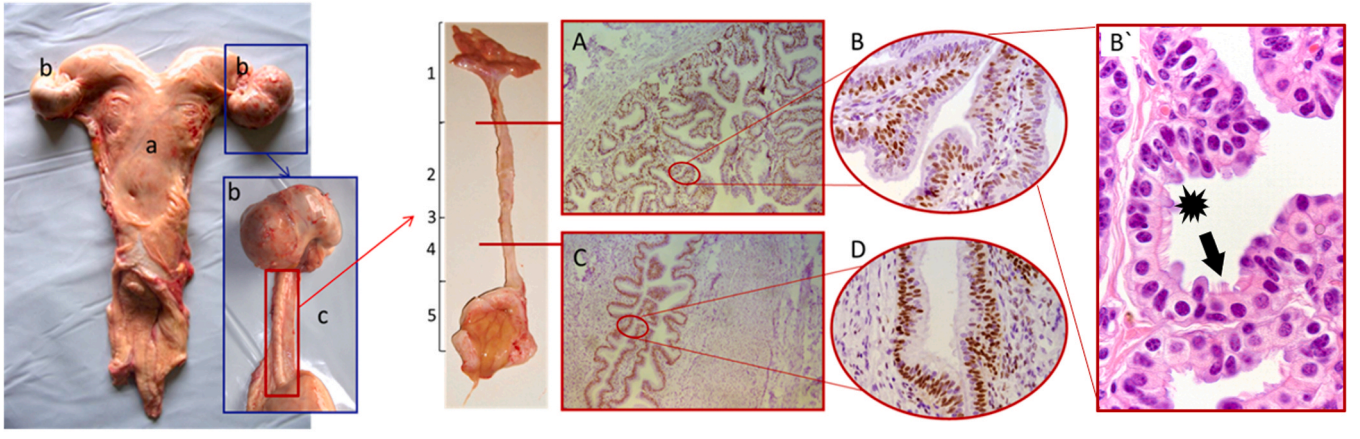

Fig. 1. Morphology and ultrastructural features of the mare`s oviduct: (a) uterus, (b) ovaries and (c) oviduct. The oviduct consists of (1) infundibulum, (2) ampulla, (2) ampullary-isthmic junction, (4) isthmus and (5) utero-tubal junction (UTJ). (A) and (B) represent ultrastructural images of the ampulla and (C) and (D) of the isthmus. B` Ultrastructural cross-section of the ampulla: arrow points to a ciliated cell, star to a secretory cell (Images kindly provided by Nelis, 2015 and Van Beek, 2018).

closure (Weber et al., 1996). The late morula- or early blastocyst-stage embryos are transported through the UTJ retrogradely into the uterus (Battut et al., 1997) under the action of prostaglandin E2 produced by the equine embryo (Weber et al., 1991). Unfertilized oocytes (UFOs), however, are retained in the oviduct because of the lack of capacity of these oocytes to produce this signal. A similar mechanism of gamete-maternal communication or interaction may affect the transport of sperm through the UTJ.

2.3. The oviduct

2.3.1. 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 (Fig. 1). 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 unwinding the loops and removing the suspending mesosalpinx, the actual length of an equine oviduct is approximately 20–30 cm. Half of the oviduct consists of the ampulla. Irregular fimbriae are present along the margin of the funnel-shaped infundibulum. Because some fimbriae are attached to the cranial pole of the ovary, the infundibulum also covers the ventrally 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 ciliated epithelium) (Fig. 1). Ciliogenesis and ciliary motion toward the uterus or oocyte are dependent on estrous 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 (Kainer, 2011).

2.3.2. An environment for capacitation and fertilization

As described previously in this manuscript, freshly ejaculated spermatozoa have to undergo a maturation process in the female reproductive tract called "capacitation" to prepare these gametes to fertilize an oocyte. Ultimately, only a small fraction of the total inseminated sperm population are transported to the site of fertilization and undergo capacitation. Based on in vitro studies, it is hypothesized that only spermatozoa with superior viability, morphology and motility are transported to the oviduct (Hunter et al., 1987; Fazeli et al., 1999).

The interaction between highly viable spermatozoa and the oviduct environmental milieu to support capacitation and fertilization has been extensively studied in several mammalian species. By contrast, in mares the oviductal environmental milieu has not been described in detail. The lumen of the oviductal ampulla, however, contains an essential environmental milieu for stallion spermatozoa to attain the ability to capacitate/fertilize. A representative in vitro model, therefore, is necessary to study the initial processes that induce sperm capacitation and fertilization, and to mimic the in vivo environmental milieu and conditions near ovulation as closely as possible. To this end, equine oviduct epithelial cell culture models, generally established with harvested ampullary epithelial cells, have been developed; these include oviduct monolayers (Dobrinski et al., 1999; Thomas et al., 1995), explants (Nelis et al., 2014) and apical plasma membranes (Leemans et al., 2016b). The effect of these models on sperm capacitation are subsequently discussed in this manuscript.

2.3.2.1. Sperm-oviduct binding. *In vivo*, sperm binding to epithelial cells in the isthmus, i.e., the caudal part of the oviduct, is likely to be essential for recruiting and storing viable, and potentially fertile, non-capacitated spermatozoa before fertilization, to establish the so-called "sperm reservoir" (Suarez, 2002). Sperm-oviduct binding supports an extension of the period of viability of the inseminated spermatozoa. This is important because the time of insemination and ovulation are not synchronized in mammals. Spermatozoa need to be maintained and nourished to bridge the time period between sperm deposition in the female tract and ovulation. Binding to the oviduct epithelium extends the lifespan of bull spermatozoa (Boilard et al., 2002) which results in a viable sperm population at the time of ovulation. Furthermore, sperm-oviduct binding at the isthmic site ensures there is selection of a high-quality sperm population (Boilard et al., 2002). These spermatozoa have the capacity for binding to the epithelium and can be found in the crypts of the oviductal folds, while spermatozoa with an altered membrane and poor viability are located in the lumen of the oviduct (Teijeiro and Marini, 2012). In cattle, sperm binding occurs mainly to ciliated oviduct epithelial cells (Sostaric et al., 2008b). Spermatozoa in the lumen can be flushed out by powerful fluid flow, while epithelium-bound spermatozoa cannot. (3) When the time of ovulation approaches, a tightly regulated small number of spermatozoa is released from the sperm reservoir, helping to prevent polyspermic fertilization. The release of too many spermatozoa would predispose to polyspermy (Hunter, 2012), while too few sperm can lead to failure of fertilization (Suarez, 2002). Multiple capacitation-related processes, such as the onset of hyperactivated motility, contribute to sperm release from the oviduct. It is hypothesized that hyperactivated motility in bull spermatozoa is suppressed in spermatozoa bound in the sperm reservoir (Hung and Suarez, 2010). Other capacitation-related processes are also suppressed to prevent polyspermic fertilization (Hung and Suarez, 2010).

In several mammalian species including cattle, hamsters and pigs, the molecular interaction between spermatozoa and the oviduct epithelium is based on a Ca^{2+}-dependent carbohydrate-lectin recognition (Suarez, 2001). In cattle, glycosaminoglycans (Sostaric et al., 2005; Gualtieri et al., 2010), S-S reductants (Gualtieri et al., 2009, 2010), and capacitation molecules such as albumin, Ca^{2+}, and HCO_3^- (Sostaric et al., 2005) alter the affinity of the spermatozoa for the oviduct epithelium and thereby induce the release of bound spermatozoa. In mares, D-galactose was previously reported to be a key-molecule for facilitating sperm-oviduct binding, on the basis of studies using a dedifferentiated oviduct monolayer model (Dobrinski et al., 1996). Indeed, galactose-binding proteins have been

isolated from the equine sperm plasma membrane (Sabeur et al., 2007). There, however, was reassessment of the potential functions of various carbohydrates, glycosaminoglycans, lectins, S-S reductants, and capacitation factors including albumin, Ca^{2+}, and HCO_3^- in equine sperm–oviduct interaction using both an oviduct explant and an oviduct apical plasma membrane model (Leemans et al., 2016b). Although N-acetylgalactosamine, N-acetylneuraminic acid (sialic acid), and D-mannose or D-glucose were present in large quantities on the oviduct epithelium, D-galactose moieties were not detected. Using a competitive binding assay and pretreatment with N-glycosidase F, equine sperm–oviduct binding was not exclusively regulated by Ca^{2+}-dependent lectin or disulphide (S-S) binding. Furthermore, the combination of the classical sperm capacitating factors albumin and HCO_3^- markedly reduced (>10-fold) the sperm binding density of the oviduct epithelium. Instead, the between-stallion spermatozoa binding affinity increased considerably, resulting in Ca^{2+}-independent head-to-head agglutination. In conclusion, combined albumin and HCO_3^- treatment induced head-to-head sperm agglutination which physically inhibits equine sperm–oviduct binding. This was also reported by Bromfield et al. (2013), as a response to equine follicular fluid. While these observations may lead to questioning of the relevance of the establishment of a sperm reservoir in horses, it can be hypothesized that head-to-head agglutination between stallion spermatozoa results in a vehicle that better facilitates transport of a population of fertile spermatozoa through the female reproductive tract towards the fertilization site. Cooperative sperm transport has been observed previously in the wood mouse (Moore et al., 2002). Once the fertilization site is reached, exposure to additional capacitation factors may induce the release of spermatozoa from the agglutination. More research is needed to understand the physiological function of head-to-head sperm agglutination between stallion spermatozoa.

While sperm-oviduct binding clearly plays a role in fertilization, this physical interaction with the epithelium may only be obligatory in the pre-ovulatory period (Leemans et al., 2016a). When spermatozoa are transported into the oviduct during the peri-ovulatory period, (i.e. if insemination is performed after ovulation), these gametes are immediately in contact with capacitation inducers. Consequently, the binding of spermatozoa to the oviduct surface is not required. This suggests that oviduct secretions, secreted primarily by the ampullary epithelium, may be equally or more relevant for the induction of capacitation in stallion spermatozoa than sperm-oviduct binding.

2.3.2.2. Oviductal fluids that create a capacitation environment. Close to 60 years ago, the time span required for spermatozoa to capacitate was investigated by oviductal insemination of the ewe (Mattner, 1963). It was concluded that a minimum of 90 min was required to achieve fertilizing capacity, and that oviduct-dependent secretions play an important role in the process of capacitation. It is now clear, at least in cattle, that oviductal fluids affect sperm function in many different ways including but not limited to capacitation (Killian, 2011).

The viscosity, volume and composition of oviductal fluid changes over time and is synchronized with the stage of the estrous cycle. Viscosity reduces after ovulation, which would facilitate an emphasized flagellar beat pattern (Hunter, 2011) and support the transport of spermatozoa to the ampulla. Viscosity does not contribute to the capacitation process directly, however, it is important for sperm transport towards the site of fertilization. The volume and composition of oviductal fluid is regulated by steroid hormones stimulating oviduct secretory cells and is highly supported during the follicular phase when estrogen is dominant.

In addition to changes in viscosity and volume, changes in molecular composition are also relevant (Killian, 2011). The lipid composition of the oviductal fluid in livestock changes mostly due to a change in phospholipid concentration. Phospholipids in oviductal fluid increase ten times in serum during estrus, and 4.5 times during the luteal phase. Spermatozoa absorb phospholipids in the oviduct facilitating changes in membrane composition. Though debated, the cholesterol concentration appears to be less in oviductal fluid, compared to serum concentrations. This induces cholesterol efflux and facilitates binding to a sterol acceptor (e.g., high density lipoproteins). In most species, the uptake of phospholipids and the efflux of cholesterol alter the composition of the plasma membrane during capacitation. The pH of the sperm cell is likely to change due to this lipid exchange and several signaling cascades can be subsequently activated (Cross, 1998; Travis and Kopf, 2002). The alteration in phospholipid:cholesterol ratio facilitates membrane fluidity and is ultimately needed for induction of the acrosome reaction and subsequent fertilization (Killian, 2011) (discussed in more detail below). Additionally, spermatozoa can be exposed to different biomolecules via extracellular vesicles (EVs). The EVs are small compartments surrounded by a bilipid membrane. The membrane contains receptors which allow the EVs to recognize their target cells, including spermatozoa. Target cells can then be altered via receptor-ligand interaction or via uptake of the EV (Mulcahy et al., 2014; Zhou et al., 2019). The EVs transfer cargo (e.g., proteins, lipids, RNAs and small non-coding RNAs) from one cell to another and this can result in a change of the physiological state of the recipient cell (Simons, 2009). The EVs have been identified both in the male and female reproductive tract, but only the EVs in the male reproductive tract have been studied extensively in horses and mice (Sostaric et al., 2008a; Aalberts et al., 2013; Zhou et al., 2019; Trigg et al., 2016; Nixon et al., 2019). The EVs in the female reproductive tract have only recently been studied in any depth. In the mouse, EVs have been identified in vaginal (Bathala et al., 2012), uterine (Griffiths et al., 2008) and oviductal fluids (Griffiths et al., 2008). The function of oviductal EVs or oviductosomes in capacitation is not yet completely understood. Results from recent studies, however, indicate EVs do contribute to the capacitation process of bull spermatozoa (Franchi et al., 2020). The interaction between oviductosomes and spermatozoa was investigated by incubating spermatozoa with oviductosomes in non-capacitating medium. Contact between bull spermatozoa and oviductosomes resulted in a progressive uptake of oviductal oviductosomes by bull spermatozoa over time, with a maximum uptake at 2 h after incubation. There was an increase in protein tyrosine phosphorylation after oviductosome uptake and this was similar to spermatozoa incubated in capacitating conditions (Franchi et al., 2020). Furthermore, murine oviductosomes are conserved in humans and play a role in sperm capacitation and fertility (Bathala et al., 2012). Because oviductosomes play a role in bull, mouse and human capacitation, it can be hypothesized that EVs may also have important functions in stallion sperm capacitation.

A change in protein composition within the oviductal fluid of cattle also occurs (Killian, 2011). It is suggested that these alterations

in protein content affect the sperm membrane and have effects on sperm functions. It is also suggested that the oviductal fluid of cattle has effects on sperm function due to either the loss or modification of sperm proteins (Miller, 2015). Until now, only very few oviduct secreted factors have been studied. The most important factors include: estrogen-dependent oviduct secretory glycoprotein (OSG) or oviductin, a unique oviduct protein conserved across many mammalian species. This protein enhances sperm-oocyte binding and

Table 1
Composition of the equine oviductal fluid compared to blood serum.

		Oviductal fluid	Blood serum	Reference
Electrolyts (mM)	Na^+	130[b]	ND	Campbell et al. (1979)
		ND	132 – 142	Rose and Hodgson (1999); Smith (2002)
	Cl^-	ND	96 – 107	Rose and Hodgson (1999); Smith (2002)
	K^+	7.9[b]	ND	Campbell et al. (1979)
		ND	3.0 – 5.9	Rose and Hodgson (1999); Smith (2002)
	Total Ca	2.3[b]	ND	Campbell et al. (1979)
		ND	2.4 – 3.3	Rose and Hodgson (1999); Smith (2002)
	Ca^{2+}	ND	1.4 – 1.7	Rose and Hodgson (1999); Smith (2002)
	Mg^{2+}	4.6[b]	ND	Campbell et al. (1979)
		ND	0.8 – 1.2	Rose and Hodgson (1999); Smith (2002)
	P	0.4[b]	ND	Campbell et al. (1979)
		ND	0.8 – 1.8	Rose and Hodgson (1999); Smith (2002)
	$HCO3^-$	ND	20 – 28	Rose and Hodgson (1999); Smith (2002)
Energy substrates (mM)	Glucose	2.84–5.92	ND	Campbell et al. (1979)
		0.18–0.57*	ND	González-Fernández et al. (2020)
		ND	3.9 – 5.6	Rose and Hodgson (1999); Smith (2002)
	Lactate	54.66–69.25*	ND	González-Fernández et al. (2020)
		ND	0.7 – 1.2	Rose and Hodgson (1999); Smith (2002)
	Pyruvate	ND	ND	Campbell et al. (1979); Rose and Hodgson (1999); Smith (2002)
Amino acids (mM)	Alanine	0.140[a,b]	0.055	Engle et al. (1984)
		2.09–3.24[a,*]	ND	González-Fernández et al. (2020)
	Arginine	0.031	0.010	Engle et al. (1984)
	Aspartic acid	0.022	0.004	Engle et al. (1984)
	Cystine	0.003	< 0.001	Engle et al. (1984)
	Glutamic acid	0.057	0.025	Engle et al. (1984)
	Glycine	0.263[a,b]	0.137[a]	Engle et al. (1984)
		2.2–5.1[a,b,*]	ND	González-Fernández et al. (2020)
	Histidine	0.020[c]	0.020	Engle et al. (1984)
		0.24–0.21*	ND	González-Fernández et al. (2020)
	Isoleucine	0.025	0.017	Engle et al. (1984)
		0.06–0.08*	ND	González-Fernández et al. (2020)
	Leucine	0.053[b]	0.029	Engle et al. (1984)
		0.26–0.39*	ND	González-Fernández et al. (2020)
	Lysine	0.053[b]	0.057[a]	Engle et al. (1984)
	Methionine	0.014[c]	0.002	Engle et al. (1984)
	Phenylalanine	0.026	0.016	Engle et al. (1984)
	Proline	0.048[c]	0.047[b]	Engle et al. (1984)
	Serine	0.051[c]	0.137[a]	Engle et al. (1984)
	Threonine	0.038	0.026	Engle et al. (1984)
	Tyrosine	0.041	0.018	Engle et al. (1984)
	Tryptophan	ND	ND	Engle et al. (1984)
	Valine	0.041	0.042	Engle et al. (1984)
		0.11–0.17*	ND	González-Fernández et al. (2020)
Metabolites (mM)	Acetylcarnitine	0.27–0.67*	ND	González-Fernández et al. (2020)
	Acetate	0.36–0.49*	ND	González-Fernández et al. (2020)
	Carnitine	2.35–2.10*	ND	González-Fernández et al. (2020)
	Choline	0.49–0.76*	ND	González-Fernández et al. (2020)
	Creatine	2.90–3.95*	ND	González-Fernández et al. (2020)
	Fumarate	0.02–0.09*,[b]	ND	González-Fernández et al. (2020)
	Histamine	0.08–0.20*	ND	González-Fernández et al. (2020)
	Malonate	ND	ND	González-Fernández et al. (2020)
	Myoinositol	19.7–15.16*	ND	González-Fernández et al. (2020)
	Phosphocholine	0.31–0.6*	ND	González-Fernández et al. (2020)
	Pyruvate + Succinate	1.29–1.5*	ND	González-Fernández et al. (2020)
Growth factors	Platelet derived growth factor (PDGF)	present	present	Eriksen et al. (1994)

[a]most prevalent amino acids; cycle-dependent significant difference ([b]: oestrus < dioestrus; [c]: 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; *: oestrus-dioestrous concentration.

penetration through the ZP, and also plays a role in the early stages of embryonic development (cattle: Boice et al., 1990; sheep: Murray, 1993; pigs: Kouba, 2000). In horses, however, Mugnier et al. (2009) reported that OSG is a pseudogene in the equine genome, which implies that this protein is not expressed. This finding, however, may have resulted from poor annotation of the equine genome. A second major protein synthesized and released by the oviduct is tissue inhibitor metalloprotease 1 (TIMP-1) which is known to be a specific inhibitor of matrix metalloproteinases like collagenases, stromelysins and gelatinases (pig: (Werb et al., 1996). Several cell functions also have been attributed to TIMP-1 including cell growth (Hayakawa et al., 1992), embryonic development (Nakanishi et al., 1986) and the maintenance and remodeling of the extracellular matrix (Sato et al., 1991). The TIMP-1 protein regulates tissue remodeling and steroidogenesis in the oviduct of sheep (Smith et al., 1994). 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. The PAI-1 protein is involved in a number of processes such as remodeling extracellular matrix, fibrinolysis, cell migration, and tumor metastasis (Andreasen et al., 1990; Murphy et al., 1992). Similar to TIMP-1, little is known about the specific actions of PAI-1 in the oviduct, but this protein may have similar functions. Other proteins identified in the oviduct of various mammals, include complement C3b, immunoglobulin A, prepro-collagen and clusterin in the pig (Buhi et al., 1996); and cytokines and growth factors in humans (Srivastava et al., 1996; Kane et al., 1997). In horses, osteopontin, atrial natriuretic peptide A (ANP A), and deleted in malignant brain tumor 1 (DMBT1) have also been identified as oviduct secretory proteins. When stallion spermatozoa were capacitated with Ca^{2+} ionophore, co-incubation of mature equine oocytes with either equine or porcine oviduct epithelial explants or monolayers increased equine IVF rates (0% compared with 9%). Although there was no significant effect of osteopontin and ANP A on fertilization, osteopontin slightly increased the IVF rates (Mugnier et al., 2009). Furthermore, Ambruosi et al. (2013) reported increased monospermic equine fertilization rates when mature oocytes were pre-incubated with DMBT1 and subsequently fertilized using procaine-activated sperm. The possibility that these results were affected by procaine-induced oocyte cytokinesis rather than fertilization by spermatozoa (cfr. (Leemans et al., 2015a) was not verified in this study. There remain numerous unidentified oviduct secreted proteins with affinity for the sperm plasma membrane that may affect the final maturation of the equine oocyte (Battut, 1995). Little, however, is known about the functions in the mare`s oviduct (Goudet, 2011).

Many other factors like electrolytes (Ca^{2+}, HCO_3^-), energy substrates, amino acids metabolites, and growth factors contribute to the composition of the oviductal fluid to create optimal fertilization conditions. The current knowledge of the composition of equine oviductal fluid compared to blood serum is summarized in Table 1. Concentrations of electrolytes in the oviductal fluid of mares tend to be similar to those in serum, except for Mg^{2+}, the concentration of which is two to five times higher in the oviduct of the mare compared to serum concentrations (Campbell et al., 1979). In contrast to glucose, lactate concentration measured in oviductal fluid was sixty times greater than in blood serum (González-Fernández et al., 2020; Rose and Hodgson, 1999; Smith, 2002). There was an increase in protein tyrosine phosphorylation in spermatozoa from two stallions when these gametes were incubated in 0.0625% and 0.125% oviductal fluid. Interestingly, there was no effect of oviductal fluid on protein tyrosine phosphorylation for a third stallion, and sperm motility and acrosome integrity did not change after spermatozoa were incubated in oviductal fluid for the spermatozoa of any of the stallions.

The acid-base balance of oviductal fluid is strictly regulated. It has been suggested that slight alkalinization of the oviductal pH has effects on stallion sperm function. Using the oviduct explant model, in vitro binding of stallion spermatozoa to the oviduct epithelium was observed which resulted in an induction of capacitation (Leemans et al., 2014). Oviduct-bound spermatozoa have a time-dependent protein tyrosine phosphorylation response that does not occur in unbound spermatozoa or when spermatozoa were incubated in oviduct explant-conditioned medium. It was previously reported that protein tyrosine phosphorylation can be induced in stallion spermatozoa using media with a relatively increased pH (González-Fernández et al., 2012; Aalberts et al., 2013). Subsequently, increased tail-associated protein tyrosine phosphorylation in oviduct epithelium-bound spermatozoa was associated with an alkaline oviductal microenvironment surrounding each oviduct explant. Secretory epithelial cells contained large, alkaline vesicles that disappeared during sperm-oviduct binding, while there was a time-dependent gradual increase in intracellular pH in oviduct-bound spermatozoa (Leemans et al., 2014). The acquisition of hyperactivated motility by spermatozoa in the peri-ovulatory period is considered in several mammalian species, such as mice and cattle, an essential step in capacitation, and this alteration in mode of motility is thought to ensure the release of bound spermatozoa from the oviduct epithelium into the lumen (Suarez, 1998). Several attempts to produce a synthetic oviduct-like medium able to induce stallion sperm capacitation, based on medium components that induce capacitation in spermatozoa from other mammalian species, were unsuccessful and did not induce hyperactivated sperm motility (Sostaric et al., 2005). Neither did the addition of glycosaminoglycans or S-S reductants to the medium induce stallion sperm hyperactivity (Sostaric et al., 2005). The media also failed to induce a release of spermatozoa from binding sites on the oviduct epithelium (Leemans et al., 2016b). Interestingly, none of pre-ovulatory and post-ovulatory oviductal fluids that were flushed from mares, 100% and 10% follicular fluid, cumulus cells, or mature equine oocytes were able to induce sperm release from oviduct explants, or induce hyperactivated sperm motility (Leemans et al., 2015b). In fact, native follicular fluid, which likely enters the oviduct during ovulation, was detrimental to sperm viability. The sperm-deteriorating component could be eliminated by heat inactivation, charcoal treatment and 30 kDa filtration, alone or in combination, indicating this component is heat sensitive, lipophilic, and has a molecular weight of 30–100 kDa. Spermatozoa in suspension containing treated follicular fluid did, however, demonstrated hyperactivated motility at a pH of 7.9 but not at a pH of 7.4. Indeed, elevated pH combined with extracellular Ca^{2+} and the heat resistant, hydrophilic, < 30 kDa component of follicular fluid induced tail-associated protein tyrosine phosphorylation, an elevated cytoplasmic Ca^{2+} concentration, and hyperactivated motility of stallion spermatozoa. Interestingly, incubation in these hyperactivating conditions induced only a limited release of oviduct bound spermatozoa (Leemans et al., 2015b). The limited sperm release, however, may be associated with avoidance of polyspermic fertilization.

3. Acquiring capacitation in vitro: molecular aspects likely leading to stallion sperm capacitation

Various aspects of stallion sperm capacitation including membrane changes, hyperactivated motility, tail-associated protein tyrosine phosphorylation and the acrosome reaction have been reviewed recently by (Leemans et al., 2019a). In this section, aspects related to the horse and not recently reviewed are discussed.

3.1. Adenylyl cyclases (ACs)

Adenylyl cyclases (ACs) are enzymes which are considered to be the major producer of cAMP (cyclic adenosine 3′,5′-monophosphate), an important second messenger discovered in 1958 (Rall and Sutherland, 1958; Sutherland and Rall 1958). The ACs have primary functions in the synthesis of cAMP by catalyzing the release of a pyrophosphate from adenosine triphosphate (ATP). The cytoplasmic effects of cAMP are mediated by protein kinase A (PKA), exchange proteins activated by cAMP (EPACs), and cyclic nucleotide regulated ion channels (Gancedo, 2013). Phosphodiesterases (PDEs) are responsible for the degradation of cAMP, thereby regulating the cAMP concentrations in the cytoplasm of the cell (Houslay, 2010; Jäger et al., 2012).

Two distinct families of ACs are described in mammalians cells with those being soluble adenylyl cyclases (sACs) and transmembrane-associated adenylyl cyclases (tmACs) (Kamenetsky et al., 2006). The sACs are present in two isoforms characterized by a single, alternatively spliced gene (AC10); a long and a short one (Buck et al., 1999). In contrast, the tmAC family includes nine genes encoding nine different isoforms (AC1–9) which have already been identified in somatic cells. The isoforms differ in structure and function, as well as in the relative abundance pattern in various tissues (Hanoune and Defer, 2001). In spermatozoa, the presence of sAC and tmAC have been confirmed in human and mouse spermatozoa (Spehr et al., 2004; Baxendale and Fraser, 2003; Wertheimer et al., 2013; Lefievre et al., 2000; Harrison et al., 2000; Tardif et al., 2004).

The tmACs are mainly regulated by heterotrimeric G proteins upon activation of G-protein-coupled receptors (GPCRs), due to the action of extracellular hormones and neurotransmitters (Sunahara et al., 2002). In contrast, sAC activity is modulated by HCO_3^- (Chen et al., 2000) and Ca^{2+} ions (Litvin et al., 2003). Furthermore, sAC activity is also sensitive to variations in intracellular ATP concentrations (Litvin et al., 2003), although proteins that might be involved in regulation of AC activity have not yet been identified.

In relation to capacitation processes, the activity of these ACs also differ from each other. It has been suggested that tmACs play a role during the mouse acrosome reaction (Wertheimer et al., 2013; Buffone et al., 2014). By contrast, production of cAMP by sAC is supposed to be required in activation of mouse, boar and bull sperm motility and hamster sperm activation and motility (Hess et al., 2005; Visconti et al., 1990 respectively), as well as in lipid-rearrangement (De Vries et al., 2003) and protein tyrosine phosphorylation (Wertheimer et al., 2013) in human spermatozoa.

Few studies about the presence and involvement of sAC in different steps of capacitation, acrosome reaction and hyperactivated motility are available for stallion sperm. It is assumed that the activation of sAC as mediated by HCO_3^- is an essential step for cAMP upregulation, thus initiating capacitation in stallion sperm (Bromfield et al., 2013). Also, in stallion sperm, capacitation-related tyrosine phosphorylation seems to be induced by sAC activation (McPartlin et al., 2011). In capacitating conditions, however, the contribution of ACs generating cAMP for the induction of hyperactivated motility is still questionable because discrete cAMP upregulation was observed under these incubation conditions without the onset of hyperactived motility (Leemans et al., 2019b).

3.2. c-AMP and the collapse of membrane asymmetry

The sperm plasma membrane is a very complex, heterogeneous and dynamic bilayer structure. The plasma membrane composition and lateral organization is responsible for the sperm's affinity for adhesion factors, solute permeability, cell signaling and fusion processes (Flesch and Gadella, 2000). This membrane is the main interface between the extracellular environment and the cytoplasm, and is a thin, flexible lipid bilayer with selective permeability to polar solutes with integral and peripheral proteins in between. It is a fluid layer due to the large proportion of long chain polyunsaturated fatty acids in the main phospholipids (Singer and Nicolson, 1972). The glycocalyx with the laterally polarized nature is also relevant for the lateral organization of the plasma membrane molecules because the carbohydrate network is in direct contact with the sperm plasma membrane via integral membrane proteins and glycolipids (Flesch and Gadella, 2000).

When there are normal conditions, the lipids are asymmetrically distributed over the lipid bilayer (Friend, 1982). In boars, phosphatidylcholine (PC), sphingomyelin (SM) and glycosphingolipids are preferentially located in the outer leaflet of the sperm plasma membrane (Gadella et al., 1999) while aminophospholipids, such as phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositols (PI) are present in the inner leaflet of the membrane. During capacitation, the plasma membrane undergoes several changes to its architecture leading to a collapse of its normal asymmetry, making it less stable and more fusogenic (Harrison, 1996). One of the early steps involved is the sAC/cAMP/PKA pathway-dependent activation of phospholipid scramblases which in turn facilitate the partial collapse of the lipid asymmetry in the plasma membrane lipid bilayer (Williamson and Schlegel, 1994; Gadella and Harrison, 2002). An increased membrane fluidity and reorganization of the lipids in the plasma membrane with the translocation of phosphatidylserine (PS) and phosphatidylethanolamine (PE) to the outer leaflet (and concomitant translocation of PC and SM to the inner leaflet) of the sperm plasma membrane was observed (Flesch and Gadella, 2000; Gadella and Harrison, 2002, Tavalaee et al., 2014). The partial collapse in membrane asymmetry associated with this initial membrane fluidity increase, with redistribution of phospholipids in the sperm head will facilitate lateral redistribution of cholesterol and cholesterol efflux from the sperm plasma membrane (Gadella and Harrison, 2000) (Fig. 2).

In the presence of capacitation factors such as HCO_3^-, supporting the increase of cellular cAMP concentrations (Gadella and

Harrison, 2000; Harrison and Miller, 2000), the increase in membrane phospholipid disorder can be observed by the fluorescent amphiphilic probe merocyanine 540 (Harrison et al., 1996). This early capacitation process has been confirmed in several species including the stallion (pig: Harrison et al., 1996; stallion: Rathi et al., 2001, Arroyo-Salvo et al., 2018; dog: Steckler et al., 2015; ram: Bernecic et al., 2021; bull: Bergqvist et al., 2006, Bergqvist et. al, 2007). The externalization of PS and PE is an important capacitation process in boars (Gadella and Harrison, 2002) and men (De Vries et al., 2003). The presence of externalized PS in viable mouse spermatozoa proved to be important in sperm-egg fusion because oocyte receptors were observed for PS (Rival et al., 2019). Currently, these processes are poorly understood in stallion sperm capacitation and will be an important subject for further research.

3.3. c-AMP and cholesterol efflux

During the sperm capacitation process, membrane proteins and lipid organization undergo marked changes (Yanagimachi, 1994; Myles and Primakoff, 1984; Harrison et al., 1996; Suzuki-Toyota et al., 2000; Cross et al., 2003; Harrison and Gadella, 2005). In the presence of capacitating factors, such as HCO_3^-, Ca^{2+} and cholesterol acceptors, a lateral redistribution of cholesterol to the apical margin of the sperm head occurs, followed by efflux of cholesterol to the extracellular environment.

In vitro capacitation-related membrane changes in boar sperm are supported by the cholesterol acceptor fatty acid free albumin and HCO_3^- (Gadella, 2008; Harrison and Miller, 2000; Flesch et al., 2001). Recently, it has been reported that there is a close association between HCO_3^-, albumin and cAMP concentrations which is essential to support capacitation processes such as cholesterol efflux in ram spermatozoa (Bernecic et al., 2019b). Under these capacitating conditions, fatty acid free albumin is able to induce a 20–40% reduction in cholesterol and desmosterol concentrations, mainly in the non-lipid raft regions of the sperm plasma membrane of boars (Boerke et al., 2013). *In vivo*, albumin resides in the female reproductive tract but it is not present in its fatty acid free form. Under these conditions, albumin has the capacity for extracting cholesterol from the plasma membrane of capacitated spermatozoa. Of more relevance, high density lipoproteins (HDLs) are present in oviductal fluid (Ehrenwald et al., 1990) and are able to facilitate reverse cholesterol transport from the sperm plasma membrane (for review see Leahy and Gadella, 2015). This has been confirmed in vitro in ram spermatozoa by Bernecic et al. (2021). Interestingly, high density lipoproteins induce both cholesterol efflux from the sperm plasma membrane and support hyperactivated motility in ram spermatozoa, whereas fatty acid free albumin did not. This indicates

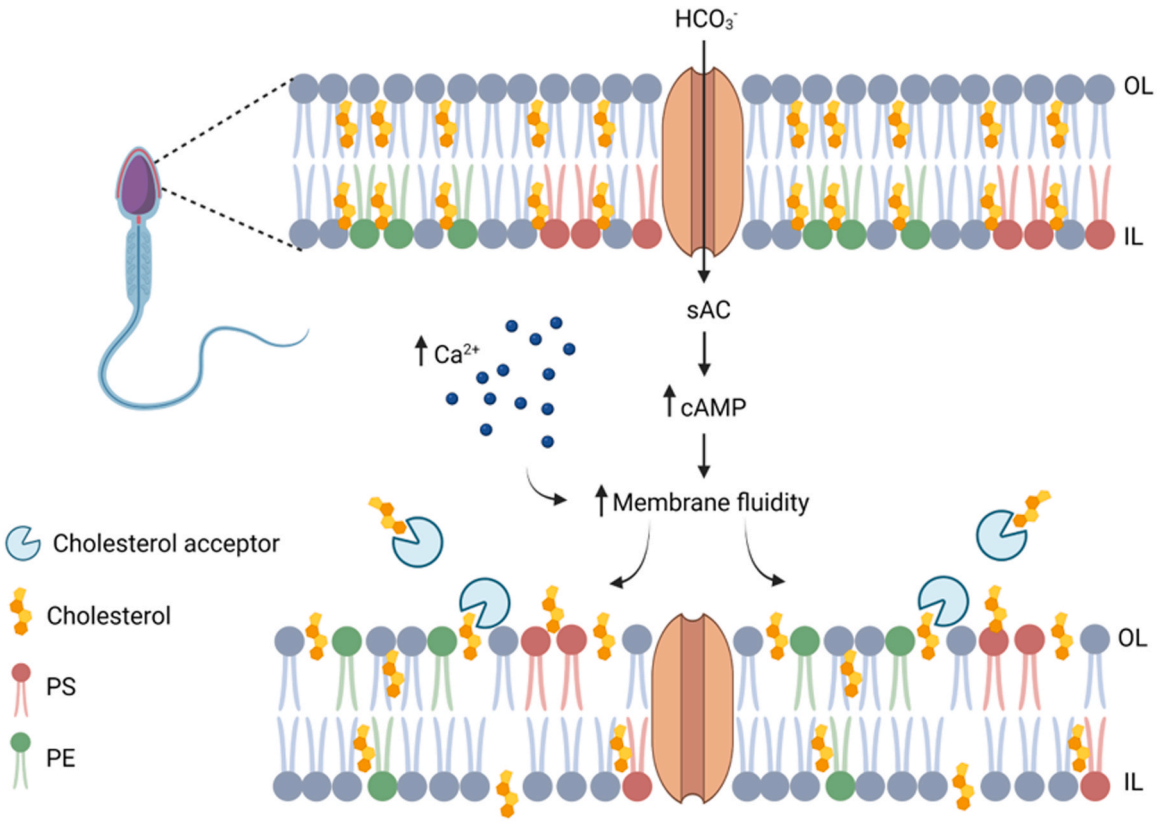

Fig. 2. Schematic overview of sperm plasma membrane remodeling during capacitation. HCO_3^- activates the sAC/cAMP/PKA pathway leading to an increase in membrane fluidity. This process is associated with a cytoplasmic Ca^{2+} increase initiating the translocation of phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the inner (IL) to the outer leaflet (OL) of the plasma membrane. Cholesterol will be more accessible for its receptor and can subsequently be extracted from the sperm plasma membrane.

that although both fatty acid free albumin and high-density lipoproteins can induce cholesterol efflux, the modes of action of these two compounds differ. It can be hypothesized that the cholesterol efflux from capacitating stallion spermatozoa is more dependent on high density lipoproteins than fatty acid free albumin. Other factors such as beta-cyclodextrins can also be used in in vitro capacitation assays. Beta-cyclodextrins have a hydrophobic-hydrophilic conformation supporting the hydrophilicity while having the capacity to bind hydrophobic compounds such as cholesterol. Careful use, however, is advised because concentrations that cause sufficient cholesterol depletion can also induce cell deterioration and death (van Gestel et al., 2005b; Leahy and Gadella, 2015).

The major function of cholesterol in the sperm plasma membrane is to promote stability of the conformational order of the membrane lipids (Leahy and Gadella, 2015). In capacitating conditions soluble adenylyl cyclase (sAC) is activated by HCO_3^- generating increased concentrations of cAMP. As a consequence, cAMP-dependent protein kinase A (PKA) is activated, resulting in cholesterol redistribution and cholesterol loss (pig: Flesch et al., 2001). A decreased plasma membrane cholesterol concentration supports membrane fluidity that facilitates aggregation of lipid-ordered microdomains or lipid rafts. These microdomains are specific arrangements of proteins and lipids contributing to sperm-oocyte interaction (Gadella et al., 2008; (Nixon and Aitken, 2009), and that have more recently been reported to contribute to the delivery of cargo from extracellular vesicles to mouse spermatozoa (Zhou et al., 2019). Caveolin-1 and flotillin-1 function as lipid raft-specific markers and accumulate in the apical part of the boar`s sperm head to fulfill a physiological function in capacitation (van Gestel et al., 2005a). Other proteins present in these lipid rafts that assist with sperm-zona pellucida binding are: angiotensin-converting enzyme and protein disulfide isomerase A6 in humans (Bromfield et al., 2016), isoforms of AQN-3 (spermadhesin), P47 (porcine homologue of SED-1), fertilin β and peroxiredoxin 5 in pigs (van Gestel et al., 2007) and the serine protease, testisin, in capacitated stallion spermatozoa (Swegen et al., 2019).

Cholesterol efflux from the sperm plasma membrane and aggregation of lipid rafts occur as a consequence of a transporter transferring cholesterol from the sperm membrane to an external cholesterol acceptor (pig: Flesch et al., 2001) and/or oxidation of membrane sterols supporting formation of oxysterols (mouse and pig: Boerke et al., 2013). The ATP binding cassette (ABC) transporters have been detected in the plasma membrane of bull (Byrne et al., 2012), dog (Palme et al., 2014) and mouse (Morales et al., 2008) spermatozoa. The localization of these transporters in the sperm head is consistent with the area of lipid rafts and cholesterol depletion in capacitated sperm (hamster: Suzuki and Yanagimachi, 1989; pig: Flesch and Gadella, 2000). The oxidation of membrane sterols, however, is a reactive oxygen species (ROS)-dependent process activated by HCO_3^- (Boerke et al., 2013). The oxidation products are oxysterols which contribute to the increase in hydrophilic characteristics and becoming more easily removed from the sperm membrane surface by cholesterol acceptors (Brouwers et al., 2011). Oxysterol formation occurs in bull (Brouwers et al., 2011), mouse and boar spermatozoa (Boerke et al., 2013). It can be hypothesized that both processes may also be required to remove cholesterol from the stallion sperm plasma membrane.

Quantification of cholesterol depletion can be performed with different degrees of difficulty and success using radioactively labelled cholesterol (Langlais et al., 1988), thin layer chromatography (Visconti et al., 1999), analysis of endogenous lipids with mass spectrometry (Flesch et al., 2001) or through the use of fluorescent dyes in flow cytometric assays (Gadella and Harrison, 2000; Bernecic et al., 2019a). Additionally, a commercially available microplate assay, i.e. the Amplex Red cholesterol assay, has been validated to quantify cholesterol depletion in stallion sperm (Macías-García et al., 2015).

Bernecic et al. (2019a) recently reported an association between cholesterol efflux (using a specific BODIPY dye) and sperm cell viability during flow cytometric analysis. These results highlight the importance of isolating only viable spermatozoa for reliable measurements of cholesterol efflux.

The relevance of cholesterol depletion in stallion sperm capacitation is unknown. *In vitro* capacitating conditions containing different concentrations of HCO_3^-, Ca^{2+} and albumin, have been examined. Cholesterol removal from the stallion sperm membrane, however, was not achieved, while varying cytoplasmic ROS concentrations were observed (Macias Garcia et al., 2015). Specifically, HCO_3^- induced an increase in ROS, which was abolished by the addition of Ca^{2+} or albumin (Macias Garcia et al., 2014). Consistent with the findings in this study, preliminary findings in our laboratory indicated the absence of cholesterol depletion in stallion spermatozoa under fatty acid free albumin capacitating conditions (data not shown). Additionally, Leemans et al. (2019b) recently reported that under similar capacitating conditions, boar spermatozoa produced markedly larger quantities of cAMP than stallion spermatozoa, however, under non-capacitating conditions no differences in cAMP concentrations were observed between the two species. If cholesterol depletion is relevant in stallion sperm capacitation, this observation may indicate that low cytoplasmic cAMP production is a limiting factor for successful cholesterol depletion from stallion spermatozoa.

3.4. cAMP and pH

Several capacitation processes in stallion spermatozoa are related to environmental pH status. Furthermore, a pH increase in the medium will induce an increase in cytoplasmic pH and facilitate protein tyrosine phosphorylation in the sperm tail (González-Fernández et al., 2012; González-Fernández et al., 2013; Leemans et al., 2015b and 2019b). Additionally, Ca^{2+}-dependent hypermotility only occurs when the medium pH is increased from 7.4 to 7.9 (Loux et al., 2013; Leemans et al., 2019b).

Interestingly, weak bases such as procaine and NH_4Cl are the most potent inducers of hyperactivated motility of stallion spermatozoa, in a Ca^{2+}-independent fashion. Instead of alkaline capacitating medium increasing only the cytoplasmic pH, weak bases act also by increasing the pH in acidic cellular organelles in a concentration dependent manner (Fois et al., 2015; Leemans et al., 2019b). Interestingly, in stallion spermatozoa hyperactivated motility was reliably induced using 2.5–10 mM procaine, whereas there was a marked cytoplasmic cAMP increase and tail-associated protein tyrosine phosphorylation at 10 mM concentration. Furthermore, 25 mM NH_4Cl did not induce the latter capacitation characteristics (Leemans et al., 2019b). Even though there were different capacitation parameters that were enhanced by increased cytoplasmic or acidic organelle pH, the acrosome reaction was not induced.

3.5. Reactive oxygen species (ROS)

The capacitated state of a sperm population is very short-lived, e.g., 1–4 h in vitro in men (Cohen-Dayag et al., 1995), due to the reactive oxygen species (ROS) generated by mammalian spermatozoa (stallion: Peña et al., 2019). Although spermatozoa are very sensitive to oxidative stress, low ROS concentrations are essential for promoting capacitation by redox regulation. A delicate balance of ROS production is extremely important during capacitation (Aitken and Drevet, 2020). Inseminated spermatozoa that are not contributing to fertilization are only protected against oxidative damage for a short time, because these spermatozoa have only a restricted amount of cytoplasm containing limited stores of antioxidants. The overlong capacitation of spermatozoa eventually results in a state of senescence and activation of an intrinsic apoptotic cascade. As a consequence, after spermatozoa have commenced capacitation these gametes become very unstable, which markedly reduces the life span of these spermatozoa (Aitken and Baker, 2004; Aitken, 2011). Species-specific effects of ROS in stallion spermatozoa have recently been reviewed by Peña et al. (2019).

Reactive oxygen species (ROS) are produced as a result of the cell`s energy metabolism (Aitken, 1995; Aitken et al., 1997). Because capacitation is a highly energy demanding process, ATP production, ROS generation and capacitation affect each other intensively (Storey, 2008). Furthermore, the production of ATP is important for several physiological processes such as motility, capacitation and the acrosome reaction and the byproducts of this process, i.e. reactive oxygen species (ROS), are also known to play a role in these capacitation signaling processes (de Lamirande and Gagnon, 1992; Griveau et al., 1995). Interestingly, oxidative phosphorylation, an aerobic energy producing process, is the most effective metabolic pathway of stallion sperm to produce ATP (Gibb et al., 2014; Gervasi and Visconti, 2016). This process occurs in the mitochondria localized in the midpiece (Storey, 2008). The most common product of ROS formation is superoxide anion (O_2^-) that subsequently is metabolized spontaneously to hydrogen peroxide (H_2O_2) or is catalyzed by superoxide dismutase (Aitken, 1995).

In various species, ROS production supported by the large energy demand are considered a significant parameter for sperm fertility (man: (De Lamirande et al., 1993; Aitken et al., 1993; mouse: Baiardi et al., 1997; horse: Baumber et al., 2003). In horses, the percentage of viable cells without oxidative damage is inversely associated with fertility. This suggests an unconventional correlation between ROS production and stallion sperm fertility. A possible explanation for this observation is that stallion spermatozoa from highly fertile stallions have a more rapid metabolism, thus resulting in an increased oxidative phosphorylation and mitochondrial membrane potential, generating larger quantities of ROS and subsequently undergoing increased lipid peroxidation (Davila et al., 2015; Akbarinejad et al., 2020; Gibb and Aitken, 2016). The sperm plasma membrane is known to contain large concentrations of polyunsaturated fatty acids (Jones et al., 1979; Wathes et al., 2007; Macías-Garcia et al., 2011) which makes stallion spermatozoa more sensitive to ROS and lipid oxidation (Storey, 1997; Gibb et al., 2014; Peña et al., 2019). Based on these observations it can be hypothesized that sperm from fertile stallions are more rapidly exhausted but will undergo sperm capacitation successfully and gain the capacity for fertilization; as summarized by Gibb and Aitken (2016), spermatozoa from highly fertile stallions 'live fast and die young'.

Whenever ROS production is excessive, plasma membrane integrity and fluidity and other capacitation processes are compromised (Ohyashiki et al., 1988; Du Plessis et al., 2015). Massive ROS production can additionally lead to the generation of cytotoxic lipid aldehydes (acrolein, 4-hydroxynonenal and malondialdehyde) from the breakdown of lipids, accelerating cell death (Aitken et al., 2012; Pizzimenti et al., 2013; Gibb et al., 2016). As a consequence, ROS production in stallion spermatozoa is commonly related to poor quality semen (Ball et al., 2001). A kinase Anchor protein 4 (AKAP4) was recently reported to be highly susceptible to adduction by 4-hydroxynonenal (4HNE) in the mouse, which resulted in a global reduction in the phosphorylation profiles following capacitation (Nixon et al., 2019). Importantly, A-kinase anchor protein 4 (AKAP4) and hexokinase 1 have been reported to be biomarkers of stallion ejaculate quality (Griffin et al., 2019).

Overall, a strictly regulated balance in capacitation-related ROS production is necessary for fertilization (Ferrusola et al., 2010). As a consequence, field studies trying to understand and reduce the major deleterious ROS effects on stallion spermatozoa caused by oxidative phosphorylation should be performed knowing that low ROS production is considered physiological in stallion sperm (Gibb and Aitken, 2016). With this perspective, the effect of the addition of antioxidants to storage or capacitation media for stallion spermatozoa has been evaluated. A few examples have been reported: L-carnitine and pyruvate, two major modulators of oxidative phosphorylation, supported improved long-term sperm storage at room temperature by reducing mitochondrial and cytosolic reactive oxygen species thereby ensuring decreased lipid peroxidation (Gibb et al., 2015). The MnTBAP (Mn(III)tetrakis(4-benzoic acid) porphyrin) supplementation to frozen-thawed stallion spermatozoa incubated in capacitating medium had a positive effect on plasma membrane integrity, membrane fluidity and ensured an increased intracellular pH (Treulen et al., 2020). D-penicillamine added to the medium during in vitro capacitation of frozen-thawed stallion spermatozoa prolonged spermatozoa`s hyperactive-like motility state (Ruiz-Díaz et al., 2020). Rosiglitazone, a molecule that diverts sperm metabolism away from oxidative phosphorylation toward glycolysis, maintained sperm motility above 60% for 6 days at room temperature (Swegen et al., 2016).

4. Conclusion

Fundamental insights into capacitation-related changes in stallion spermatozoa in the female reproductive tract during the periovulatory period are reviewed in this manuscript. It is clear that in vitro capacitating media for stallion spermatozoa should be adjusted to pH 7.9 due to the effect of alkaline pH on sperm capacitation processes, including increases in cytoplasmic Ca^{2+} and pH, tail-associated protein tyrosine phosphorylation, and hyperactivated motility. A repeatable, standardized IVF protocol has not yet been established for horses because current capacitating conditions still lack the factors required to alter sperm plasma membrane dynamics to enable induction of the acrosome reaction. It is possible that non-physiological capacitation triggers, such as fatty acid free albumin, fail to mimic the capacitation-inducing properties of high-density lipoproteins in horses. Beyond this, factors that are essential for

inducing stallion sperm capacitation may be lacking. The unidentified capacitation factors that induce capacitation are likely present in follicular and/or oviductal fluid and need to be identified so that these can be utilized to design a defined equine capacitating medium that is efficacious for conducting IVF with equine gametes.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartially of the research reported.

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