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REVIEW ARTICLE

Reproductive tract modifications of the boar sperm surface

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The sperm cell has a unique, polarized, and segregated surface that is modified extensively by the changing environments in both the male and the female reproductive tracts. The sperm cannot refresh its surface, as protein translation and membrane recycling by intracellular vesicular transport have ceased upon its maturation. So, how is the sperm surface modified in the reproductive tracts and how do these processes affect fertilization? This review traces these modifications as boar sperm travels from their liberation from the Sertoli cell into the lumen of seminiferous tubules of the testis to the site of fertilization in the ampulla of the oviduct in the sow, via an artificial insemination route. The effect of sperm dilution for artificial insemination, as well as more extensive sperm processing for in vitro fertilization, cryopreservation, or sex sorting, are also discussed with respect to how these procedures affect sperm surface organization and fertilization capacity.

KEYWORDS
epididymal maturation, fertilization, oviduct, seminal plasma, sperm surface

[A] better understanding of how the sperm surface is modified during all stages from sperm maturation to fertilization will provide better strategies to minimize sperm processing effects that currently handicap artificial insemination [in pigs].

1 | INTRODUCTION

The sperm surface is extensively modified as they travel from the testis to the site of fertilization. This review follows the voyage of the newly liberated boar sperm from its nourishing Sertoli cell into the lumen of the seminiferous tubules until ejaculation from the male genital tract, and then tracks the inseminated sperm from the cervix toward the ampulla of the oviduct, where some of them will fertilize the ovulated eggs. Throughout this journey, sperm encounter a constantly changing environment that will modify their surface and trigger changes to their behavior. For example, sperm will interact with diverse epithelial linings of both reproductive tracts, and will release or adsorb soluble components as well as extracellular vesicles that reside in reproductive tract fluids (reviewed by Gadella, 2014). Each of these natural changes at the sperm surface can influence the fertilization capacity of sperm.

As assisted reproductive technologies become more commonplace on breeding farms, particular attention must be paid to alterations of the sperm surface that may be imposed by sperm processing—including sperm dilution, washing, freeze/thaw cycle, flow cytometric sorting, and activation (for in vitro capacitation research as well as in vitro fertilization of pig oocytes). The second part of this review focuses on the impact of these artificial surface alterations on the fertilization capacity of boar sperm, and discusses the techniques that modify the sperm surface to improving their longevity and/or fertilization capacitation. For brevity and clarity, details on the specific sperm surface proteins were excluded, as they are covered in other publications (e.g., Brewis & Gadella, 2010, 2017).

2 | THE JOURNEY

2.1 | Testicular sperm morphology, with relation to the sperm surface

The process of spermatogenesis in the testis leaves sperm cells with specific morphological features that belie their ontogeny. All the major cell structures and overall organization of the sperm are predetermined during spermatogenesis, and remain essentially unaltered from the time the sperm separate from Sertoli cells. A typical sperm can be divided into a head, a mid-piece, and a tail (Figure 1) (for details see Edddy & O'Brien, 1994).

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2.1.1 | The sperm head

The dimensions of the boar sperm head are 5 by 8 by 0.8 μm (length, breadth, and thickness, respectively). This structure contains two large organelles, the nucleus and the acrosome. The nucleus encloses the male haploid genome, which is highly condensed by protamines that almost completely replace histone proteins during spermatid elongation in the testis (Ward, 2010). Super condensation of sperm chromatin into DNA toroids not only minimizes the size of the sperm head, but protects the genome against damage and allows for extreme flattening, in the case of boar sperm (thickness of 0.4 μm; the volume of the nucleus is only ~10% of that of a diploid somatic cell nucleus, which represents a fivefold reduction in volume). This minimal chromatin volume also facilitates sperm penetration of the structures surrounding the egg by allowing for compaction of the head itself. Protaminated sperm DNA is also generally silenced of gene expression by the late spermatid phase (Boerke, Dieleman, & Gadella, 2007), which is consistent with its nuclear envelope being almost completely devoid of nuclear pores (Franke, 1974).

The acrosome overlaps the apex of the nucleus (Flesch, Voorhout, Colenbrander, van Golde, & Gadella, 1998). This organelle is a condensation product of exocytotic vesicles that are shed from the trans-Golgi network, which has everted its topology toward the nucleus during spermatid differentiation, forming a large head-cap vesicle over the anterior nuclear envelope (Clermont, Hermo, Rambourg, & Thorne-Tjomsland, 1989; Martínez-Menárguez, Geuze, & Ballesta, 1996). The acrosome is a large, secretory vesicle with one continuous membrane; the part covering the nucleus is called the inner acrosomal membrane whereas the part underlying the plasma membrane is called the outer acrosomal membrane. The acrosome membrane may also contain factors involved in gamete fusion (Gadella & Evans, 2011). The lumen of the acidic acrosome (pH 5.0) is filled with a matrix of hydrolytic, glycosidic, and proteolytic enzymes (Thomas, Garner, DeJarnette, & Marshall, 1997); acrosome exocytosis releases these contents into the local environment, which is usually in the proximity of the egg. In rodents, the acrosome reaction appears to be induced by the associations with the cumulus layer (Jin et al., 2011), whereas ultrastructural analysis showed that the acrosome reaction is induced by the zona pellucida in pigs and cows (Hyttel, Greve, & Callesen, 1989; Michelmann, Rath, Töpfer-Petersen, & Schwartz, 2007; Russell, Peterson, Blumershine, & Freund, 1980). In the pig, "primary" surface binding to the zona pellucida of the egg (Russell et al., 1980; van Gestel, Brewis, Ashton, Brouwers, & Gadella, 2007) triggers the acrosome reaction, and the released enzymatic contents are involved in "secondary" binding of the sperm to the zona pellucida and in digestion of the zona pellucida itself (see Gadella, 2010, 2012). The acrosome of rodent sperm is larger and its enzyme matrix is more condensed, allowing for a slower release of enzymes compared to the pig and the cow.

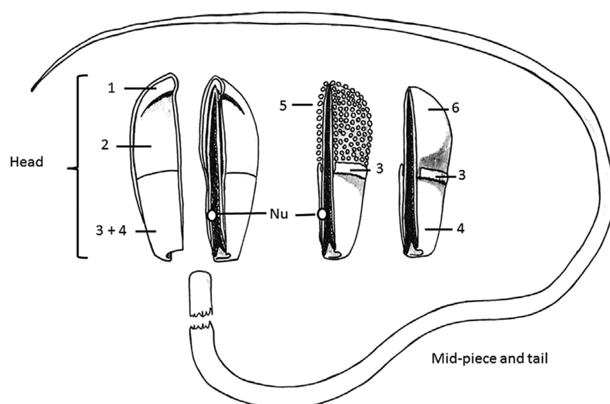

FIGURE 1 Schematic representation of pig sperm indicating the functional differentiation of domains in the sperm head. (1) The apical ridge area is the specific site of the sperm head that, after sperm capacitation recognizes, the zona pellucida; this is also where acrosome docking takes place. (2) The pre-equatorial area, together with the apical ridge, is where the plasma membrane fuses with the underlying outer acosomal membrane to form hybrid vesicles (5). (3 + 4) The equatorial and post-equatorial sperm plasma membrane do not fuse with intracellular membranes. After the acrosome reaction (3), the equatorial segment is formed, which is specifically involved in the binding and fusion of sperm with the egg (see Figure 2). Note that the inner acrosomal membrane (6) takes over the function of the plasma membrane (1 + 2), when the hybrid vesicles are removed from the zona pelludica-penetrating sperm (acrosomal shrout). The inner acrosomal membrane is connected to the equatorial segment by a small portion of non-fused outer acrosomal membrane that forms a hairpin structure (see text for further details)

2.1.2 | The sperm mid-piece

The mid-piece is the nucleus-adjacent portion of the flagellum, and contains a battery of some 100 mitochondria that are coiled around the flagellum; by contrast, the sperm head and distal portion of the flagellum do not contain mitochondria. The mitochondria in the mid-piece are involved in aerobic metabolism, and the ATP produced is consumed both for sperm homeostasis (for instance, for maintaining the Na^+/K^+ ion gradient) as well as for sperm motility. After fertilization, the egg selectively eliminates these sperm mitochondria (Song, Yi, Sutovsky, Meyers, & Sutovsky, 2016), which could be in response to their lower or absent mitochondrial DNA copy numbers (Luo et al., 2013).

2.1.3 | The sperm tail

The sperm tail is the region of the flagellum not covered by coiled mitochondria, and is the only part of the sperm in which no intracellular organelles are present. With the exception of the end piece, a large portion of the tail is covered by a fibrous sheath and outer dense fibers. The tail is present exclusively for sperm movement. Microtubules in the sperm tail efficiently slide over each other, resulting in bending or stretching of the tail. Tail movement is an energy-consuming process, fed by ATP aerobically produced in the mitochondria of the mid-piece or by glycolysis in the tail, which occurs in the fibrous sheath (Feiden, Stypa, Wolfrum, Wegener, & Kamp, 2007; Westhoff & Kamp, 1997). The principal piece of the sperm tail possesses elastic properties required for the forward-propelling movement of ejaculated boar sperm.

2.1.4 | Cytoplasmic droplet

Sperm newly liberated from the apical surface of Sertoli cells contain a cytoplasmic droplet at the neck region, a remnant of the cytoplasmic

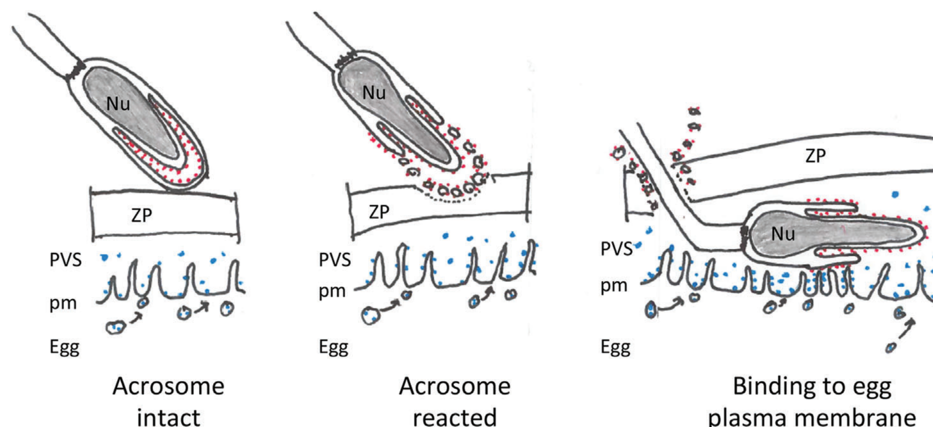

FIGURE 2 Possible sources of adsorbed proteins at the equatorial segment of sperm. The middle panel depicts sperm bound to the zona pellucida (ZP), which is undergoing the acrosome reaction. The intra-acrosomal proteins (red) may diffuse toward the equatorial segment. The right panel depicts a sperm cell that has complete zona pellucida penetration, and now resides in the perivitelline space (PVS), where it can interact with the egg plasma membrane (pm) and proteins within this space (blue). Whether or not these hypothesized surface rearrangements at the equatorial segment take place and/or functional fertilization-related protein(s) is(are) added is not known (discussed by Gadella & Evans, 2011). Nu, sperm nucleus. The figure is based on a schematic from Ito et al. (2010)

bridges that retained a group of spermatids in a syncytium to synchronize their development and differentiation. This cytoplasmic droplet is removed during epididymal maturation (see section 2.3).

2.2 | General aspects of the sperm cell

The sperm retains a minimal volume of cytosol that is devoid of other organelles. This exclusion of cytoplasm has three important consequences for the sperm: (i) The sperm head does not contain ribosomes or rough endoplasmic reticulum, so no de novo protein synthesis occurs (Boerke et al., 2007); (ii) no vesicle-mediated transport occurs, so exocytosis or endocytosis are absent (Flesch & Gadella, 2000); and (iii) the sperm surface is always in close proximity to intracellular structures such as the acrosome, the nuclear envelope, the mitochondria, and the fibrous sheath (Flesch, Brouwers, et al., 2001; Peterson & Russell, 1985). The first two features imply that surface recycling and renewal of intracellular and intrinsic membrane proteins does not occur efficiently in mature sperm, therefore sperm proteins that originated from the testis remain at the surface for a long time (>2 weeks) and damaged or defective proteins remain at the sperm surface. By contrast, somatic cells utilize surface recycling by vesicle-mediated transport and de novo protein production to constantly renew the cell surface and to remove defective proteins by sorting them to lysosomal or proteasome degradation pathways. The third feature, together with the highly polarized structure of sperm, define discrete surface domains based on specific organelle or cytoskeletal interactions underlying the sperm plasma membrane.

A newly liberated sperm in the seminiferous tubule lumen displays a clearly segregated sperm surface, based on the distribution of surface proteins (James, Wolfe, Ladha, & Jones, 1999). A tight-junction-like protein structure called the distal ring, located between the sperm head surface and the mid-piece surface, prevents the mixing of membrane components from and to the sperm head, while a protein structure called the annular ring segregates the surface of the mid-piece with the distal tail. Each of the separated sperm surface domains overlying the head, mid-piece, and tail are further subdivided (Edddy & O'Brien, 1994; Peterson & Russell, 1985). The boar sperm head surface, for instance, contains an apical surface involved in zona pellucida recognition; a pre-equatorial surface involved in the zona-induced acrosome reaction; an equatorial surface that forms after the acrosome reaction, called the equatorial segment, where specific gamete fusion occurs; and the post-equatorial surface, which does not have a direct function in fertilization but may indirectly participate in organizing the above interactions (Figure 1). The function and possible reorganization of surface subdomains in the mid-piece and tail are not well-studied.

2.3 | Epididymal maturation

Testicular sperm are unable to fertilize an egg. This is partly due to the presence of the cytoplasmic droplet, which can be seen as a clump foot attached to the sperm that hinders efficient sperm transit through the extracellular vestments of the egg (i.e., the cumulus mass and zona pellucida). More importantly, testicular sperm is immotile (Dacheux, Castella, Gatti, & Dacheux, 2005) and lacks surface factors and protein complexes that are required for fertilization (Burkin & Miller, 2000).

Released testicular sperm must be transported through the rete testis and into the epididymal duct for maturation. Sperm maturation in the epididymis takes approximately 2 weeks in boars, and transits the sperm form caput to cauda regions of this organ (Dacheux et al., 2005). Substantial rearrangement of the sperm surface is accomplished during this process, resulting in (i) removal of the cytoplasmic droplet; (ii) a change in metabolism, which allows for sperm to acquire motility; and (iii) removal and adsorption of peripheral proteins on the sperm surface (Guyonnet et al., 2011), which are particularly relevant to fertility as proteins involved in zona pellucida recognition originate from the epithelial lining of the epididymis (Kuo, Li, Maeda, Gadella, & Tsai, 2016; van Gestel et al., 2007). Sperm in the cauda epididymis are able to fertilize an egg in vitro (Matás et al., 2017), which indicates their state of maturation. However, epididymal boar sperm are highly sensitive to the female genital tract, especially the cervix, making them too fragile to

reach the oviduct in vivo and too fragile for use in in vitro fertilization (Harkema, Colenbrander, Engel, & Woelders, 2004).

2.4 | Seminal plasma

During ejaculation, mature epididymal sperm are pumped past the vas deferens and through the remaining male genital tract. Secretions of the seminal vesicles, the bulbo-urethral gland, and the prostate dilute the sperm population, while providing metabolic substrates, ions, and factors that stabilize the gametes (Harkema et al., 2004). Some of these seminal proteins adsorb to the sperm surface, dampening premature sperm activation (Johnson, Weitze, Fiser, & Maxwell, 2000; Leahy & Gadella, 2011a, 2011b; Maxwell & Johnson, 1999), which normally occurs in the oviduct during a process known as capacitation (Gadella, 2008a, 2008b; Gadella & Boerke, 2016). The sperm-stabilizing factors in the seminal plasma, which are also known as decapacitation factors, form a surface-stabilizing extracellular coating that is retained during ejaculation (Kawano, Ito, Kashiwazaki, & Yoshida, 2010). The factors coating the sperm as well as those free in seminal plasma may be associated with male fertility (Strzeżek et al., 2005).

Seminal plasma also affects sperm passage through the uterus as they proceed toward the oviduct (Abney & Williams, 1970; Rath, Knorr, & Taylor, 2016). Entry and residence within the oviduct, however, results in the loss of these male-derived, stabilizing surface factors (Calvete & Sanz, 2007), thus re-exposing the epididymal proteins that are required for interacting with and penetrating the cumulus matrix, the zona pellucida, and the egg (Boerke, Tsai, Garcia-Gil, Brewis, & Gadella, 2008; Gadella, 2008b; Gadella & Van Gestel, 2004; Tsai & Gadella, 2009).

2.5 | Dilution for artificial insemination

Natural insemination presently being overtaken by artificial insemination on pig breeding farms. In general, young male pigs are held in separate stables for initial selection based on desired genotype and phenotype. Further selection takes place after puberty, based on the boars' health (absence of pathogens) and sperm production (quantity as well as quality). Only high-performing boars are selected as artificial insemination donors, which requires an average ejaculate collection frequency of 2–3 times a week. In the Netherlands, predominantly sperm-rich fractions are collected, followed by filtration through gauze to remove the gel fraction that is secreted by the bulbourethral gland. The sperm are then diluted 5- to 10-fold with an isotonic dilution buffer (originally marketed as Beltsville Thawing Solution) to 200–400 million sperm per milliliter, slow-cooled to 17°C, and kept at that temperature in 80-ml portions for insemination for up to 2 days (Broekhuijse, Feitsma, & Gadella, 2012). Insemination is performed with a pipette that is placed in the cervix (Broekhuijse et al., 2012). Although the exact effect of sperm dilution and cooling has not been investigated, they may affect the organization of the sperm surface (Leahy & Gadella, 2011a, 2011b). Media and cooling protocols other than based on Beltsville Thawing Solution are used—for instance, special media for extended semen storage—and may differentially affect sperm, but are not reviewed here. Ultimately, the dilution medium reduces the concentration of seminal components and decapacitation factors associated with the ejaculated sperm; the lower temperature likely reduces lateral diffusion of membrane components. Both may influence the function and integrity of the sperm surface. Nevertheless, routine pig artificial insemination yields larger litters and higher non-return rates of sows compared to natural mating (Broekhuijse et al., 2012).

2.6 | Cervix/uterus

The vast majority of sperm inseminated into the cervix will not enter the site of fertilization. Part of the inseminate rapidly drains from the female genital tract by back flow (note that the floor of the pig stable will be contaminated with fluids containing high levels of wide-spectrum antibiotics, which increases the probability that multi-resistant bacteria will be generated at the breeding farm); most of the remaining sperm are eliminated by slower drainage from the cervix or by phagocytosis with the cervix or uterus (Hawk, 1983; Katila, 2012; Matthijs, Engel, & Woelders, 2003). Transport of sperm through the cervix and uterus, however, is fast, allowing for the rapid removal of unbound seminal plasma factors from the sperm surface.

Reduction in sperm quantity may be a consequence of simple dilution or an active selection process that favors a superior subpopulation, thus removing aberrant or dysfunctional sperm. A largely reduced, but apparently fixed, number of sperm reach the tip of the uterine horn, the utero-tubal junction, and the oviduct within 1 hr post-insemination (Hawk, 1983). This efficient rate of sperm transport is made possible by smooth muscle contractions and ciliary beating within the female reproductive tract, resulting in the accumulation of a sperm reservoir at the tip of the uterine horn at the utero-tubal junction within 1 hr following insemination; only a tiny portion of this reservoir population will enter the oviduct (Bracken, Safranski, Cantley, Lucy, & Lamberson, 2003; Brüssow, Egerszegi, & Rátky, 2014; Langendijk et al., 2002; Mburu, Einarsson, Lundeheim, & Rodriguez-Martinez, 1996, Mburu, Rodriguez-Martinez, & Einarsson, 1997; Sumransap, Tummaruk, & Kunavongkrit, 2007). Of the 1.5 to 3 billion inseminated sperm, approximately 0.1–0.3 million sperm make up the sperm reservoir at the utero-tubal junction (Langendijk, Soede, & Kemp, 2005), and some 2,000 sperm will enter the oviduct; similar quantities of sperm were measured within the sperm reservoir and the oviduct when low-dose intra-uterine insemination is performed (Sumransap et al., 2007; Tummaruk, Sumransap, Techakumphu, & Kunavongkrit, 2007). These consistent numbers suggest that sperm residing at the utero-tubal junction and the oviduct have actively escaped cervix and uterine interactions, as opposed to being passively transported, and that sperm interact with or adsorb secreted factors from the cervix or the uterus, and likewise with the mucous/epithelial cells of the uterus and/or with infiltrating leukocytes entering the uterine fluid (Taylor, Rath, Zerbe, & Schuberth, 2008; Taylor, Schuberth, et al., 2009; Taylor, Zerbe, Seyfert, Rath, Baulain, et al., 2009; Taylor, Zerbe, Seyfert, Rath, Schuberth et al., 2009).

The various interactions that occur between sperm and the environment of the female reproductive tract prepare the sperm surface for fertilization and/or to reduce the quantity of sperm that

reach the oviduct (reviewed by Rath et al., 2016). Comparison of changes observed from in vitro or ex vivo experiments or materials flushed from the uterus with what is observed in vivo within the uterus (Taylor et al., 2008; Taylor, Schuberth, et al., 2009; Taylor, Zerbe, Seyfert, Rath, Baulain, et al., 2009) are needed to determine if these interactions are at all involved in sperm selection. One challenge, however, is keeping uterine epithelial cells and leukocytes obtained from uterine fluids in functionally unaltered states. One observation that supports an active selection process requiring the interaction of the sperm surface with the epithelium is that release of sperm bound at the sperm reservoir appears to depend on hormonal changes peri-ovulation (Mburu et al., 1997).

2.7 | Oviduct

Sperm that enter the oviduct bind to cilia of ciliated epithelial cells (Mburu et al., 1997; Suarez, Redfern, Raynor, Martin, & Phillips, 1991). After a certain period of time, especially around ovulation, sperm are liberated and exhibit characteristics of capacitation, such as hyper-activated motility, allowing them to reach the ampulla, where they can fertilize the just-ovulated egg (Talevi & Gualtieri, 2010). Secretory cells of the porcine (Hunter, Petersen, & Greve, 1999) and bovine (Sostaric et al., 2008) isthmus and ampulla are stimulated to secrete factors around the time of ovulation, and these factors are thought to take part in the release and activation of sperm. Most of the knowledge on sperm capacitation, zona pellucida binding, and induction of the acrosome reaction are based on in vitro studies, as is detailed below.

2.7.1 | 1. Induction of surface changes by in vitro fertilization media that resemble oviduct fluid

Pig in vitro fertilization protocols are mostly derived from human and bovine protocols. Sperm is diluted and pipetted on a density cushion (often a discontinuous Percoll gradient), and centrifuged to remove immature sperm, epithelial cells, and white blood cell contamination as well as seminal plasma and loosely associated peripheral membrane proteins from the mature sperm (Gadella & Luna, 2014). The loose pellet containing the mature sperm is then diluted with synthetic oviduct fluid, a medium that resembles the ionic and metabolite composition of the oviduct—i.e., >15 mM bicarbonate, >1 mM Ca^{2+}, and delipidated albumin (Boerke et al., 2013; Gadella & Boerke, 2016). When incubated at 38.5°C, 5% CO_2 for 2 hr in this medium, sperm will show signs of capacitation and will then be primed to fertilize an egg.

The three capacitation factors elicit synergistic effects on sperm: Bicarbonate enters the cell via a Na^+/HCO_3^- symport transporter (Gadella & Visconti, 2006), and binds at a 1:1 stoichiometry to soluble Adenylate cyclase (sAC), thus activating it for cyclic adenosine monophosphate (cAMP) production (Okamura, Tajima, Soejima, Masuda, & Sugita, 1985). Enhanced cAMP levels in turn activate a cAMP-dependent Protein kinase A (PKA) (Gadella & Luna, 2014), which provokes tyrosine phosphorylation of proteins (Flesch, Colenbrander, van Golde, & Gadella, 1999; Gadella & Visconti, 2006) and stimulates glycolysis and hyper-activated motility in the sperm tail (Eddy, Toshimori, & O'Brien, 2003). PKA activation and its down-stream signaling also cause a rapid increase in membrane fluidity and a rearrangement of membrane proteins and lipids in the sperm head (Harrison & Gadella, 2005), which are Ca^{2+}-dependent responses. A slower response is the induction of reverse cholesterol transport to a cholesterol-accepting factor and a concomitant lateral rearrangement of sperm surface components (Boerke et al., 2008; Flesch, Brouwers, et al., 2001; Gadella, Gadella, Colenbrander, van Golde, & Lopes-Cardozo, 1994; Gadella, Lopes-Cardozo, van Golde, Colenbrander, & Gadella, 1995; Gadella, Tsai, Boerke, & Brewis, 2008; Tsai & Gadella, 2009; van Gestel, Brewis, et al., 2005). One cholesterol-accepting factor within the oviduct is high-density lipoprotein (HDL), but for in vitro fertilization HDL can effectively be replaced in synthetic oviduct fluid with either bovine serum albumin or cyclodextrin (Leahy & Gadella, 2015; Salmon, Leclerc, & Bailey, 2016; van Gestel, Helms, Brouwers, & Gadella, 2005). The outcome of bicarbonate, Ca^{2+}, and albumin on sperm is the removal of approximately 20–40% of its cholesterol and a redistribution of sphingomyelin and cholesterol-enriched lipid-ordered membrane micro-domains from the plasma membrane overlying the acrosome to the acrosome ridge area of the sperm head (Boerke et al., 2008; Tsai et al., 2007; van Gestel, Brouwers, Ultee, Helms, & Gadella, 2016; van Gestel, Helms, et al., 2005).

During cholesterol depletion, both the apical ridge plasma membrane effectively dock with the outer acrosome membrane by the stable formation of multiple trimeric soluble *N*-ethhylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (Tsai, Brewis, van Maaren, & Gadella, 2012; Tsai et al., 2007; Tsai, van Haeften, & Gadella, 2010) and, in the same surface area, the formation of a zona pellucida-recognizing protein complex along the extracellular surface of the apical head (Flesch, Wijnand, et al., 2001; Gadella, 2008a, 2010, 2012; van Gestel et al., 2007). These changes partly depend on mild lipid peroxidation of cholesterol and desmosterol (Boerke et al., 2013; Brouwers et al., 2011; Brouwers, Silva, & Gadella, 2005) and activation of phosphatidylionsotol-specific phospholipase C (PI-PLC), which removes some of the glycosylphophatidylinositol (GPI)-anchored proteins and thus promotes the lateral reordering of membrane components (Boerke et al., 2014) that allows for acrosome docking and/or formation of the zona pellucida-recognition protein complex (Gadella, 2014). Sperm binding to the zona pellucida induces an influx of Ca^{2+} from outside the sperm, thus initiating the Ca^{2+}-dependent reconfiguration of the *trans-* to *cis-* SNARE complexes that fuses the sperm plasma membrane and acrosomal outer membrane (Tsai et al., 2012). This results in a sperm that has lost its apical plasma membrane and the apical region of the outer acrosomal membrane; the hybrid vesicles that result from acrosome fusion are also referred to as the acrosomal shroud, and are shed from the sperm head (see Figures 1 and 2).

The acrosome-reacted sperm cell actively penetrates the zona pellucida by concerted action of drilling movement (hyperactivated motility due to the increased ATP production by the elevated glycolytic activity) and activity of the enzyme matrix from the acrosome on the constituents of the zona pellucida. Penetration of the zona pellucida binding allows the sperm to reach the perivitelline space, where it can interact with the ooplasma via its remaining plasma membrane surrounding the tail as well as the distal and equatorial surface. The plasma membrane at the equatorial region is connected to the remainder of the outer acrosomal membrane and to the inner

acrosomal membrane in a hairpin-like shape; these membranes must now perform the function of separating the cellular and extracellular environments. The equatorial segment also contains proteins that recognize, bind, and fuse with the oolemma (Gadella & Evans, 2011). Which protein(s) is (are) directly involved in gamete fusion is not clear, nor is whether functional exposure of such membrane fusion proteins requires proteins or protein complexes at the equatorial segment, additional membrane proteins from the acrosome membranes adjacent to the equatorial segment, or/and proteins from the oolemma or the perivitelline space (Figure 2) (Ito et al., 2010).

The primary result of gamete fusion is the egg's uptake up of the entire sperm, including the introduction of a soluble sperm factor, phospholipase Cζ, that activates the second meiotic division, enabling zygote formation, and embryo development. Phospholipase Cζ also activates a polyspermy block by stimulating calcium waves and the cortical reaction (Swann & Lai, 2016). Nevertheless, polyspermy is a major problem during pig fertilization, occurring at a higher rate for gilts compared to adult sows, and at a rate of >30% in in vitro-matured oocytes used for in vitro fertilization (Davis, 2004; Tsai et al., 2011). In vitro fertilization protocols are therefore not commercially used for the pig. Additionally, since polyspermic zygotes often arrest at early cleavage stages and a minimum number of pig embryos must be recognized maternally and must implant to achieve pregnancy, pregnancy failure rates tend to be higher following the transfer of in vitro fertilized zygotes or mated gilts. This outcome compromises litter sizes for efficient and economic meat production, so improvements must be made before in vitro fertilization becomes routine on pig farms (Romar, Funahashi, & Coy, 2016).

2.7.2 | Effect of sperm processing using oviduct fluid or oviduct explants

The relatively high incidence of polyspermic fertilization during in vitro fertilization prompted researchers to study how the oviduct affects the incidence of polyspermy. The group, led by Coy, established that exposure to oviduct fluid or allowing sperm to passage for some time either in the oviduct, in oviduct explants, or oviduct cultures significantly reduces the incidence of in vitro polyspermic fertilization (Coy, Cánovas, et al., 2008; Coy, Grullon, et al., 2008; Canovas, Romar, Grullon, Aviles, & Coy, 2009; Mondéjar, Avilés, & Coy, 2013; Romar et al., 2016). This implies that sperm-oviduct interactions and the presence of bioactive components in the oviduct fluid are able to suppress polyspermy. For this reason, we are establishing an oviduct-on-chip system for the pig, similar to what we made for the cow (Ferraz et al., 2017; Ferraz, Henning, Stout, Vos, & Gadella, 2016) and the mare (Leemans et al., 2016), in order to develop a better mimic of the oviduct. This will ultimately produce better embryos as well as reduce polyspermic fertilization and the incidence of parthenogenic activation.

3 | SPERM PROCESSING

The sperm surface is clearly a dynamic environment under normal in vivo circumstances, and human interference imposes additional stresses on it. Indeed, dilution of ejaculated sperm in Beltsville Thawing Solution or related sperm extenders, followed by cooling down to 17°C, likely affect sperm—yet these changes are do not ultimately influence artificial insemination litter sizes. Additional handling must be performed to ship or to select sperm (for sex, for example), which can further impact sperm performance. For example, cryopreservation of sperm involves mixing the gametes with milk and egg yolk lipid vesicles plus combinations of cryoprotectants, transferring the mixtures to straws, cooling them further using a computer-controlled traject, and then plunging these straws into liquid nitrogen—only to be thawed and separated from the cryoprotectants before insemination (Benson, Woods, Walters, & Critser, 2012; Yeste, 2016). During sex sorting, diluted sperm are incubated with a viability stain and a DNA stain, processed through a flow cytometer in which the sperm head is probed as it passes through an ultraviolet laser beam, and sorted for an X or Y chromosome based on total DNA content in the sperm head. The process also requires that sperm pass through a piezo electric crystal that forms air droplets for deflection and sorting into collection tubes, at a speed of 70 km/hr (Rath et al., 2016). Thus, methods that reduce the potential physical damage are needed to improve the collected sperm. Also, whether or not X and Y sperm carry different surface proteins is not certain (Almiñana et al., 2014; Hendriksen, Welch, Grootegoed, Van der Lende, & Johnson, 1996). If surface differences do exist between X and Y chromosome-bearing sperm, then much less stressful immune-conjugated paramagnetic bead selection could be implemented for sorting.

Both cryopreserved as well as sex-sorted sperm may have commercial advantages, such as carrying superior genetic information or a preferred sex chromosome, but both methods lead to severe membrane stress (Leahy & Gadella, 2011b). Such stress often mimics the outcome of capacitation—indeed, the sperm phenotype following cryopreservation has been called "cryocapacitation" (Salmon et al., 2016)—and leads to lower fertilization potential. This can be explained, in part, by the fact that the membrane changes imposed by sperm processing are irreversible and do not allow the proper sequence of steps required for fertilization to occur (van Gestel, Helms, et al., 2005). Also, the oviduct or in vitro fertilization environment required to capacitate fresh sperm may be too hostile for more-fragile, processed sperm, leading to premature cell deterioration. Thus, stabilizing the sperm surface prior to processing, thereby limiting destabilization to only after insemination or in vitro fertilization, will be an important achievement for increasing the efficiency of assisted reproduction for pigs (a thorough discussion can be found in Leahy & Gadella, 2015). Some approaches that are promising toward meeting this goal include freezing sperm with stabilizing seminal factor components or with cholesterol-enriched albumin or cyclodextrin (Salmon et al., 2016; Tomás, Blanch, Cebrián, & Mocé, 2013); such approaches still need to be tried with sex-sorted boar sperm.

4 | CONCLUSION

A complex series of surface modifications occur over the 2 weeks that sperm resides in the rete testis and the male genital tract, and during the <24-hr period between sperm ejaculation into the cervix and their progress through the female genital tract to the egg in the ampulla of the

oviduct. Current protein identification, membrane biochemistry, and cell biology techniques have provided insights to some of the membrane processes required for in vitro fertilization, but a reliable in vitro system that mimics the pig oviduct is not yet at hand, so the high incidence of polyspermic fertilization and the demand for large litter size are preventing the commercial feasibility of in vitro fertilization approaches. Sperm processing during sperm cryopreservation and/or sex sorting likely impacts the sperm surface, which also lowers fertilization potential of the processed sperm. Thus, a better understanding of how the sperm surface is modified during all stages, from sperm maturation to fertilization, will provide better strategies to minimize sperm-processing effects that currently handicap artificial insemination and/or future applications of in vitro fertilization in the pig.

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