The physiological roles of the boar ejaculate

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During ejaculation in the boar, sperm cohorts emitted in epididymal cauda fluid are sequentially exposed and resuspended in different mixtures of accessory sex gland secretion. This paper reviews the relevance of such unevenly composed fractions of seminal plasma (SP) *in vivo* on sperm transport and sperm function and how this knowledge could benefit boar semen processing for artificial insemination (AI). The firstly ejaculated spermatozoa (first 10 ml of the sperm-rich fraction, SRF [P1]) remain mainly exposed to epididymal cauda fluid and its specific proteins i.e. various lipocalins, including the fertility-related prostaglandin D synthase; than to prostatic and initial vesicular gland secretions. P1-spermatozoa are hence exposed to less bicarbonate, zinc or fructose and mainly to PSP-I spermadhesin; than if they were in the rest of the SRF and the post-SRF (P2). Since the P1-SP is less destabilizing for sperm membrane and chromatin, P1-spermatozoa sustain most *in vitro* procedures, including cryopreservation, the best. Moreover, ejaculated firstly, the P1-spermatozoa seem also those deposited by the boar as a vanguard cohort, thus becoming overrepresented in the oviductal sperm reservoir (SR). This vanguard SR-entry occurs before the endometrial signalling of SP components (as PSP-I/PSP-II and cytokines) causes a massive influx of the innate defensive PMNs to cleanse the uterus from eventual pathogens, superfluous spermatozoa and the allogeneic SP. The SP also conditions the mucosal immunity of the female genital tract, to tolerate the SR-spermatozoa and the semi-allogeneic conceptus. These *in vivo* gathered data can be extrapolated into procedures for handling boar spermatozoa *in vitro* for AI and other biotechnologies, including simplified cryopreservation.

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Introduction

Modern pig production is vertically integrated, with breeding done through AI. This first-line, easy, practical and highly effective biotechnology serves two major purposes (i) avoid spreading venereal (or otherwise semen-shed) diseases and, (ii) maximise dissemination of genetic material to large numbers of sows. With proper detection of oestrus (twice daily) and AI correctly performed (80-100 mL, cervical, 2-3 times/oestrus) with high-quality semen (> 2 x 10^9 motile spermatozoa), > 90% of farrowing rates and mean litter sizes of 12–14 piglets are reached, comparable with those achieved with natural mating (reviewed by Rodríguez-Martínez 2007a). With such results, it is not surprising that pig AI became an essential breeding tool, increasing worldwide from ∼ 7% at the start of the 1980s (Reed 1985) to more than 80% 20 years later (Wagner & Thibier 2000). In the European Union (EU) ∼ 84% of all sows/gilts are bred by AI (Feitsma, personal communication).

Improvements in the design of media, AI-catheters and moment of AI, have led to > 99% of all AIs done around the world being made with liquid semen (Wagner & Thibier 2000). Frozen-thawed (FT) semen is, however, rarely used (∼ 1%, Thibier, personal communication), since cryopreservation is still considered "quasi experimental". However, breeding enterprises are interested in further developing cryopreservation, since FT-semen could be used in situations in which the widely used liquid semen can not, such as the international exchange of genetic lines without transporting livestock, the long-term conservation of superior genetic individuals in genetic resource banks, or the testing for presence of pathogens before use. Yet, boar spermatozoa show low cryosurvival (∼ 40%) and a shortened lifespan among surviving spermatozoa. This obviously leads to lower farrowing rates and smaller litter sizes compared to liquid semen (reviewed by Rodríguez-Martínez 2007a). Moreover, cryopreservation procedures are cumbersome, time-consuming and yield few doses/ejaculate, all of which deter from its wider use.

Ongoing research aims to widen AI-use, by decreasing sperm numbers per AI-dose (increasing extension), designing novel media (chemically-defined) or quicker cryopreservation methods, and implementing alternative procedures for semen deposition (intra-uterine). Despite gains thus far (rev by Rodríguez-Martínez 2007a), innate characteristics of the boar ejaculate such as the specifics of the sperm structure and of the seminal plasma (SP), yet hinder full success. The SP is not just a sperm vehicle, but as relevant modulator for sperm function, for sperm transport post-breeding and as inductor of both innate and adaptive immunological responses by the female that would ensure reproductive success. Considering the current focus for novel semen extension and sperm treatments for AI, the present paper attempts to review aspects of the characteristics of the boar ejaculate in relation to sperm transport in the female genitalia *in vivo*, and how this knowledge could benefit boar semen processing for AI.

Ejaculation in the pig

Ejaculation is a highly coordinated physiological process involving neurological and muscular events that build two distinct phases; (i) emission (the formation and deposition of semen [spermatozoa and seminal fluid] in the urethra and (ii) the forthcoming ejection of the semen through the penis urethra (the so-called expulsion, or ejaculation proper). In boars, emission and ejaculation repeat as waves for 5-10 min, during which the complete ejaculate (250-300 ml in a mature boar) is sequentially verted into the female cervix lumen or it is manually collected *ex-corpore* into a recipient (Senger 2005). In the latter case, waves can be regarded by the operator as a sequence of three major fractions. These fractions are classically called *pre-sperm* (PSF, with a clear seminal fluid, some gel and a heavy degree of contamination of cell debris, urine and smegma from the preputium), *sperm-rich* (SRF, easily recognised by its creamy-white colour) and *post sperm-rich* (PSRF, that goes from greyish to watery

in aspect) and whose increasing amounts of "tapioca-like" flocula, signals the end of the ejaculation process, accompanied by a fading of the penile erection. Interestingly, a second ejaculation sequence can be manually stimulated with brief, firm, pulsating hand pressure applied to the penis, upon which a new, but smaller, SRF is often collectable (Mann & Lutwak-Mann 1981).

The ejaculate of the boar

The ejaculate is a sperm suspension ($\sim 50-90\times10^9$ in 9 month-old boars to $\sim 70-110\times10^9$ when they reach maturity, i.e. after 12 months of age; Flowers 2008, Wallgren, unpublished results) in a SP composed of the mixture of the contents of the tails of the ductus epididymides and the secretions of the accessory sexual glands. The latter vary in content, volume and occurrence of excretion, building fractions of different composition. The PSF-SP contains mainly secretion of the urethral and bulbourethral glands, as well as of prostate; the SRF-SP is a blend SP where the emitted epididymal fluid in which spermatozoa (see Fig. 1) originally bathe, is diluted in vesicular gland and prostate secretions. Lastly, the PSRF-SP suspends few spermatozoa, being a fluid primarily derived from the increasing secretion of the vesicular glands, the prostate and, by the end of the ejaculation, the bulbourethral glands (Einarsson 1971, Mann & Lutwak-Mann 1981). The latter delivers a tapioca-like flocular secretion that coagulates in contact with the vesicular fluid the SP, as seen when the entire ejaculate is collected in an open recipient. The role of this process is, *in vivo*, to retain the ejaculate *in utero*, minimizing the transcervical backflow commonly seen at AI with liquid semen, where the gel component is consistently filtered away (Viring & Einarsson 1981). Spermatozoa are, therefore, ejaculated with a maximum peak concentration in the first portion of the SRF (i.e. the first 10–15 ml, called Portion 1 or P1, an easily collectable portion; Rodriguez-Martinez *et al.* 2005, Fig. 1), decreasing thereafter in numbers along this fraction to virtually disappear by the PSRF alongside with increasing secretion of the vesicular glands.
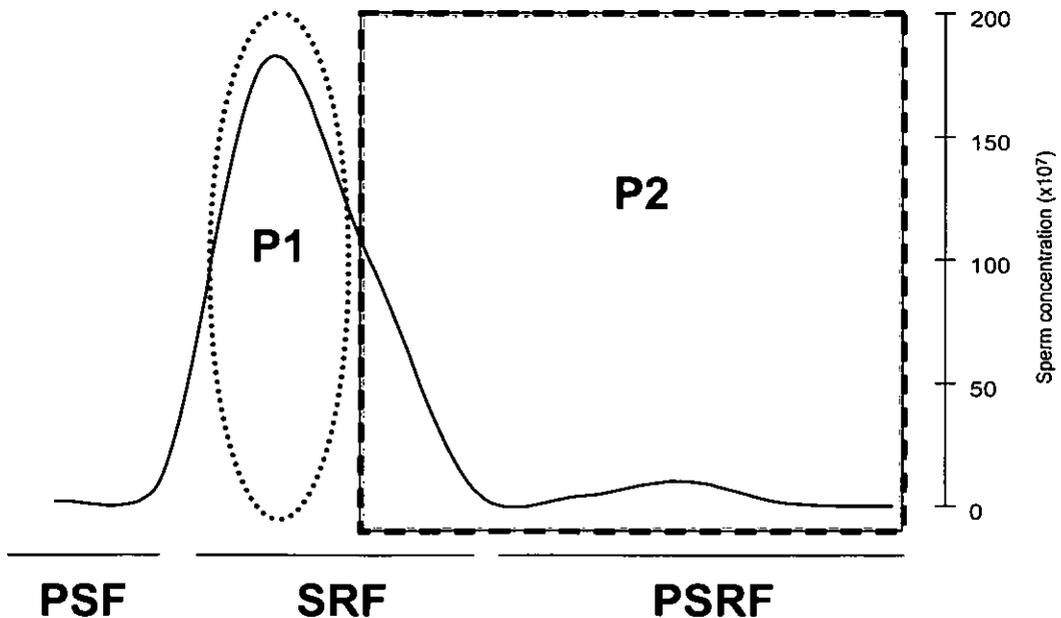

Fig. 1 Relative sperm concentrations (——) define ejaculate fractions in the boar ejaculate (PSF: pre-sperm fraction, SRF: sperm-rich fraction, PSRF: post sperm-rich fraction (includes gel component). Portions of the ejaculate can also be defined; P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF.

The boar seminal plasma, a heterogenous fluid

Most studies of the boar SP have been done as bulk fluid (since this is the way the ejaculate has been sampled), but there are excellent studies of fractionated samples (Einarsson 1971, Lavon & Boursnell 1975), which have shown that, owing to its sequential formation, the SP contains many components, all of which interact with spermatozoa and the surrounding female environment. The PSF has mostly electrolytes (mainly Na and Cl); the SRF mainly proteins but also steroid hormones (Claus et al. 1987, Claus 1990), glycerophosphorylcholine, fructose, glucose, inositol, citrate, bicarbonate and zinc, while the PSRF has increasing amounts of proteins, bicarbonate, zinc, Na, Cl and sialic acid (summarised by Mann & Lutwak-Mann 1981).

The source of the steroid hormones found in the boar ejaculate varies. Most testosterone derives from the accessory sexual glands, while oestrogens are mainly (80–90%) introduced in semen by the epididymal contents (originally testicular; Claus 1990 and references therein). Oestrogens, which in the boar can reach > 10 μg/ejaculate, stimulate the myometrium directly or indirectly (through induction of endometrial $PGF_{2\alpha}$), as well as by influencing the release of LH (Claus 1990). Therefore, SP-oestrogens are considered important, along with the behavioural, neuronal release of oxytocin; for the coordinated, long-lasting uterine motility during oestrus which issues the rapid phase of sperm transport in the female (Langendijk et al. 2005) and, ultimately, fertility (Claus et al. 1989).

Proteins are a major component of the boar ejaculate (39.4 $\pm$ 13.45 mg/ml, Rodriguez-Matinez et al. 2005), 80-90% of vesicular gland origin and 75-90% of them belonging to the spermadhesin lectin family. This family comprises three members; the Alanine-Glutamine-Asparagine proteins AQN (-1 and -3), the Alanine-Tryptophan-Asparagine proteins [AWNs] and the Porcine Seminal Plasma proteins I and II [PSP-I and PSP-II] (Töpfer-Petersen et al. 1998). Spermadhesins are multifunctional 12-16 kDa glycoproteins whose biological activities depend on their sequence, grade of glycosylation or aggregation state, as well as their ability to bind heparin (the AQN-1, AQN-3 and AWN being grouped as heparin-binding proteins [HBPs]) or not (PSPs), as they attach to the sperm plasma membrane to various degrees from the testis to the ejaculate. Collectively, they have been related to multiple effects on spermatozoa including membrane stabilisation, capacitation, and sperm-oviduct or zona pellucida (ZP) interplay. HBPs seem to stabilise the supra-acrosomal plasmalemma prior to capacitation in vivo (Calvete et al. 1997). AWN-epitopes have been detected on boar spermatozoa bound in vivo to the ZP, strongly suggesting AWN is a bona fide sperm surface-associated lectin, mediating sperm-ZP interactions at fertilisation (Rodriguez-Martinez et al. 1998). In vitro, however, HBPs failed to promote sperm survival (Centurión et al. 2003), while PSPs, also binding to the sperm surface (Töpfer-Petersen et al. 1998), display protective action on highly-extended and processed spermatozoa (Caballero et al. 2004, 2006, 2008 and references therein). The PSP-I and PSP-II account for > 50% of all SP-proteins, forming a non-heparin-binding heterodimer of glycosylated spermadhesins (Calvete et al. 2005) which depict immunostimulatory activities in vitro (Leshin et al. 1998) and in vivo (Rodríguez-Martínez et al. 2005). The various SP proteins originate from the testis, the epididymides and the sexual accessory glands (García et al. 2008), and their relative concentration vary among ejaculate fractions (see Fig. 2). Expectedly, the amounts of proteins increase 4-fold alongside the secretion of the vesicular glands, the relative concentrations thus being lowest P1, to increase (HBPs and, particularly the PSPs) towards the bulk of the PSRF (Rodríguez-Martínez et al. 2005), implying that the major proportion of spermatozoa is not immediately suspended in high amounts of SP-proteins in vivo, particularly not those firstly ejaculated.

Bicarbonate, an ion considered highly relevant for sperm motility, for induction of destabilisation changes in the plasma membrane and, ultimately, for in vitro and in vivo capacitation (reviewed by Rodríguez-Martínez 2007b), is present in the bulk boar ejaculate (20–23 mM/l). Concentration varies among fractions, from 14–17 mmol/l in the PSF and SRF, respectively, to the double i.e.

> 30 mmol/l, in the PSRF (Rodríguez-Martínez *et al.* 1990b). Bicarbonate levels are lowest in the P1 (~ 13 mmol/l) (Saravia *et al.* unpublished, Fig. 3), e.g. the first cohort of ejaculated P1-spermatozoa does not bathe in a SP with high levels of bicarbonate.
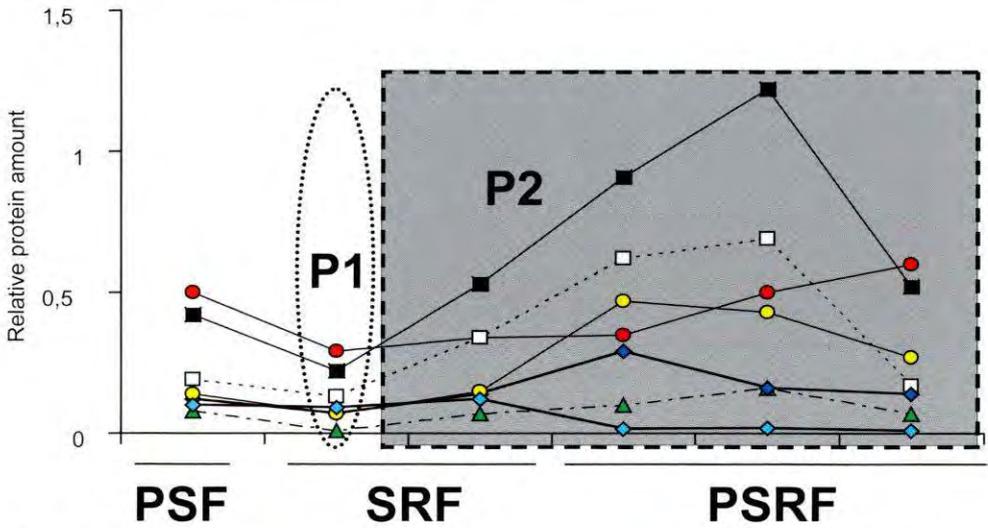

Fig. 2 Temporal relative amounts of seminal plasma proteins (●: PSP-I, ■: PSP-II, □: AQN-1, ▲: AQN-3, ◆: AWN-1, ○: AWN-2, ◆: inhibitor of acrosin/trypsin) in consecutive samples of the boar ejaculate (PSF: pre-sperm fraction, SRF: sperm-rich fraction, PSRF: post sperm-rich fraction (includes gel component)(modified from Rodriguez-Martinez *et al.* 2005).
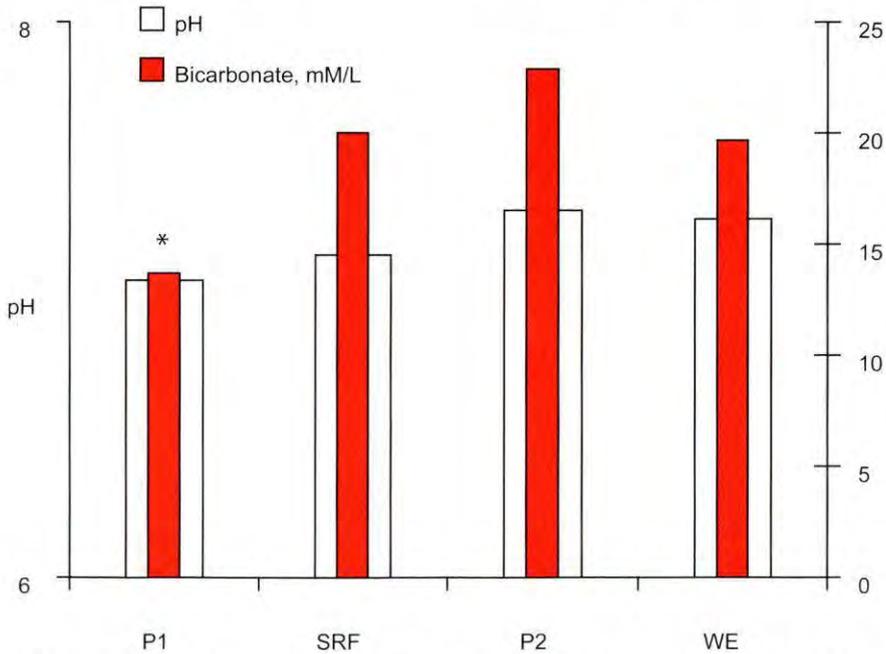

Fig. 3 Mean bicarbonate concentration (mM/L) and pH in different portions (P1 and P2), the sperm-rich fraction (SRF) of the ejaculate or the whole ejaculate (all fractions) of the boar (n = 20 boars; P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF, WE: whole ejaculate, including PSF, SRF and PSRF. *: $P < 0.05$ (modified from Saravia *et al.*, 2009b).

The cation zinc, unusually abundant in boar SP (Massanyi et al. 2003) is also of utmost importance for sperm function, and it is secreted in the SP alongside sperm and fructose emission, as shown in Fig. 4. Zinc stabilises, among other functions, sperm chromatin in human (Björndahl & Kvist 2003) and pigs (Kvist et al. 1987, Björndahl et al. 1990). In vivo, depletion of nuclear zinc by secretions decreases stability, being followed by a non-zinc, but disulphide-bridge dependent superestabilisation, which counteracts normal descondensation during fertilisation (Rodriguez-Martinez et al. 1990a). Such zinc-dependent stability can be challenged in vitro, by exposure to the detergent sodiumdodecylsulphate (SDS) and the zinc-chelating agent EDTA (a customary component of boar semen extenders, 6 mM) for 60 min at 60° C, which decondenses the sperm nucleus (see Fig. 4). As depicted, boar spermatozoa ejaculated in the later expulsed sperm-bearing portions (PSRF, where zinc levels are significantly higher) become more superstabilised than those expelled in the first portions, particularly the P1, since they clearly diminished their capacity to decondense in vitro alongside ejaculation. However, the total SP-zinc concentration as such is not an indicator of the free zinc amount that equilibrates with the sperm zinc content. A concomitant increase in citrate, having three binding sites for zinc at slightly alkaline pH, or presence of zinc-binding proteins could result in a zinc-chelating environment (Kvist et al. 1990).
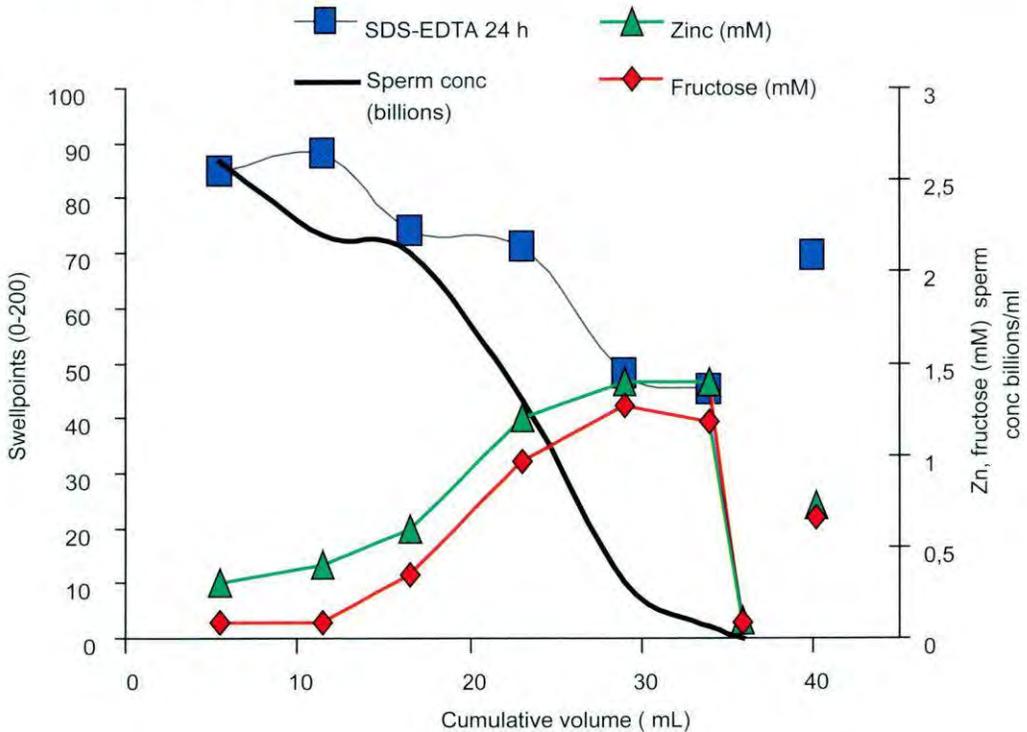

Fig. 4: Chromatin decondensation in vitro (of boar spermatozoa collected in 5 mL samples (n = 9) within the porcine sperm-rich fraction (SRF) and the post-sperm-rich fraction (PSRF) and an independently collected whole ejaculate (at 40). Zinc (mM), fructose (mM) and sperm numbers ($x10^9$/ml) are also depicted as markers. Decondensation was quantified as sperm nuclear swell-points* (i.e. the sum of % moderately decondensed sperm heads x 1 and the % grossly decondensed sperm heads x 2, maximal swellpoints are 200) after exposure to SDS-EDTA at 24h. (Kvist et al. unpublished).

The change from a zinc-dependent into a disulphide-dependent superstabilisation occurs upon storage of both cauda and ejaculated boar spermatozoa (Kvist *et al.*, unpublished results) and enhanced by prior exposure to a zinc-chelating environment (Björndahl *et al.* 1990), as depicted in Fig. 5 for immature (from testis, rete testis, caput or corpus epididymides) or mature spermatozoa (from cauda epididymides, whole ejaculate and consecutive aliquots of the SRF [as A1-A4] and the PSRF [A5-A8]). Sperm chromatin decondensation in SDS-EDTA at 0 or 24 h post-collection was more readily seen in immature than in mature spermatozoa. While storage (up to 20 d) promotes the disulphide-bridge dependent chromatin superstabilisation in immature spermatozoa, mature spermatozoa can sustain short storage (24 h) without becoming superstabilised, provided they are not pre-exposed to zinc-chelating treatment (Björndahl *et al.* 1990). Zinc also seems to play a major role in maintaining the stability of the spermadhesin PSP-I/PSP-II heterodimer, an effect that can be reversed by EDTA or at acidic pH, as found in the sperm reservoir (SR), i.e. a 1–2 cm segment of the utero-tubal junction (UTJ) and the adjacent isthmus of each oviduct (Rodríguez-Martínez *et al.* 2005), suggesting the female also regulates the extent of its action (Campanero-Rhodes *et al.* 2005).
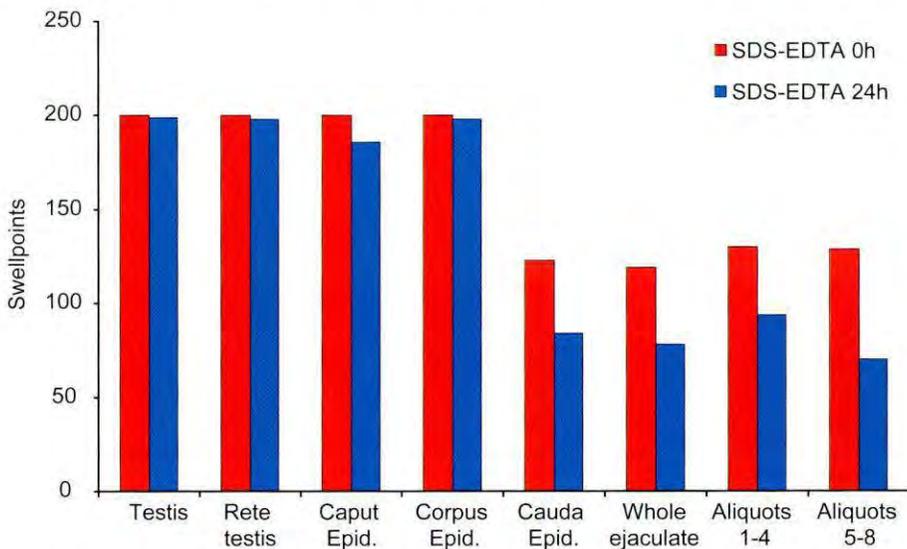

Fig. 5 Ability of the chromatin of boar spermatozoa, collected from various sources (testes, epididymides, whole ejaculate and consecutive aliquots of the SRF [Aliquots 1-4] and the PSRF [Aliquots 5-8]) to decondense in vitro (quantified as sperm nuclear swell-points*, i.e. the sum of % moderately decondensed sperm heads x 1 and the % grossly decondensed sperm heads x 2, maximal swellpoints are 200) after exposure to SDS-EDTA at 0 or 24h (Kvist et al. unpublished).

Distribution of the ejaculated spermatozoa in the female

During natural mating, the boar sequentially deposits the various fractions of the ejaculate in the cervical canal and elicits, by distending the uterine cavity, a stretching response from the myometrium. Both distention and SP-oestrogens, induce production/release of endometrial $PGF_{2\alpha}$ to, ultimately, provoke rhythmic, antiperistaltic (ad-ovarian) and peristaltic (ad-cervical) myometrial contractions. Antiperistalsis dominates the rapid phase of sperm transport through the

female internal genital tract and transports –within minutes– a small subpopulation of spermatozoa towards the SR (Rodríguez-Martínez et al. 2005). Ad-cervical peristaltis effectively mixes the uterine sperm suspension since the cervical canal is plugged by the ejaculated gel fraction. At AI with gel-free liquid semen, these contractions cause a large retrograde flow (up to 35-40% of the volume introduced, holding up to 20-25% of spermatozoa) within 30 min (Viring & Einarsson 1981, Steverink et al. 1998).

When semen is deposited (via mating or AI) pre-ovulation, enough spermatozoa (10^5 to 10^8) reach and colonise the functional tubal SR, to ensure successful fertilisation of all ovulated oocytes. Although this so-called 2nd phase of sperm transport mainly occurs between 5 and 60 min from insemination, the SR replenishment can take longer, depending more on sperm numbers inseminated than on the number of following matings or AIs (see review by Rodríguez-Martínez 2007b and references therein). The tubal SR is immunologically-privileged (Bergqvist et al. 2005), where sperm viability and fertilising capacity are preserved (Rodríguez-Martínez et al. 2001, Tienthai et al. 2004) for the entire preovulatory period. From the SR, restricted sperm numbers (10^2-10^3) are gradually, but apparently continuously released towards the presumed site of fertilisation at the ampullary-isthmic junction (AIJ), thus defining the 3rd phase of sperm transport, particularly when ovulation is approaching or has occurred (Rodríguez-Martínez 2007b). Not all spermatozoa are trapped in the SR, and trans-oviductal passage occurs during the preovulatory period (Viring 1980) but it is most evident post-ovulation, when spermatozoa do not need a long SR-storage. In either case, sperm numbers are importantly reduced (to hundreds) at the AIJ, a fact that strongly contributes to the physiological ratio (1:1) between spermatozoa and oocytes during fertilisation _in vivo_ (Hunter & Rodríguez-Martínez 2004).

Which spermatozoa colonise the sperm reservoir?

Theoretically, anyone; provided they are potentially capable of interacting with the SR (Viring 1980). However, the sequentiality of the entry through the cervico-uterine lumen during mating has led to the hypothesis that the 1st ejaculated sperm sub-population (in P1) is, by reaching first the SR, overrepresented there. P1- and P2- (last portion of the SRF and the PSRF) spermatozoa were collected from fertile boars. The P1-spermatozoa were loaded with the fluorophore Hoechst 33252, while P2 spermatozoa were kept unstained until conventional cervical AI of equal sperm numbers ($\sim 10 \times 10^9$ spermatozoa) per portion was performed _ad modum_ 12 h after onset of oestrus in weaned sows; either mixing P1 and P2 aliquots (control, $n = 5$) in a single AI flask (90–mL dose), or testing (a) a sequential order (P1-P2, Treatment A, $n = 5$) with P1 (10 mL) inseminated first, immediately followed by deposition of 80 ml of P2-semen or (b) an inverse order (P2-P1, Treatment B, $n = 5$). The sows were euthanized ~ 3 h later and the SRs flushed to recover the spermatozoa, which were accounted for as stained and unstained. While the number of spermatozoa flushed from the SRs did not differ between groups nor between boars (NS, ranging 0.9 to 2×10^9), the proportion of stained P1-spermatozoa significantly ($P < 0.05$) differed between groups, but not between boars. The highest proportion of P1-spermatozoa in the SRs (59.8 ± 5.66 %, means $\pm$ SEM) was found when a sequential order (P1-P2, Group TA) of insemination was issued (see Fig. 6). Reversing this order (Group TB, P2-P1) dramatically decreased the proportion to $15.6 \pm 2.1\%$, much lower than when a mixed suspension (control) was inseminated at one time ($36.9 \pm 2.70\%$). The hypothesis tested proved valid; when spermatozoa were inseminated in the same order as ejaculated _in vivo_, they were overrepresented in the SR. It remains to be determined whether such proportionality is maintained at the site of fertilisation.
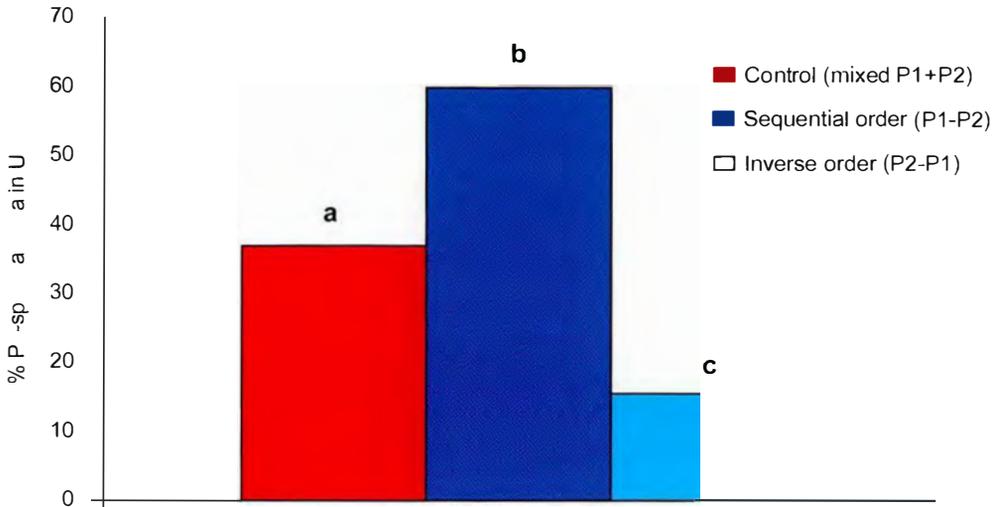
Fig. 6 Mean proportions of P1-spermatozoa present in the uterotubal junction (UTJ) of oestrous sows (n = 15, equally allotted) 3 h past-AI with a sequential order (P1-P2), an inverse order (P2-P1) or a mixture (P1 + P2, control) of ejaculate portions (P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF); a-c: different letters denote significant differences, P < 0.05 (Rodriguez-Martinez *et al.* unpublished).

What happens with the spermatozoa that do not enter the sperm reservoir?

While ~ 40% of the volume of the AI-dose is lost rather quickly (within 30 min) via vaginal reflux, this fluid only contains ~ 22-25% of the inseminated spermatozoa. The remaining ones have either entered the SR "sanctuary" or are still in the uterine lumen. The uterine cavity is, ~ 10 min after AI, invaded by inflammatory polymorphonuclear granulocytes (PMNs) which migrate from the lamina propia (i.e., subjacent to the lining epithelium, where they accumulate after extravasation, presumably a result of the high levels of oestrogens that dominate pro-oestrus in pigs), through the lining epithelium (Lovell & Getty 1968, Rodríguez-Martínez *et al.* 1990c), see Fig. 7a-c. Massive numbers luminal PMNs are first detected by 30 min (Lovell & Getty 1968), to sustain entry for the following 2–3 h (Viring & Einarsson 1981), surpassing the number of inseminated spermatozoa (Matthijs *et al.* 2003). This dramatic uterine PMN-influx is accompanied by accumulation of macrophages, granulocytes and lymphocytes in the endometrial stroma and, to a lesser extent, into the base of the lining epithelium (Rodríguez-Martínez *et al.* 1990c, Bischof *et al.* 1994, Kaeoket *et al.* 2003, Robertson 2007); a picture not seen in the oviduct, except for the mesothelial-covered infundibulum (Jiwakanon *et al.* 2006) and for the presence of lymphocyte-like cells in the base of the SR and the adjoining isthmus (Rodríguez-Martínez *et al.* 1990c). The resulting mass of leukocytes and sperm/SP-debris are, during this hourly period, eliminated from the lumen by continuous vaginal discharge but also by epithelial phagocytosis (Rodríguez-Martínez *et al.* 1990c) so that a new inseminate can enter a cleansed uterine lumen, free from redundant spermatozoa or semen-associated micro-organisms. The lumen must, moreover, be ready to host and nurture the semi-allogeneic early embryos when they enter the uterus by 48 h after ovulation.
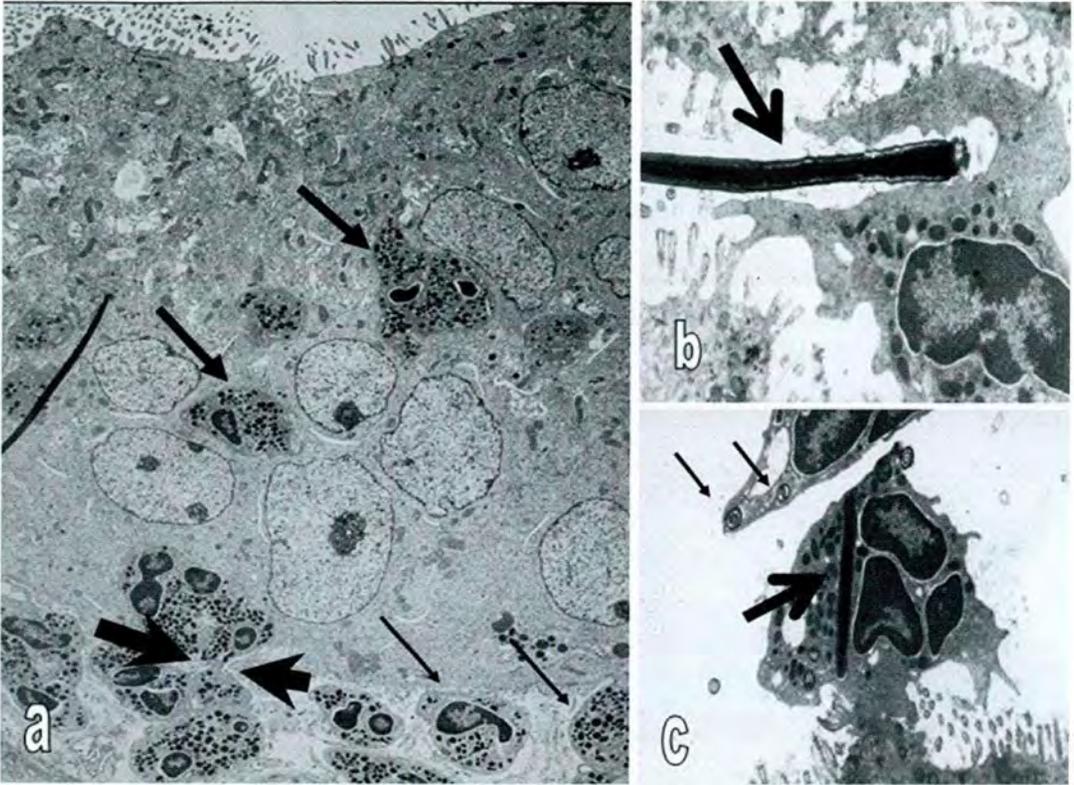
Fig. 7a-c: Entry of polymorphonuclear leukocytes (PMNs) into the uterine lining epithelium (a) and lumen (b,c) of oestrous sows, following AI of neat semen. The TEM-micrographs show the migration of PMNs from the lamina propria (a, thin arrows), through the basal membrane (a, between thick arrows) to the lining epithelium (a, medium-arrows) and the lumen (b,c) where they phagocytose individual spermatozoa (large arrows: sperm heads in b and c, small arrows: sperm tails in c)(micrographs H Ekwall, SLU).

Does the seminal plasma modulate the genital immunology of the female?

Or do the "*per natura*-antigenic" spermatozoa also play a role? While sows do not show changes in cellular immune reactivity after AI (Veselsky et al. 1981), gilts exposed to sperm and/or seminal antigens increased litter size in a following fertile mating (Murray & Grifo 1983, Flowers & Esbenshade 1993). AI with washed boar spermatozoa caused a greater influx of PMNs into the uterine cavity of gilts compared to SP or whole semen (Rozeboom et al. 1998, 1999), suggesting their chemotactic character (Rozeboom et al. 2001a). In contrast, AI of sperm-free SP increased by 5.4-fold the number of PMNs infiltrating the porcine uterine lumen (O´Leary et al. 2004).

Most data now suggest that the SP ensures reproductive success by promoting the survival of some spermatozoa, and conditioning the female immune response to tolerate paternal antigens. SP initiates –by interaction with the luminal epithelium- a cascade of downstream immunological effects, including the advancement of ovulation (Waberski et al. 2006), the production of progesterone (O´Leary et al. 2006) and other defence/immune responses. SP upregulates MHC class II and interleukin-2 (IL-2) receptor expression (Bischof et al. 1994), induces the transitory expression of pro-inflammatory (PMN-attractants) soluble cytokines (as

IL-1) and cyclo-oxygenase-2 (O´Leary *et al.* 2004) and the expression of interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), and the monocyte attractant protein-1 (MCP-1) which, towards early pregnancy, leads to a transition of leukocyte phenotypes, PMNs being replaced by macrophages and dendritic cells (Robertson 2007). Boar SP also contains high levels of the immunosuppressive transforming growth factor-β (TGF-β, Robertson *et al.* 2002), a very potent, multifunctional cytokine group which, in the pig, regulates T-cell differentiation to reach a state of functional, adaptive immune tolerance to male antigens by the female (O´Leary *et al.* 2004, Robertson 2007). This would explain why priming to seminal antigens improves fertility in pigs in later oestrous events (Murray & Grifo 1983, Flowers & Esbenshade 1993, Rozeboom *et al.* 2000). Moreover, inter-boar differences in cytokine SP-content might lead to different adaptation levels by the females (Robertson 2007), thus suggesting the SP-induction of maternal tolerance might relate to the differences in embryo survival often observed among sires (e.g. innate fertility).

SP-infusion studies have, however, lead to -at first sight- paradoxical results, probably owing to differences in animal categories, methods and experimental design used. For instance, SP either induced (Bischof *et al.* 1994, O´Leary *et al.* 2004, Rodríguez-Martínez *et al.* 2005) or attenuated (Veselsky *et al.* 1991, Rozeboom *et al.* 1999, 2001b, Taylor *et al.* 2008, 2009) the inflammatory response of the pig endometrium during mating or AI. It is possible that we are simply facing several steps, firstly a transitory inflammatory response initiated by the SP and a secondary recruitment of antigen-presenting cells (macrophages and dendritic cells), prerequisite for the generation of paternal antigen-specific T-cells (Schuberth *et al.* 2008).

SP-spermadhesins such as the PSP-I/PSP-II have shown capacity to bind to (Yang *et al.* 1998), and to enhance pig lymphocyte proliferation (Leshin *et al.* 1998). We tested, therefore, whether pig HBPs and PSPs, isolated from the SP of SRF samples collected from mature, fertile boars (Calvete *et al.* 1996, Rodriguez-Martinez *et al.* 2005) could recruit PMNs and different lymphocyte subsets into the lining epithelium of the pig uterus *in vivo*. Consecutive biopsies were taken (2-120 min) under narcosis and treated histologically and by immunohistochemistry (IHC) using mAbs against CD2, CD4 or CD8 lymphocyte subsets. Compared to controls (saline-infused uteri), exposure to the PSP-I/PSP-II heterodimer significantly ($P < 0.05$) induced the migration of PMNs (Log_{10}) to the surface epithelium, within 10 min of infusion (Fig. 8), a recruitment that was sustained over the experimental period, becoming 5-fold by 30 min and 7-fold higher from 60 min onwards ($P < 0.001$). PMNs were detected in the uterine lumen by 30 min and thereafter. The infusion of a similar dose of HBPs had no significant effect (NS). Regarding the IHC (Fig. 9), saline infusion did not significantly increase the number of CD2, CD4 nor CD8 positive (+) cells in the epithelium or the lamina propria over time (NS). CD2 + cell numbers were only significantly increased (4 to 7-fold from 10 min onwards) by infusion of PSP while no significant effect of any treatment was seen on CD4 + cells. CD8 + cell numbers increased significantly (3-fold) only after 60 min of the infusion of PSP. A 1–2 fold higher amount of CD8 + cells was seen during the entire period following the infusion of HBPs (NS). In sum, the heterodimer PSP-I/PSP-II induced an increase in the numbers of some uterine lymphocytes (such as CD2 + [Tk, NK, cytokine-rearing cluster] and CD8 + [cytolytic] cells, Gerner *et al.* 2009) *in vivo*. Numbers increased earlier for CD2 + (from 10 min onwards) than for CD8 + (60 min), suggesting that this immunostimulatory effect could be of primary nature. However, albeit being done *in vivo*, and registering changes over time (2–120 min), this experiment was done under narcosis, on few animals and testing single CD-subsets rather than combinations, which would have provided a more accurate quantification of the processes. On the other hand, PSP-I/PSP-II was confirmed as leukocyte chemoattractant in pigs, confirming previous *in vitro* (Leshin *et al.* 1998) and *in vivo* studies (Rodriguez-Martinez *et al.* 2005). AI of SP-PSPs in conscious oestrus sows, at doses 5-fold lower (3 mg/ml, 100-ml dose) than those present in the

boar ejaculate, led to a substantial (6-fold higher than in controls) recruitment of PMNs to the uterine cavity (35.4 ±12.56 vs 5.83 ±4.62 million PMN/ml, mean ±SD, P < 0.001). It remains to study, however, whether any portion of the ejaculate can induce this PMN entry.
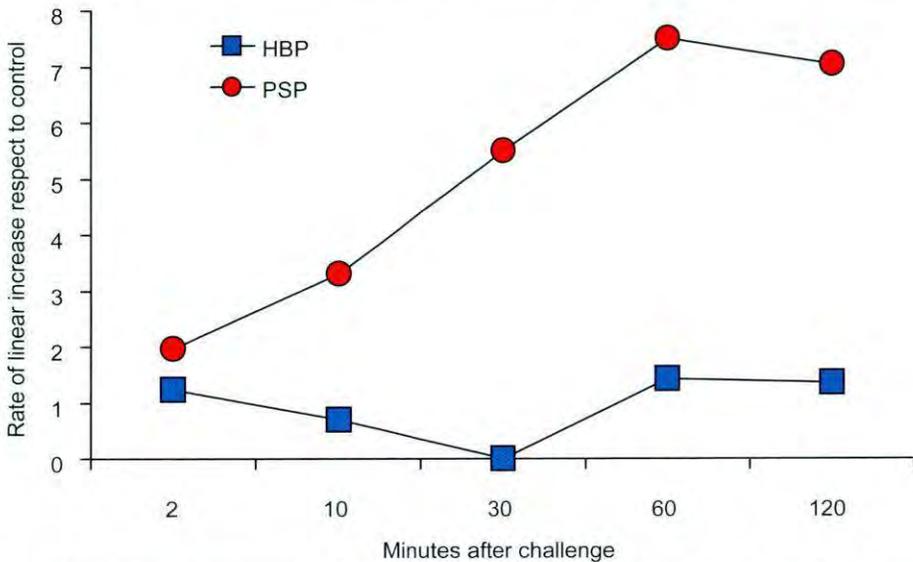

Fig. 8 Rate of linear increase rate for PMN recruitment (compared to control i.e. sham, saline infused uterine horn) to the uterine surface epithelium in oestrous sows (n = 6) at various times (2-120 min) after intrauterine infusion of porcine SP-HBP (HBP) or SP-PSPI/PSP-II (PSP) spermadhesins (3 mg/ml, 100-mL dose). The identity and purity of the protein preparations were assessed by N-terminal sequence analysis and Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight (MALDI-TOF) mass spectrometry while amino acid analysis was used to quantify the amount of either protein (which averaged 15 mg/ml). Sows were, under narcosis, unilaterally infused with spermadhesins, respectively saline and endometria removed for biopsy at various intervals. The tissues were fixed (2% paraformaldehyde) and processed for histology and manual counting of PMNs at x400 using an ocular reticulum on coded slides. The relative number of cells (Log10) was quantified in treatment vs control tissues at each time interval (Rodriguez-Martinez et al., unpublished).

Why are there sperm subpopulations in the boar ejaculate?

Many studies, including our own, have described the presence of sperm subpopulations in the boar ejaculate either by chromatin stability, morphology, motility or membrane resistance variables (Rodriguez-Martinez et al. 1987, Cremades et al. 2005, Peña et al. 2005, Saravia et al. 2007b and references therein, Druart et al. 2009) or even by fertilizing capacity in vitro (Xu et al. 1996, 1998; Zhu et al. 2000). These sperm subpopulations are apparently distributed either along the entire ejaculate, or within major fractions, such as the SRF. We therefore focused, from 2001, on portions of the SRF and PSRF, the so-named P1 and P2, seeking for differences in resilience between spermatozoa fortuitously ejaculated in one of these portions. Spermatozoa bathing in P1 cope better with different handling, such as storage at room temperature, cooling, or freezing-thawing, than P2-spermatozoa (Sellés et al. 2001, Peña et al. 2006, Saravia et al. 2007a, 2009a and references therein). Obviously, these differences would not be specifically allotted to differences among spermatozoa, since spermatozoa collected in the entire SRF (as praxis) are able to sustain handling as well. We have now established that it is the SP from
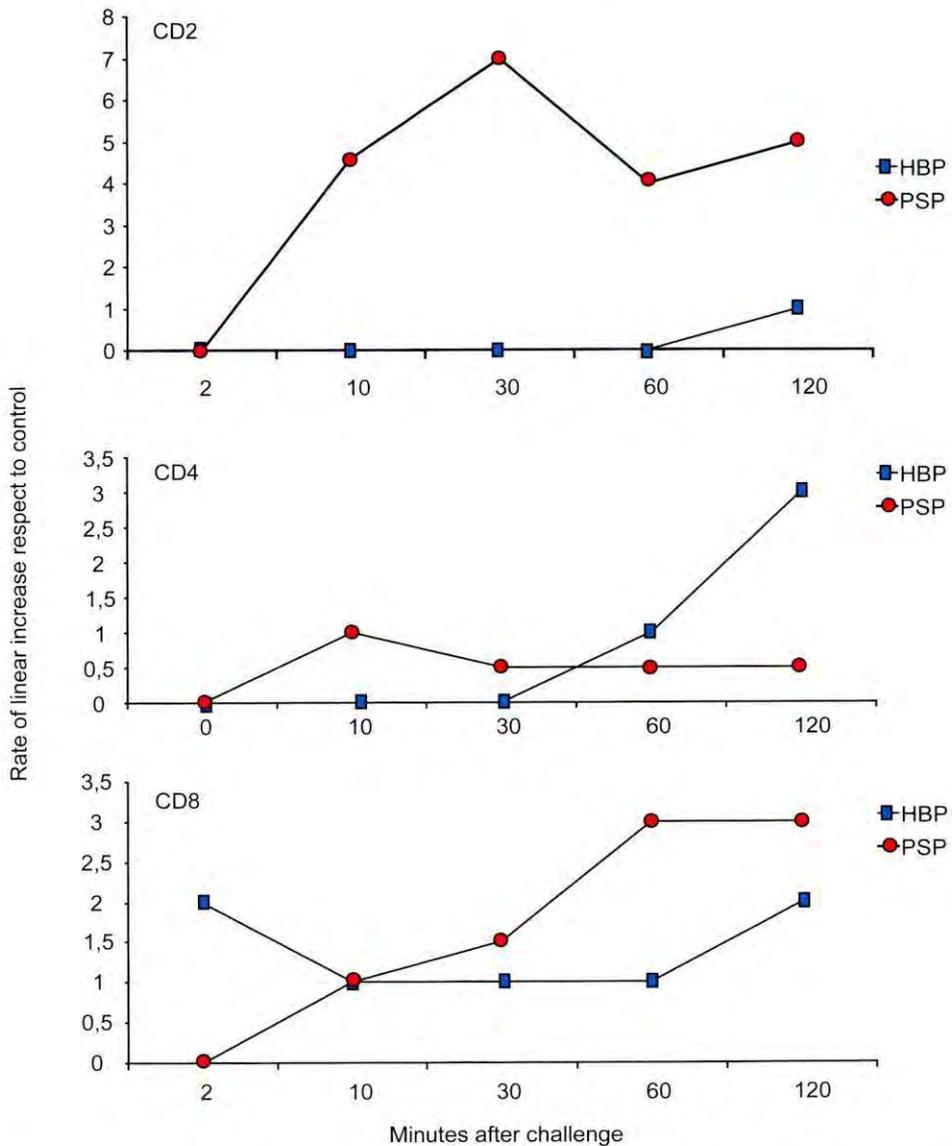
Fig. 9 Rate of linear increase rate for lymphocyte subsets (CD2, CD4 and CD8) recruitment (compared to control) to the uterine surface epithelium in oestrous sows (n = 6) (2-120 min) after intrauterine infusion of porcine SP-HBP (HBP) or SP-PSPI/PSP-II (PSP) spermadhesins (3 mg/mL, 100-ml dose). For description of the identity and purity of the protein preparations and general procedures see Fig. 8. Immunohistochemistry (IHC) using microwave-effected antigen retrieval was done with a standard avitin-biotin immunoperoxidase technique and primary mouse mAbs (VRMD, Pullman, WA, USA). Counting of IHC-marked T-cells in the tissue for biopsy was done at × 400 using an ocular reticle (tissue area: 0.0625 mm2), on coded slides. Particular attention was taken to the lining epithelium and the subjacent lamina propia. The relative number of cells (Log[10]) was quantified in treatment tissues against control tissues, at each time interval (Rodriguez-Martinez *et al.*, unpublished).

these ejaculate portions (P1-SP or P2-SP) that differently influences sperm kinematics of those fortuitously P1- or P2-contained spermatozoa from individual boars, primarily or secondarily exposed (i.e. following cleansing and re-exposure) to pooled P1-SP or P2-SP from the same males during 60 min. Spermatozoa were subjected to controlled cooling and thawing in MiniFlatPacks™ (MFPs) and examined for motility (CASA) at selected stages of processing. A higher proportion of P1 spermatozoa than of P2 spermatozoa incubated in their native SP portion were motile from collection to post-thawing. When P1-spermatozoa were cleansed from their original SP and re-exposed to pooled P2-SP, sperm kinematics deteriorated from extension to thawing. By contrast, cleansed P2 spermatozoa increased motility to P1 levels, especially after thawing (Fig. 10) when re-exposed to pooled P1-SP. This influence of SP on sperm kinematics was not sire-dependent and presumably related to different concentrations or either SP proteins or other factors in the different SP-portions (Rodríguez-Martínez et al. 2008, Saravia et al. 2009a).
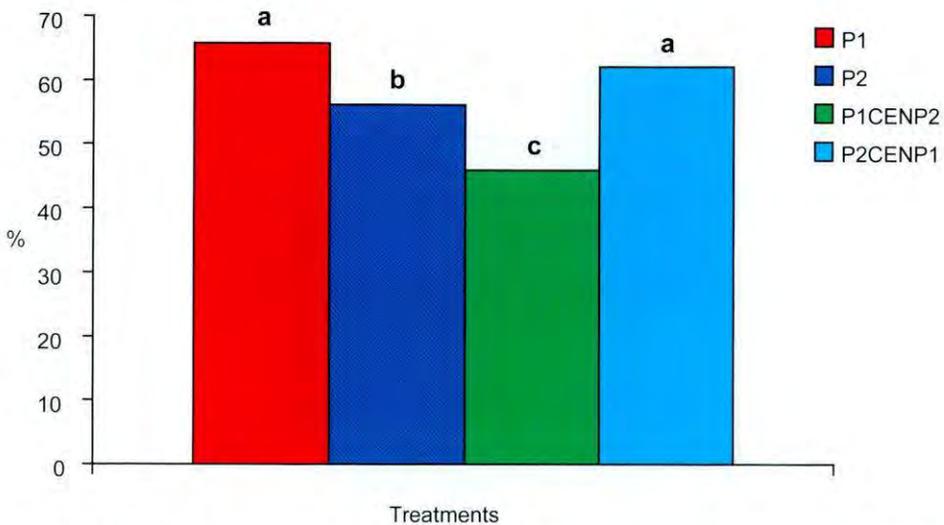

Fig. 10 Cryosurvival (% motility post-thawing) of boar spermatozoa either held in their "native" seminal plasma (P1-SP or P2-SP) for 60 min at room temperature or cleansed by centrifugation and incubated for 60 min t room temperature in the "other SP", as P1CENSP2 (spermatozoa from P1 cleansed and exposed to SP from P2) or P2CENSP1 (spermatozoa from P2 cleansed by centrifugation and exposed to SP from P1) (n = 25; P1: 1st 10 ml of the SRF, P2: the rest of the SRF and the PSRF), a-c: different letters denote significant differences, $P < 0.05$ (modified from Saravia et al. 2009a).

What are the differences between the P1-SP and P2-SP? Several, many of these already enumerated, such as they differ in zinc and bicarbonate amounts, the latter being 2-fold lower in P1-SP than in P2-SP (Fig. 3). In relation to this, the pH of P1-SP was 3 pH units lower than that in P2-SP. The ion bicarbonate plays important roles in sperm physiology, both by maintaining intracellular pH (ipH) and the homeostasis of the cell, and by modulating sperm motility and membrane stability through its effects on the sperm adenylyl cyclase (Henning et al. 2008). It is responsible for the initiation of motility at ejaculation (Rodríguez-Martínez 1991), and it is also considered the main effector of changes within the lipid bilayer of the sperm plasma membrane that are associated with sperm capacitation in vivo in the pig (Tienthai et al., 2004). A lower concentration of bicarbonate (and lower pH) in P1-SP would modulate the ipH of the surrounding spermatozoa and thus its kinematics (Gatti et al. 1993, Saravia et al. 2009a).

P1-SP and P2-SP significantly differ also in their contents of total protein (the P1-SP having only 17-18% of the total, Rodriguez-Martinez et al. 2005). In addition, the P1-SP and P2-SP have clearly distinct protein profiles, the P2-SP having the highest concentration of spermadhesins, in particular glycoforms of PSP-I/PSP-II heterodimer of high mass (Rodriguez-Martinez et al. 2005). Using one- and two-dimensional SDS-PAGE electrophoresis to separate proteins in P1 and P2, followed by in-gel enzymatic digestion, mass fingerprinting and collision-induced dissociation tandem mass spectrometry (CID- MS/MS) for peptide sequencing, we have recently confirmed that the P2-SP mostly contained spermadhesins, while the P1-SP contained, besides PSP-I, actin and proteins of the Lipocalin family, namely the lipocalin-type Prostaglandin D-synthase (L-PGD-S), Epididymal Secretory Protein-1 (ESP-1) and Lipocalin (Table 1)(Calvete et al. unpublished). Lipocalins are multifunctional proteins, involved in retinol transport, pheromone-binding and transport and prostaglandin synthesis (Flower 1996, Marchese et al. 1998). As major epididymal proteins secreted in the caput segment, both EPS-1 and L-PGD-S are relevant for sperm maturation (Fouchecourt et al. 2002, Leone et al. 2002) and sperm quality (Chen et al. 2007). L-PGD-S, primarily involved in transporting retinoids and other lipophilic ligands, binds to the sperm apical ridge being strongly related to sire fertility, including pigs (Gerena et al. 1998, Fouchecourt et al. 2002, Flowers 1995).

Table 1. Identification of protein spots in boar seminal plasma portions P1 (first 10 mL of the SRF) and P2 (the rest of the SRF and the PSRF), separated by one (1D) and two (2D) dimensional SDS-PAGE electrophoresis followed by in-gel enzymatic digestion, mass fingerprinting and collision-induced dissociation tandem mass spectrometry (CID- MS/MS) to sequence selected peptide ions (Calvete et al., unpublished).

1D SDS-PAGE		2D SDS-Page		Peptide ion		MS/MS-derived sequence	Protein
Ejaculate portion				m/z	z		
P1	P2	P1	P2				
1		1		488.4	2	AGFAGDDAPR	Actin [Q6QAQ1]
2				675.3	2	GFTEDGIVFLPR	Lipocalin-type Prostaglandin D synthase [Q765P8]
3				569.3	2	(214.2)TVVATDYR	Unknown
4				744.7	2	(258.1)CTYFCDXPR	Unknown
		2		584.6	2	GTPXANGDXAXK	Lipocalin [~ XP_001917526]
		3, 4		508.4	2	SGINCPIQK	Epididymal Secretory Protein-1 [O97763]
		5-8	1-7	524.3	2	LDYHACGGR	PSP-I [P35495]
			8	528.3	2	GSDDCGGFLK	AQN-3 [P24020]
			9	508.8	2	INGPDECGR	PSP-II [P35496]
			10, 11	925.8	3	ASFHIYYYADPEGPLPFPYFER	AWN [P26776]

Is there any relation between ejaculate characteristics and fertility?

The obvious answer would be yes. However, to determine which characteristic is most relevant and, particularly, if it could be measured *in vitro* remains elusive (Popwell & Flowers 2004) since we are unable to relate results of sperm function to the fertility of pigs, which is not binary as in bovine, but combines pregnancy/farrowing and prolificacy (litter size). Moreover, by inseminating excessive sperm numbers, we mask the relations between measurements of sperm attributes (measured singly [motility, membrane integrity etc] or collectively [ZP-binding, IVF etc], Rodriguez-Martinez 2007a, Foxcroft et al. 2008).

As already enumerated, the SP plays major roles, at the level of the spermatozoa they cover and interact with, and for the signalling they excert towards the female genital tract, which has

consequences beyond our current understanding. Seminal antigens have proven beneficial for fertility improvement *in vivo*, with substantial variation among boars (Murray & Grifo 1983, Flowers & Esbenshade 1993, Rozeboom *et al.* 2000), suggesting that the SP of a boar differs somehow from that of another boar. A logical immediate difference is a variation in the type and the relative amounts of SP-proteins, of which some have been correlated to *in vivo* fertility and thus collectively named "fertility-asociated", such as the L-PGD-S (Flowers 1995, 1997, review by Foxcroft *et al.* 2008), clearly present in the P1-SP (Table 1). However, when these proteins have been tested *in vivo* or *in vitro*, results have varied, once more probably caused by the excessive sperm numbers used. Another major question remains; which is the mode of action of these SP-proteins? One tempting hypothesis has been already launched (Robertson 2007), where established differences in SP cytokine contents would lead to different degrees of maternal tolerance by the female and thus attain differences in embryo survival, leading to variation in fertility and, particularly, prolificacy between boars. It is hoped that this line of research will be followed.

Can we use this knowledge for boar semen processing or porcine AI?

Porcine SP from the SRF influences sperm physiology (reviewed by Rodríguez-Martínez *et al.* 2008). On the other hand, SP from solely the PSRF, with a higher amount of bulbourethral gland fluid reduced semen fertility (Iwamoto *et al.* 1992), presumably by the relatively increasing presence of AQN-3 in this portion (Iwamoto *et al.* 1995, see Table 1). Removal of SP (by extension in a buffer and further centrifugation/re-extension) is, therefore, customary during conventional cryopreservation of boar semen. However, SP-exposure is not solely negative. Addition of bulk SP to boar semen was initially considered to ameliorate the effects of sperm processing (from extension to sex-sorting) on sperm survival and the fertility after AI, mostly using empirical approaches (review by Caballero *et al.* 2008 and references therein). Addition of bulk SP (12.5% v/v) to semen for AI attenuated endometrial post-breeding reactions *in vitro* (Rozeboom *et al.* 2001b, Taylor *et al.* 2009) and *in vivo* (Rozeboom *et al.* 1999, 2000), but individuals still reacted when foreign proteins (such as BSA) were present (Taylor *et al.* 2009). Bulk SP-addition (~ 10%) of bulk SP increased sperm longevity post-thaw (Einarsson & Viring 1973), apparently by preserving membrane stability (Vadnais *et al.* 2005), as it occurs after flow cytometry for sex-sorting (review by de Graaf *et al.* 2008). AI-fertility could be improved adding 25-30% v/v of SP (Crabo & Einarsson 1971, Larsson & Einarsson 1976) but not when ~ 10% SP was added (Abad *et al.* 2007, Kirkwood *et al.* 2008). Positive effects (motility, viability and *in vitro* fertilising capacity) of adding exogenous SP (5% v/v) to semen during cooling were only seen when the supplement SP was derived from boars judged to have good semen freezability, irrespective of the total SP protein amount or profile (Hernández *et al.* 2007). This evident variation in results when using SP-supplementation is not surprising, since SP-addition was between 5% and 100% v/v, the SP used was either from the whole ejaculate (all fractions), different fractions, or solely from the SRF, from individual boars or from pooled sources, disregarding the large variability in SP-composition seen among sires, owing to age, breed and most likely having a genetic background (Roca *et al.* 2006). Exposure of highly-extended spermatozoa to low doses (1.5 mg/ml) of well identified SP-proteins gave different results. HBP-spermadhesins, which play major roles during sperm transport and fertilisation (Calvete *et al.* 1997, Rodríguez-Martínez *et al.* 1998), were not beneficial for sperm viability *in vitro* (Centurión *et al.* 2003). PSPs, on the other hand, provided substantial protection and maintained fertilising capacity (Caballero *et al.* 2004, 2006, 2008 and references therein). However, such benefits of PSPs could not be shown when handling high sperm concentrations

(as praxis when handling boar semen for AI) nor during cryopreservation (Hernández et al. 2007), probably because the spermatozoa had already been in contact with higher amounts of the SRF-SP after collection.

SRF-boar spermatozoa incubated at room temperature in their own SP became more resistant to cold shock (Pursel et al. 1973) and had, when pre-incubated for 3-20 h, higher post-thaw survival and fertilising capacity (Eriksson et al. 2001). Such incubation post-ejaculation with their surrounding SP is, therefore, nowadays customary in our laboratory, during the temperature decrease from 35–30°C to room temperature, before first extension with BTS for 30-60 min (Saravia et al. 2007a, 2009a).

Removal of the vesicular glands did not affect either the freezability or the fertility of frozen-thawed boar spermatozoa (Moore & Hibbitt 1977), possibly by abolishing the documented induction of chromatin superstabilisation (Kvist et al. 1987), or the reduction of sperm binding to the oviduct (Summers & Pena 2008) caused by the vesicular gland fluid. Considering this would place the P1-spermatozoa in advantage, we recently attempted to simplify the customary freezing protocol for boar semen. We used solely P1-spermatozoa, holding them in their "native" SP for 30 min, extended with Lactose-EggYolk (LEY) before cooling to +5°C within 1.5 h, prior to being mixed with LEY + glycerol and orvus-es-paste (LEYGO), packed into MFP, and customarily frozen. The entire procedure, here named "simplified freezing (SF)" lasted 3.5 h compared to the "conventional freezing (CF)" that was used as control procedure, which lasted 8 h (Saravia 2008, Saravia et al. 2009b). As controls, spermatozoa from the SRF were compared to P1-spermatozoa. The P1-SF-processed semen showed similar proportions of sperm motility (and kinematics), plasma membrane and acrosome intactness post-thaw, to the SRF-semen customarily frozen (SRF-CF). Mean post-thaw sperm motility ranged from 56% to 69%, the highest percentages being among the P1-SF. Interestingly, there was barely any variation between sires or within-sire for P1-derived variables, in contrast to SRF, independent of the handling method (CF or SF). The reason behind a maintained P1-sperm survival after this shorter freezing process is yet unknown, but the particular milieu of the P1-SP (protein levels and types, lower bicarbonate, zinc and fructose levels etc), together with the use of the cryobiologically well-suited MFP are highly relevant. We are now awaiting for intended fertility trials to determine whether the fertilizing capacity of the processed semen was also preserved by the P1-SP as most *in vitro* results indicate.

Acknowledgments

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