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Influence of seminal plasma on the kinematics of boar spermatozoa during freezing

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Abstract

Sperm motility is, for its relation to cell viability and fertility, a central component of the spermiogram, where consideration of motion patterns allows discrimination of sub-populations among boar spermatozoa. Extension and cryo-preservation imposes changes in these patterns in connection to handling, additives, temperature changes and the removal of boar seminal plasma (BSP) which apparently makes spermatozoa susceptible to oxidative stress, thus affecting survival and motility post-thaw. Detailed kinematic analyses during sperm cooling are sparse, particularly when considering the instrumentation and settings used for analyses, the effect of extenders, and of the BSP the processed spermatozoa are exposed to. Spermatozoa present in the first collectable 10 mL of the sperm-rich fraction of the ejaculate (portion 1, P1-BSP), have shown an increased ability to sustain motility during and after cryo-preservation than spermatozoa immersed in the rest of the ejaculate (portion 2, P2). When P2-spermatozoa were cleansed from their BSP and exposed for 60 min to pooled P1-BSP, their motility post-thaw increased to similar levels as P1-spermatozoa. This BSP-influence is sire-dependent, presumably related to the protein concentration in the different ejaculate portions, and apparently unrelated to changes in membrane integrity or membrane stability through conventional, controlled cooling.

Keywords: Sperm kinematics; CASA; Sperm populations; Seminal plasma; Boar

1. Introduction

Sperm motility is the parameter most frequently used to measure boar sperm viability in the ejaculate, during/after the process of cryo-preservation and, indirectly, to provide cues on the potential fertility of the spermatozoa [1]. The latter has, however, proven inconsistent [2], probably owing to the methodology used for motility assessment. In most cases, subjective visual examinations are performed. Despite this approach being practical, cheap, quick and with a proven correlation to more objective motility determinations using computer-assisted sperm analysis (CASA) [3], they still fail to accurately predict fertility [4,5]. Use of CASA instruments have, by providing detailed information on sperm motility patterns retrieved simultaneously from large

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sperm numbers, yielded significant, although varying, relationships with fertility results [6–9]. It is likely that differences in power and rationale exist among CASA systems which, together with operator-driven differences in handling of settings and of samples, have contributed to this variability [10]. However, the analyses of motility have provided valuable insight on how spermatozoa move and how their motility changes under physiological conditions [11] or when handled for use in assisted reproduction techniques (ARTs). ARTs-related procedures such as semen extension, fluorophore loading, ultraviolet and laser illumination, high-speed sorting, cooling, and cryo-preservation, are potentially harmful to the functional structures of the spermatozoon, and might thus impose different degrees of change in sperm function, including motility [12]. However, there is a lack of systematic studies aimed at determining movement patterns during sperm processing [13]. Moreover, since boar spermatozoa present in an ejaculate constitute a heterogeneous population in terms of their patterns of movement [14,15], CASA-analyses of motility patterns have made possible the discrimination of sperm subpopulations both in fresh ejaculates [16], post-thaw and during selected steps of semen processing [9,17] thus helping categorisation of boars for their ability to sustain freezability of their semen [18,19]. Whether changes – quantitative as well as qualitative – in these subpopulations relate solely or concertedly to sire, ejaculate, processing set-up or analytical instrumentation power, remains to be studied in detail. There are indications that – for instance – removal of seminal plasma causes changes in motility patterns [17]. Boar seminal plasma (BSP), the complex mixture of fluids from the cauda epididymides and the accessory sex glands, does not only influence the female genital tract [20,21] but also exerts major effects on sperm resistance to cold shock [22], oxidative stress [23,24], and resistance to processing [25]. Spermatozoa present in specific fractions of the ejaculate exhibit different effects [26–32], most probably owing to the different nature of the BSP components that they are exposed to [20]. The latter is noteworthy, considering that during natural mating in pigs, the spermatozoa do not usually stay in prolonged contact with the BSP. Actually, *in vivo*, they are absolutely not in contact with the major volume of BSP that is, in fact, ejaculated after the spermatozoa. Therefore, since detailed kinematic analyses during sperm cooling are sparse, particularly in relation to the instrumentation and settings for analyses, the effect of extenders, and of the BSP the processed spermatozoa are exposed to, this presentation intends to review this area, focusing on the influence of certain portions of the BSP on the kinematics of boar spermatozoa during freezing.

2. Sperm motility, the most studied semen attribute

The analysis of the collected ejaculate in boars comprises the immediate observation of aspect, volume and density, followed by the determination of sperm concentration and motility. For the latter, phase contrast light microscopy is most commonly used to determine the percentage of spermatozoa depicting a pattern of progressive, rectilinear movement, a movement perceived as ''normal''. This estimation is either done ''subjectively'' (albeit often by an experienced operator) or ''objectively'', using CASA instruments, which are now less costly and therefore more widespread. The subjective assessment is practical, inexpensive and allows for the elimination of ejaculates or processed semen depicting poor sperm motility, which is a good clinical indicator for potential sub- or infertility. However, we most often deal with boars whose spermatozoa are highly motile, depicting acceptable motility during processing, and even post-thaw. It is here where CASA instruments appear more valuable, since they provide much more data with a degree of objectivity and detail which would hopefully reveal subtle differences in motility not perceivable by the human eye. Most of these instruments digitise microscope images of sperm trajectories accounting for speeds of translation and lateral movement of the spermatozoa, describing patterns of motility and the relative proportions of cells showing these, as well as other re-calculated kinematic variables. Newer computerized sperm analysers (such as the Qualisperm™, Biophos, Pfäffikon, Switzerland, http://www.biophos.com), on the other hand, determine the number of particles (spermatozoa) crossing pre-determined fields of view, yielding a regression fluctuation algorithm of sperm numbers and translation classes. The results yielded by the older instruments, extensively used for research purposes, are often based on comparatively few (hundreds) spermatozoa analysed per sample, which are followed in most cases for a few seconds of their trajectory. The latter instrumentation (Qualisperm™), which appears more suitable for routine use, analyses thousands of spermatozoa per sample, restricting the output of information to numerical sperm classes based on their velocity of translation in a large field. Compared to subjective assessment [4,5], the outcome of motility analyses using CASA analyses have been related to the fertility of the semen used, or

even of the boar (at least when using some instruments), but yet consistently failed to accurately predict fertility outcome [6–9]. This inability for prediction can well be caused by the use of unsuitable programming of settings. Another common reason might be the use of the same settings for freshly ejaculated or processed spermatozoa which has resulted in some CASA instruments/settings yielding better results after the selective death of weak spermatozoa caused by the processing than before, a clear yet paradox pseudo-enhancement of the values of sperm kinematics retrieved [33]. This constitutes a major concern for bias, calling for detailed studies of sperm kinematics during sperm handling and processing, which not only should use species-specific threshold values but also should focus on absolute kinematic parameters such as sperm velocities and the lateral displacement of the sperm head (the so-called LDH), rather than on derived, re-calculated parameters [33].

3. Spermatozoa are able to change their pattern of motion under different conditions

Freshly ejaculated boar spermatozoa basically display one type of physiological motility, the so-called ''activated'' motility, a name derived from the sudden activation displayed by the spermatozoa leaving the acidic caudae epididymal fluid at ejaculation, when they are confronted with the BSP, a fluid 10-fold richer in bicarbonate compared to the caudae [34,35]. In an activated spermatozoon, the tail displays a symmetrical lower-amplitude waveform that drives the cell in a more-or-less straight line when swimming in relatively non-viscous media, such as the BSP or semen extenders [36]. Deviations from this activated linear motility exist in every ejaculate, from spermatozoa depicting non-linear motility, with long turns; circular motility (circles of varying radius) or even local motility (often vibrating on site, non-translational). CASA-instruments have been designed to cover this spectrum of patterns, the velocity of translation and also to pin-point spermatozoa showing deviations of the sperm head relative to the neck (LDH). Spermatozoa with activated motility acquire, after a sequential exposure to the environment of the uterus and the oviduct (review by [37]), another type of motility; the so-called ''hyperactivated pattern'', which can be seen in spermatozoa recovered from the site of fertilization [11]. In these spermatozoa, the pattern of the tail beat has changed to one that is asymmetric and of higher amplitude, depicting a circular or a figure-8 trajectory with associated changes in LDH in spermatozoa suspended in seminal plasma or semen extender [38]. It is postulated that the hyperactivated spermatozoa swim, however, in a relatively straight-line when exposed to more viscous fluids, such as the intraluminal tubal fluid. This difference presumably aids oviductal spermatozoa to detach from the tubal epithelium, to swim towards the site of fertilization, and to assist the penetration of the zona pellucida (review by [39]). Although hyperactivated motility is often seen in association with the onset of capacitation, it is now clear that these two pathways are separate and divergent because hyperactivation and capacitation can also occur independently of each other [40]. Hyperactivated motility-like sperm behaviour has also been described after *in vitro* induction using surplus Ca^{2+}, a condition characterized by spermatozoa holding a curvilinear velocity (VCL) >97 μm/s and an average LDH >3.5 μm [41], similar to that depicted by boar spermatozoa if exposed *in vitro* to peri-ovulatory fluid collected from the porcine site of fertilization [42].

4. Analyses of motion patterns allows discrimination of sperm sub-populations

Over the years, the boar ejaculate has been considered and analyzed as an entity, and the spermatozoa viewed as forming a single sperm population. The boar ejaculate is, however, delivered in fractions where spermatozoa are present in variable numbers embedded in BSP with variable amounts of fluids and proteins, suggesting they may behave differently (see review by [20]). Moreover, the application of CASA-assessment and a subsequent multivariate pattern cluster analysis of the CASA-derived data (Pattern Analysis statistical computer program, PATN, CSIRO, Canberra, Australia), has led to the knowledge that the boar ejaculate usually contains three-to-four sub-populations of motile spermatozoa [14,16,43–45]. Furthermore, additional sub-populations can be identified by repeated PATN-analyses of the initial clusters, often of the major one [17]. Although the eventual relationship of sub-populations to fertility outcome has been explored and found relevant [16,46], there remains significant variation between ejaculates within sire and also within ejaculate over time, dependent on the processing conditions, since spermatozoa change from one sub-population to another, indicating that the ejaculate consists of discrete sub-populations with different tolerance to cryo-handling. Therefore, it is not surprising that the relationship between sperm sub-populations and sperm quality, particularly the ability of

spermatozoa to sustain cryo-preservation, has been studied the most [9,17,45].

5. Cryo-preservation imposes changes in sperm motility

For cryo-preservation, boar spermatozoa are collected as a bulk sample, albeit often restricted to the sperm-rich fraction of the ejaculate. These spermatozoa are extended in suitable extenders containing substances considered to protect them from chilling and freezing injuries; but they are also centrifuged, cooled, exposed to harmful cryoprotectants, dehydrated during freezing and re-hydrated during thawing. The process of cryo-preservation induces thus both a loss of sperm viability and an impairment of functionality of the spermatozoa surviving thawing, mostly associated to membrane damage [47]. Under the best conditions, ~50% of boar spermatozoa survive cryo-preservation [31,48–50]. Sperm motility seems clearly affected by the procedures, especially the concentration of cryo-protectant, the container used and the temperature/speed of thawing [31,50,51]. This is easily determined by the pattern of movement of the surviving spermatozoa which, immediately post-thaw, is mostly circular and less vigorous compared to that of liquid-preserved semen or extended semen before freezing [51]. This pattern changes to a more linear one already by 30 min post-thaw at 38 °C [31,32,51]. With increasing length of incubation (often not longer than 120 min), spermatozoa slow down, but still maintain their linear motility [31,32]. Such changes are of interest for future studies, since observations in human spermatozoa have shown that cryo-tolerant cells usually show less ''linear'' movement compared to cryo-sensitive cells, which usually depict slow, straight velocity [33]. A major problem is to determine the different populations in the processed sample. Circular motility is often associated with hyperactivation, following the definition reported by Schmidt and Kamp [41], but applying these parameters to the conventionally frozen–thawed boar semen in any of our laboratories, it would mean most spermatozoa are hyperactivated post-thaw regarding VCL but seldom regarding LDH. Obviously, the definition of hyperactivation, based solely on CASA-assessments, needs to be revised.

As already explained, the spermatozoa present in a boar ejaculate constitute a heterogeneous population in terms of their patterns of movement [14]. Being that motility is affected by cryo-preservation, a study was designed to determine whether boar spermatozoa clustered in sub-populations would react – motility-wise – to the process, including thawing [17]. Those boar spermatozoa deemed motile by CASA were allotted to different sperm populations according to their individual kinematic parameters using a series of multivariate pattern cluster analyses (PATN [14]). As well, the progress of these primary sperm populations, and of the sub-populations derived from the largest primary sperm population, was evaluated through the different steps (extension at 22 °C, following removal of BSP and re-extension with lactose-egg yolk at 17 °C, after addition of cryoprotectant at 5 °C, and, post-thaw (incubated at 37 °C for 30 or 150 min)) of a cryo-preservation protocol. A first cluster analysis showed three motile sperm populations (P), the major one being progressive and/or vigorous (P1, 90.4%). The size of these populations remained constant throughout the five steps of the cryo-preservation set-up. Sorting among the P1-spermatozoa with a second PATN, revealed this population was comprised of three sperm sub-sets, namely sP1, cells with progressive and vigorous movement (58.7%); sP2, progressive cells only (24.6%); and sP3, cells with vigorous movement, hyperactive-like (16.7%). While sP1 remained unchanged during the process, both sP2 and sP3 varied significantly through the freezing–thawing procedure, particularly during the removal of the BSP and the cooling to 5 °C, when increases in ''hyperactivated-like'' movement became evident. Perhaps the most important finding was that most spermatozoa showed progressive and vigorous movement, and that this sub-population remained constant during cryo-preservation, albeit its proportion differed among boars [17]. Similar results have been recently found in dogs, where ejaculates having a dominant sub-population of spermatozoa with vigorous movement, best survived cryo-preservation [52,53]. Therefore, use of such a sperm sub-population as a marker would aid in identifying boars with good freezability and, hopefully, good fertility.

6. Does boar seminal plasma affect sperm motility patterns during cryo-preservation?

The ejaculated spermatozoa are immersed in BSP, which owing to the nature of its emission, is ejaculated in rather specific fractions during natural mating [54]. Although these fractions can be distinguished and retrieved manually, boar semen for artificial insemination is usually collected either as bulk (whole) ejaculate (all fractions but the gel-fraction) or, because it holds most spermatozoa, as the sperm-rich fraction [20]. As mentioned above, BSP affects sperm physiology by

increasing their motility (action of bicarbonate, see review by [35]), resistance to cold-shock [22], destabilization of the plasmalemma [55], and cryo-induced DNA-damage [56]. Some BSP fractions play a specific function *in vivo*, such as the gel fraction providing a cervical plug after semen deposition. However, since it clots the rest of the BSP if the ejaculate is collected as a bulk, this particular fraction is, therefore, customarily removed in practice. The contact of the spermatozoa to all the BSP is not always beneficial, nor does it naturally occur. As such, a reduction of fertility follows a lengthy exposure of boar spermatozoa to bulk BSP [57], apparently related to low-molecular weight BSP-components, which compromise sperm function [58]. Therefore, the removal of BSP (by centrifugation or extension with a buffer) is practiced *in vitro*, and considered critical to ensure maximal sperm viability in the majority of protocols of semen cryo-preservation. However, the incubation of boar spermatozoa in homologous sperm-rich fraction BSP at room temperature for up to 20 h did not cause detrimental changes in post-thaw sperm survival [51] nor in the ability to penetrate oocytes *in vitro* [59], indicating that the deleterious changes caused by BSP may be specific to some fractions, which are not necessarily included in the cryo-preservation protocol. Using boar spermatozoa collected in the sperm-rich fraction, adverse decreases in sperm motility were detected after removal of the BSP by centrifugation [17], with these spermatozoa also showing a significant increase in intracellular ROS formation, whose generation causes oxidative stress and impairs sperm survival [60].

The spermatozoa contained in the first jets of the ejaculated sperm rich-fraction (for instance in the first 10 mL of fraction P1) seem to be the ones that mainly contribute to building the oviductal sperm reservoir at the female utero-tubal junction [20]. Concomitantly, the spermatozoa from this particular P1-portion have been found to be less sensitive (or have better resistance) to the stress normally induced (particularly to the plasma membrane) during the cryo-preservation process, compared to the spermatozoa retrieved from the rest of the ejaculate (portion 2, P2) [27–29,31], probably owing to the different nature of the seminal plasma that they are exposed to [20]. For instance, the total protein concentration in the BSP of the P1-ejaculate portion is six-fold lower than in the rest of the ejaculate (P2), while the P1-fraction contains ~50% of the total sperm number ejaculated. Following these findings, where differences were seen between spermatozoa fortuitously present in different fractions of the boar ejaculate, a study was set-up to determine whether sperm kinetics (monitored using CASA at 38 °C) were different between the above two ejaculate portions during cryo-preservation and post-thawing [32]. Semen from mature boars ($n = 4$, 5 replicates) was frozen and thawed in MiniFlatPacks (MFP, 0.5×10^9 spermatozoa/dose). Evaluation was done at 4 specific stages: S1 = after collection (suspended in BTS+®), S2 = at 15 °C (suspended in lactose-egg yolk, LEY), S3 = at 5 °C (suspended in LEY–glycerol–Equex) and, S4 = 30 min post-thaw. The total proportion of motile spermatozoa was always significantly higher among spermatozoa from P1-BSP than the P2-BSP, both during cooling and post-thaw (S1–S4). Within a portion of the ejaculate, total sperm motility did not vary during cooling, but was clearly reduced in S4. Among the motile spermatozoa, the majority displayed non-linear motility similarly in both portions (P1 and P2), but differed between stages of cooling and post-thaw within a portion. The proportion of spermatozoa showing linear motility was similar between ejaculate portions (P1 vs. P2) with a significant increase post-thaw (S4, ~45%). Sperm velocities differed between portions in S1, but they remained similar thereafter (S2–S4), independent of the portion. Values for LDH were higher in P1 than P2 during cooling, but equalised post-thaw, without denoting any hyperactivated-like motility. The data suggested that sperm kinematic parameters were maintained during controlled cooling but altered at thawing. Motility was better maintained for P1 than for P2 at all stages, confirming previous findings of the beneficial nature of this particular portion of the BSP regarding preservation of sperm motility in pigs.

7. Can these effects be confirmed?

In order to confirm that such beneficial effects were due to the hereby named P1-fraction of the BSP, a follow-up study was devised (Saravia et al., unpublished), where sperm motility was checked using two computer-assisted motility analysers, a conventional CASA-instrument (SM-CMA; MTM Medical Technologies, Montreaux, Switzerland) and the Qualisperm™ (Biophos). Aliquots of spermatozoa were collected from P1 (in P1-BSP, positive control), P2 spermatozoa (in P2-BSP, negative control), and also after spermatozoa from P2 were cleansed from ''their'' BSP (P2-BSP) either by washing and centrifugation (CEN, $800 \times g \times 10$ min $\times 2$) or by Percoll density gradient filtration (PER, 30/60, $900 \times g$ for 12 min), followed by a subsequent exposure to pooled (minimum four boars) P1-BSP for 60 min. Sperm kinetics from the four

aliquots of each split ejaculate (five boars, five replicates) were assessed during the complete cooling/freezing and thawing protocol, at the four stages described above, viz. S1 = suspension in BTS (P1 and P2) or BSP from P1 (CEN and PER) at room temperature after a 60-min holding period; S2 = suspension in LEY at 15 °C; S3 = mixing with the third extender (LEY-glycerol-Equex) after cooling to 5 °C for 2 h; and S4 = post-thaw. Special attention was paid to have an approximately similar concentration for fresh, cooled and frozen–thawed spermatozoa. The spermatozoa were packaged at concentrations of 1×10^9 spermatozoa/mL, frozen and thawed in MFPs according to Saravia et al. [31]. Moreover, samples from S1 and S4 were analysed at time 0, i.e. immediately after collection and post-thaw respectively, and also after 30 min of incubation at 38 °C to check for differences over time of incubation. The BSP (P1 and P2) was collected from the same five boars following regular rest periods of 3 or 4 days between collections. The BSP was centrifuged five times ($3000 \times g \times$ 20 min each time) and filtered through 0.2 μM filters (Filtropur S 0.20, Sarstedt AG & Co, Nümbrecht, Germany), and frozen at −20 °C until use.

The output of the computer-aided instruments was, *a priori*, expected to be different. While the SM-CMA-instrument provides the proportion of total motile spermatozoa and, within this population, the proportions (%) of different patterns of motility (as linear, non-linear, circular and local motility) as well as sperm velocities (in μm/s, as curvilinear [VCL], straight-line [VSL] and average [VAP]) and the LDH (μm); the Qualisperm™ only yields the total proportions of motile and immotile spermatozoa, and, for the former, a breakdown of sperm velocity classes, mainly corresponding to local-motile (10.1–25 μm/s) and motile (>25 μm/s) spermatozoa, which roughly corresponded to the settings of the SM-CMA-instrument. As already explained, a major difference between the instruments was the number of spermatozoa analysed, which was significantly higher in the Qualisperm™ (∼4000) than in the SM-CMA (∼250), thus leading to a significantly lower variation per analysed sample for the Qualisperm™ compared to the SM-CMA. However, when comparing the proportions of total motile spermatozoa yielded by the two equipments, the outputs were similar ($P > 0.05$) for all sperm sources (P1, P2, CEN or PER), irrespective of the stage of processing considered (S1–S4), with a significant ($P < 0.05$), albeit low correlation ($r = 0.38$) between instruments. Interestingly, either instrument was able to disclose differences in the pattern of movement of the post-thawed spermatozoa which, immediately post-thaw (time 0), was mostly circular, to become more linear after a 30-min incubation at 38 °C, thus confirming previous studies [31,32,51]. As well, either instrument could detect the presence of highly linear, lower-speed spermatozoa in the PER-cleansed sperm samples.

These similarities of preliminary results suggest the Qualisperm™ might become a suitable instrument for routine use. However, the conventional CASA equipment, where more kinematic variables could be screened, rendered more information than the Qualisperm™ both within the objectives of the study and for comparisons with other studies. For example, the results of the SM-CMA showed the sperm controls (P1 and P2) along S1–S4 depicted consistently similar kinematic changes as in previous studies [32], including the presence of significant differences between sires ($P < 0.001$). The overall results obtained in the present study indicated that the spermatozoa exposed to the P1-BSP-fraction tolerated best the cryo-handling compared to P2-spermatozoa, particularly considering post-thaw values (see Table 1) in terms of absolute kinematic parameters (velocities) and the proportion of total motile spermatozoa. The number of surviving spermatozoa post-thaw differed ($P < 0.001$) within portion as well as between portions, being higher in P1 ($P < 0.05$–0.001) than in P2, thus confirming previous findings [27–29,31,32]. The major sub-population among these total motile spermatozoa in P1–P2 and CEN+P1-BSP depicted non-linear motility (48–70%) throughout the cryo-preservation stages (S1–S4, n.s.). The second sperm sub-population showed a linear motility (20–40%). The PER+P1-BSP spermatozoa, on the other hand, showed a lower proportion of non-linear motile spermatozoa ($P < 0.01$) and a higher ($P < 0.003$) proportion of linearly motile spermatozoa, particularly post-thaw (S4). Cleansing of P2-spermatozoa by CEN, followed by exposure to pooled P1-BSP, increased their total motility to levels similar to the P1-controls, particularly at S4 (post-thaw; see Table 1); but the P2-spermatozoa cleansed by Percoll density gradient (PER) did not reach similar levels, despite being exposed to P1-BSP, probably owing to the type of sperm selection done by Percoll, as shown above for the proportions of linearly motile spermatozoa. This sperm population was always the highest for the PER-procedure at all stages (S1–S4; 46–70%), concomitantly showing the lowest VCL (81–96 μm/s) and LDH values (1.7–2.3 μm). The results confirm the capacity of Percoll density gradients to primarily separate spermatozoa with the highest linearity and straightness, features that could be picked up by computer-assisted

Table 1
Percentages (means ± S.E.M.) of total motility for spermatozoa collected from two different portions of the ejaculate (portion 1, P1 = the first 10 mL of the sperm-rich fraction; portion 2, P2 = the rest of the ejaculate); and of P2-spermatozoa, cleansed either by washing and centrifugation (CEN) or by filtration through Percoll density gradient (PER) and thereafter exposed for 60 min to pooled P1-boar seminal plasma (P2 + P1-BSP)

Processing stage[†]	Source of spermatozoa			
	P1*	P2*	P2-CEN + P1-BSP*	P2-PER + P1-BSP**
S1	90.0 ± 1.41^{aA}	80.5 ± 2.61^{aAB}	87.9 ± 1.44^{aA}	75.8 ± 3.03^{aB}
S2	88.8 ± 1.37^{aA}	83.6 ± 1.78^{aA}	83.1 ± 1.71^{aA}	68.7 ± 3.61^{aAB}
S3	92.4 ± 0.83^{aA}	82.4 ± 1.56^{aB}	80.1 ± 2.43^{aB}	64.4 ± 4.27^{bC}
S4	61.8 ± 2.70^{bA}	53.1 ± 2.65^{bB}	61.6 ± 2.62^{bA}	41.7 ± 2.37^{cC}

The spermatozoa were subjected to extension, cooling, freezing and thawing [31] and the motility assessed using computer-assisted sperm analysis (CASA; SM-CMA) following 30 min of incubation at 38 °C, at different stages of the cryo-preservation process (S1 = after collection/extension in BTS, S2 = at 15 °C, S3 = at 5 °C, and S4 = post-thaw (five boars, five replicates)). Values with different superscripts indicate significant differences ($P < 0.05$–0.001) between stages of cryo-preservation$^{(a–c,\ \text{within column})}$ or between sperm sources$^{(A–C,\ \text{within row})}$.

[†] For S1–S4: most (48–70%) motile spermatozoa in P1–P2-CEN* depicted a non-linear motile kinematic pattern (n.s.), while those assessed after PER** showed mostly (46–70%) a linear motile pattern, highest at S4 ($P < 0.003$).

instrumentation (SM-CMA or Qualisperm^{TM}) despite their different methodological rationale, and clearly showing the characteristics of an ''activated'' pattern, not hyperactivated [41]. A problem with the use of Percoll (or other density gradients) is the low recovery rate (<10%), implying substantial cell losses [61] and perhaps biasing the results [33].

Among the other sperm sub-sets, LDH and VCL were at border levels for the so-called ''hyperactivation'' according to Schmidt and Kamp [41], but it is difficult to conceive that most spermatozoa would become hyperactive by simply diluting (S1) or removing (S2) the BSP, since availability of Ca^{2+} seems the most relevant trigger [38,41], and Ca^{2+}-chelating agents are present in the extender used. Taken together, these preliminary results suggest that, irrespective of the spermatozoa considered, exposure to P1-BSP led them to sustain better motility even after a complete protocol for cryo-preservation compared to the P2-BSP. The results of PER pre-treatment are, in this respect, confusing, but might relate to the effects of the procedure *per se*, rather than on the effect of the P1-BSP.

8. What might be causing this difference between BSP-portions?

Tentatively, the type of BSP present in the portions studied may cause a difference in motility patterns, either because they had a different constitution of inorganic or organic components (for instance, bicarbonate or Zn^{2+} differ among portions of the ejaculate), or proteins, of which spermadhesins (12–16 kDa) are majority [62,63]. Regarding spermadhesins, the portions P1 and P2 of the BSP hereby tested differ significantly in their relative content of total protein, which is larger in P2 (32.4 ± 12.7 g/L) than in P1 (7.0 ± 1.3 g/L), with clear differences in electrophoresis separation on agarose gels [20]. Further analyses obtained by combination of reverse-phase HPLC and MALDI-TOF mass spectrometry have shown that the protein compositions of P1-BSP and P2-BSP also differ, with P1-BSP being characterized by major components of 5, 7 (acrosin inhibitor), and 10 kDa, which are not present in P2-BSP (Calvete et al., unpublished). The major components of P2-BSP are the spermadhesins (including the glycosylated heterodimer PSP-I/PSP-II), which are almost absent in P1-BSP. Perhaps it is the higher concentration of spermadhesins that confers on the P2-spermatozoa a poorer motility than those exposed to P1-BSP. Low concentrations of particular spermadhesins (such as the non-heparin binding PSP-I/PSP-II heterodimer) have been shown to preserve sperm motility in boar spermatozoa either highly extended, cryo-preserved or sorted by high-speed flow cytometry [64–66]. Another possible explanation is the larger amount of heparin-binding spermadhesins in P2-BSP which have a documented detrimental effect on sperm motility *in vitro* [67], or the presence in this portion of low-molecular weight components that restrain motility, such as the anionic peptide of 5.7 kDa sperm motility inhibiting factor (SMIF) [68]. Further studies are needed to determine which factors in the BSP are responsible for these differences in sperm kinetics. Provided that isolation can be done and properly tested, they may offer advantages in enhancing sperm motility.

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References

[1] Rodriguez-Martinez H. Can we increase the estimative value of semen assessment? Reprod Domest Anim 2006;41(Suppl 2): 2–10.
[2] Rodriguez-Martinez H. State of the art in farm animal sperm evaluation. Reprod Fertil Dev 2007;19:91–101.
[3] Vyt P, Maes D, Rijsselaere T, Dejonckheeere E, Castryck F, Van Soom A. Motility assessment of porcine spermatozoa: a comparison of methods. Reprod Domest Anim 2004;39:447–53.
[4] Popwell J, Flowers W. Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. Anim Reprod Sci 2004;81:97–113.
[5] Gadea J, Sellés E, Marco M. The predictive value of porcine seminal parameters on fertility outcome under commercial conditions. Reprod Domest Anim 2004;39:303–8.
[6] Holt C, Holt WV, Moore HDM, Reed HCB, Curnock RM. Objectively measured boar sperm motility parameters correlate with the outcomes on-farm inseminations: results of two fertility trials. J Androl 1997;18:312–23.
[7] Tardif S, Laforest JP, Cormier N, Bailey J. The importance of porcine sperm parameters on fertility in vivo. Theriogenology 1999;52:447–59.
[8] Gadea J. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology 2005;63:431–44.
[9] Gil MA, Roca J, Cremades T, Hernández M, Vázquez JM, Rodríguez-Martínez H, et al. Does multivariate analysis of post-thaw sperm characteristics accurately estimate in vitro fertility of boar individual ejaculates? Theriogenology 2005;64:305–16.
[10] Mortimer ST. CASA—practical aspects. J Androl 2000;21: 515–24.
[11] Suarez SS, Dai XB, DeMott RP, Redfern K, Mirando MA. Movement characteristics of boar sperm obtained from the oviduct or hyperactivated in vitro. J Androl 1992;13:75–80.
[12] Rodriguez-Martinez H, Barth AD. In vitro evaluation of sperm quality related to in vivo function and fertility. In: Juengel JI, Murray JF, Smith MF, editors. Reproduction in domestic ruminants VI. Nottingham, UK: Nottingham University Press; 2007. p. 39–54.
[13] Holt WV, Medrano A, Thurston LM, Watson PF. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology 2005;63:370–82.
[14] Abaigar T, Holt WV, Harrison RAP, del Barrio G. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessment. Biol Reprod 1999;60:32–41.
[15] Abaigar T, Barbero J, Holt WV. Sperm heterogeneity as revealed by a new approach of sperm motility function. Reprod Domest Anim 2005;40:332.
[16] Quintero-Moreno A, Rigau T, Rodriguez-Gil JE. Regression analyses and motile sperm subpopulation structure as improving tools in boar semen quality analysis. Theriogenology 2004;61: 673–90.
[17] Cremades T, Roca J, Rodriguez-Martinez H, Abaigar T, Vazquez JM, Martinez EA. Kinematic changes during the cryopreservation of boar spermatozoa. J Androl 2005;26:610–8.
[18] Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol Reprod 2002;66: 545–54.
[19] Roca J, Rodriguez-Martinez H, Vazquez JM, Bolarín A, Hernandez M, Saravia F, et al. Strategies to improve the fertility of frozen–thawed boar semen for artificial insemination. In: Ashworth CJ, Kraeling RR, editors. Control of pig reproduction VII. Manor Farm, Thrumpton, UK: Nottingham University Press; 2006. p. 261–75.
[20] Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vázquez JM, et al. Boar spermatozoa in the oviduct. Theriogenology 2005;63:514–35.
[21] Robertson SA. Seminal fluid signalling in the female reproductive tract: lessons from rodents and pigs. J Anim Sci 2007;85: E36–44.
[22] Pursel VG, Schulman LL, Johnson LA. Effect of dilution of seminal plasma and interaction period on cold shock susceptibility of boar spermatozoa. J Anim Sci 1973;37:528–31.
[23] Roca J, Gil MA, Hernández M, Parrilla I, Vazquez JM, Martinez EA. Survival and fertility of boar spermatozoa after freeze–thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:91–9.
[24] Roca J, Rodriguez MJ, Gil MA, Carvajal G, Garcia EM, Cuello C, et al. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15–24.
[25] Caballero I, Vazquez JM, Centurion F, Rodriguez-Martinez H, Parrilla I, Roca J, et al. Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. Reprod Domest Anim 2004;39:370–5.
[26] Xu X, Ding J, Seth PC, Harbison DS, Foxcroft GR. In vitro fertilization of in vitro matured pig oocytes: effects of boar and ejaculate fraction. Theriogenology 1996;45:745–55.
[27] Sellés E, Wallgren M, Gadea J, Rodriguez-Martinez H. Sperm viability and capacitation-like changes in fractions of boar semen after storage and freezing. Proceedings of the 6th International Conference on Pig Reproduction, vol. 1, Missouri, USA; 2001. p. 51.
[28] Peña FJ, Johannisson A, Wallgren M, Rodríquez-Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 2003;78:85–98.
[29] Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. Zygote 2004;12:117–24.
[30] Peña FJ, Saravia F, Núñez-Martínez I, Johannisson A, Wallgren M, Rodriguez-Martinez H. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Anim Reprod Sci 2006;93:101–13.
[31] Saravia F, Wallgren M, Nagy S, Johannisson A, Rodríguez-Martínez H. Deep freezing of concentrated boar semen for intrauterine insemination: effect on sperm viability. Theriogenology 2005;63:1320–33.
[32] Saravia F, Hernández M, Wallgren MK, Johannisson A, Rodríguez-Martínez H. Cooling during semen cryopreservation does

not induce capacitation of boar spermatozoa. Int J Androl 2007;30:485–99.
[33] Katkov II, Lulat AG. Do conventional CASA-parameters reflect recovery of kinematics after freezing? "CASA paradox" in the analysis of recovery of spermatozoa after cryopreservation. CryoLetters 2000;21:141–8.
[34] Rodriguez-Martinez H, Ekstedt E, Einarsson S. Acidification of the epididymal fluid in the boar. Int J Androl 1990;13:238–43.
[35] Rodriguez-Martinez H. Aspects of the electrolytic composition of boar epididymal fluid with reference to sperm maturation and storage. In: Boar Semen Preservation II, Johnson L, Rath D, editors. Reprod Domest Anim 1991;Suppl 1:13–27.
[36] Turner RM. Moving to the beat: a review of mammalian sperm motility regulation. Reprod Fertil Dev 2006;18:25–38.
[37] Rodriguez-Martinez H. Role of the oviduct in sperm capacitation. Theriogenology 2007;68:138–46. doi: 10.1016/j.theriogenology.2007.03.018.
[38] Ho HC, Suarez SS. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction 2001;122:519–26.
[39] Suarez SS. Sperm interactions with the female tract: inspiration for assisted reproduction. Reprod Fertil Dev 2007;19:104–10.
[40] Marquez B, Suarez S. Different signalling pathways in bovine sperm regulate capacitation and hyperactivation. Biol Reprod 2004;70:1626–33.
[41] Schmidt H, Kamp G. Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. Reproduction 2004;128:171–9.
[42] Nichol R, Hunter RHF, De Lamirande E, Gagnon C, Cooke GM. Motility of spermatozoa in hydrosalpingeal and follicular fluid of pigs. J Reprod Fertil 1997;110:79–86.
[43] Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 1996;31:17–24.
[44] Holt C, Holt WV, Moore DM. Choice of operating conditions to minimize sperm subpopulation sampling bias in the assessment of boar semen by computer-assisted semen analysis. J Androl 1996;17:587–96.
[45] Thurston LM, Watson PF, Mileham AJ, Holt WV. Post-thaw functional status of boar spermatozoa cryopreserved using three controlled rate freezers: a comparison. Theriogenology 2003;60:101–3.
[46] Satake N, Elliot RMA, Watson PF, Holt WV. Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. J Exp Biol 2006;209:1560–72.
[47] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60–61:481–92.
[48] Eriksson BM, Rodriguez-Martinez H. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in large 5 ml packages (FlatPack). Anim Reprod Sci 2000;63:205–20.
[49] Carvajal G, Cuello C, Ruiz M, Vazquez JM, Martinez EA, Roca J. Effect of centrifugation before freezing on boar sperm cryosurvival. J Androl 2004;25:389–96.
[50] Hernández M, Roca J, Gil MA, Vázquez JM, Martinez EA. Adjustments on the cryopreservation conditions reduce the incidence of boar ejaculates with poor sperm freezability. Theriogenology 2007;67:1436–45.
[51] Eriksson BM, Vazquez JM, Martinez E, Roca J, Lucas X, Rodriguez-Martinez H. Effect of holding time during cooling and of type of package on plasma membrane integrity, motility and in vitro oocyte penetration ability of frozen–thawed boar spermatozoa. Theriogenology 2001;55:1593–605.
[52] Núñez-Martínez I, Morán JM, Peña FJ. Two step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J Androl 2006;27:596–603.
[53] Núñez Martinez I, Moran JM, Peña FJ. A three step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reprod Domest Anim 2006;41:408–15.
[54] Mann T, Lutwak-Mann C. Male reproductive function and semen. In: Themes and trends in physiology, biochemistry and investigative andrology. Berlin: Springer-Verlag; 1981. pp. 495.
[55] Maxwell WM, Johnson LA. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology 1999;52:1353–62.
[56] Fraser L, Strzezek J. Effect of different procedures of ejaculate collection, extenders and packages on DNA integrity of boar spermatozoa following freezing–thawing. Anim Reprod Sci 2007;99:317–29.
[57] Iwamoto T, Tsang A, Luterman M, Dickson J, de Lamirande E, Okuno M, et al. Purification and characterization of a sperm motility-dynein ATPase inhibitor from boar seminal plasma. Mol Reprod Dev 1992;31:55–62.
[58] Fraser L, Dziekonska A, Strzezek R, Strzezek J. Dialysis of boar semen prior to freezing–thawing: its effects on post-thaw sperm characteristics. Theriogenology 2007;67:994–1003.
[59] Suzuki K, Eriksson B, Shimizu H, Nagai T, Rodriguez-Martinez H. Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized in vitro. Int J Androl 2000;23:13–21.
[60] Hernandez M, Vasco D, Vazquez JM, Martinez EA, Roca J. Oxidative stress and its relationship to membrane instability through the freezing-thawing process of boar spermatozoa. Reprod Domest Anim 2007;42(S2):94–5.
[61] Rodriguez-Martinez H, Larsson B, Pertoft H. Evaluation of sperm damage and techniques for sperm clean-up. Reprod Fertil Dev 1997;9:297–308.
[62] Calvete JJ, Sanz L, Dostàlovà Z, Töpfer-Petersen E. Spermadhesins: sperm-coating proteins involved in capacitation and zona pellucida binding. Fertilität 1995;11:35–40.
[63] Strzezek J, Wysocki P, Kordan K, Kuklinska K. Proteomics of boar seminal plasma—current studies and possibility of their application in biotechnology of animal reproduction. Reprod Biol 2005;5:271–90.
[64] Caballero I, Vazquez JM, Calvete JJ, Roca J, Sanz L, Parrilla I, et al. Low doses of the seminal plasma PSP-I/PSP-II heterodimer spermadhesin influence pig oocyte maturation in vitro and decrease sperm penetration. J Androl 2004;25:1004–12.
[65] Caballero I, Vazquez JM, Rodriguez-Martinez H, Gil MA, Calvete JJ, Sanz L, et al. Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction. Zygote 2005;13:11–6.
[66] Caballero I, Vazquez JM, García EM, Roca J, Martínez EA, Calvete JJ, et al. Immunolocalization and possible functional role of PSP-I/PSP-II heterodimer in highly-extended boar spermatozoa. J Androl 2006;27:766–73.
[67] Centurión F, Vazquez JM, Calvete JJ, Roca J, Sanz L, Parrilla I, et al. Influence of porcine spermadhesins on the susceptibility of boar spermatozoa to high dilution. Biol Reprod 2003;69:640–6.
[68] Kordan W, Holody D, Eriksson B, Fraser L, Rodriguez-Martinez H, Strzezek J. Sperm motility inhibiting factor (SMIF). A plasmatic peptide with multifunctional biochemical effects on boar spermatozoa. Reprod Domest Anim 1998;33:347–54.