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Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct

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Summary

When spermatozoa from two or more boars are mixed and females inseminated, the resulting litters are often skewed in favour of one male but there is currently no satisfactory physiological explanation for this effect. However, to reach the oocytes, the spermatozoa must enter the oviduct where they are exposed to factors that modulate their activity. They either become sequestered within the oviductal sperm reservoir or bypass the reservoir and proceed towards the oocytes. The oviduct may therefore hold the key to mammalian sperm selection, thereby explaining why laboratory tests of sperm function, performed on whole ejaculates, are unable to account for the boar-specific skewing effects. We have previously shown that boar sperm motility is highly stimulated by bicarbonate, a naturally abundant component of oviductal fluid. Using motility-based sperm subpopulation analysis, we show here that the relative sizes of bicarbonate-responsive and unresponsive sperm subpopulations vary between individual boars. Proteins derived from oviduct epithelial plasma membranes suppress the activation response and modify sperm movement trajectories in a subpopulation-specific and dose-dependent manner. The suppression response varies between boars and some spermatozoa remain unsuppressed in the presence of oviductal proteins. When boars are ranked according to their susceptibility to bicarbonate-induced stimulation, rankings differ depending upon the presence or absence of oviductal proteins. The suppression response is not caused by inhibition of bicarbonate uptake; on the contrary this is enhanced by oviductal proteins. We suggest that the boar-specific and sperm subpopulation-specific interactions between sperm motility activation and suppression responses are likely to result in sperm selection before the spermatozoa meet the oocytes.

Key words: bicarbonate, sperm motility, sperm transport, intracellular pH, fallopian tube.

Introduction

Sperm motility is widely recognised as a significant and general determinant of male reproductive success. However, species differences in the events preceding fertilisation have led to the evolution of sophisticated motility control mechanisms that influence the relative reproductive success of males. In freshwater fishes, sperm motility is activated by sudden exposure to water and spermatozoa must reach and fertilise eggs within a very short activation time. In this situation the ability to produce faster spermatozoa provides a fertility advantage, as demonstrated by Gage et al. in studies of Atlantic salmon (Gage et al., 2004). In similar vein, Creech et al. showed that nitric oxide produced near the micropyle of fathead minnow oocytes stimulates sperm velocity, providing a further advantage to those spermatozoa with potential for faster motility (Creech et al., 1998). It is also becoming clear that modification of flagellar beating patterns in response to external signalling factors is the basis of sperm chemotaxis across a wide range of species, from species with external fertilisation such as sea urchins and siphonophores to those whose fertilisation occurs internally (Ralt et al., 1994; Sun et al., 2005; Xiang et al., 2004; Xiang et al., 2005).

In species with internal fertilisation, the significance of sperm motility is made more complex because the spermatozoa encounter different environments within the male and female reproductive tract, and are exposed to factors with cell signalling capabilities. Moreover, the initial stages of sperm transport are mediated by the female tract itself. Sperm transport from the vagina, through the cervical canal and towards the oviduct, is mediated by a combination of intrinsic sperm motility and peristaltic movements of the female reproductive tract. However, the entrance to the oviduct, the uterotubal junction, acts as a selective barrier to sperm transport. Several studies have indicated that in order to reach the oviductal environment, spermatozoa must be physically intact [i.e. possess intact plasma membrane and acrosome

(Ellington et al., 1999; Esponda and Moreno, 1998)], functionally competent at the molecular level (Nakanishi et al., 2004) and appropriately motile (Herrmann et al., 1999; Olds-Clarke, 1996).

Heterospermic insemination studies using mixed semen from two or more males have consistently demonstrated that conception rates are skewed in favour of certain individuals [cattle (Beatty et al., 1969; Beatty et al., 1976; Stewart et al., 1974), pigs (Berger et al., 1996; Stahlberg et al., 2000), rabbits (Vicente et al., 2004)] even though equivalent numbers of live sperm have been used. This situation may mimic the processes of sperm competition, a phenomenon that occurs among many species, including mammals (Birkhead, 1995; Parker, 1970). Physiological explanations for this paternity skewing effect still remain unsatisfactory when interpreted in terms of standard sperm function tests such as motility and viability.

In her study of heterospermic insemination and skewed paternity in pigs, Berger et al. found a correlation between dominance in conception and sperm-egg binding ability (Berger et al., 1996). This implies that ability to reach the eggs should be correlated with ability to traverse the uterotubal junction and to negotiate the oviduct; it is not yet clear whether these abilities are actually correlated. Detailed studies of sperm motility in mice have, nevertheless, shown that the uterotubal junction effectively blocks the transit of spermatozoa with defective flagellar function (Herrmann et al., 1999; Olds-Clarke, 1986).

These findings emphasise the importance of intrinsic sperm motility in populating the oviductal reservoir. Upon ejaculation, mammalian spermatozoa are briefly exposed to seminal plasma but then enter the uterine environment prior to reaching the oviduct. Once within the oviductal isthmus, significant modification of motility occurs as this region is involved in storing spermatozoa for periods of varying duration prior to fertilisation (Birkhead and Moller, 1993). Observations of spermatozoa taken from the isthmic reservoir of several species have consistently shown that their motility is suppressed (e.g. Bedford and Breed, 1994; Burkman et al., 1984; Overstreet and Cooper, 1975; Overstreet et al., 1980) and motility modulation has also been observed in spermatozoa exposed to oviductal fluid (McNutt et al., 1994). The sperm reservoir within the oviductal isthmus therefore contains a selected and highly fertile subpopulation of the original ejaculate (Ellington et al., 1999; Gualtieri and Talevi, 2003; Suarez, 1998) and it is unsurprising that measures of motility based on whole ejaculates show little correlation with fertility under competitive conditions such as heterospermic insemination.

Previous experiments have shown that boar spermatozoa are highly sensitive to the presence of bicarbonate, responding with rapid motility activation (Harrison et al., 1996a; Harrison and Miller, 2000; Holt and Harrison, 2002; Okamura et al., 1985; Tajima et al., 1987) and modification of membrane lipid architecture (Harrison, 2004). When semen samples are washed using a bicarbonate-free Percoll density gradient the viability of the resultant population is typically 80–90%; however, the cells are often quiescent. Adding >5 mmol l^{-1} bicarbonate/CO_2 to the sperm suspension without changing external pH, rapidly (<2 min) stimulates the motility of some, but not all, spermatozoa. This mimics the situation within the porcine female reproductive tract, where entry to the oviduct is accompanied by an elevation of bicarbonate concentration from <5 mmol l^{-1} in the uterine horns to approximately 35 mmol l^{-1} in oviductal fluid (Rodriguez-Martinez et al., 2005) (J. M. Vazquez, personal communication). Here we investigate the relative proportions of such bicarbonate-sensitive spermatozoa in different ejaculates and examine the possibility that these might represent the functionally privileged population selected for entry into the oviduct. However, if sperm motility suppression is induced in the oviduct, the model would be incomplete without also investigating the interaction with oviductal factors. We therefore tested the effects of a soluble fraction of oviductal epithelial cell apical plasma membrane proteins (APM), which is known to enhance sperm survival *in vitro* (Elliott et al., 2001; Fazeli et al., 2000; Fazeli et al., 2003; Holt et al., 2005), on the degree of motility stimulation induced by bicarbonate/CO_2.

To examine these effects in detail we analysed trajectory data from individual spermatozoa using pattern analysis (Abaigar et al., 1999); this analysis allows us to examine and compare the behaviours of sperm subpopulations within ejaculates and to see how the population responses vary between individual boars. In essence, the study confirms the existence of a bicarbonate-sensitive sperm subpopulation within each ejaculate and shows that the oviductal protein fraction suppresses sperm activation in a dose-dependent manner. Interestingly, the oviductal proteins also modify the nature of sperm motion, higher doses leading to increasingly linear trajectories. We also confirmed that the oviductal proteins do not suppress motility by blocking bicarbonate/CO_2 transport; on the contrary it was found that they caused enhanced intracellular alkalinisation, a process associated with the onset of capacitation (Zeng et al., 1996).

Materials and methods

Collection and washing of spermatozoa

Sperm-rich fractions of semen were collected from fertile boars (*Sus scrofa* Linnaeus 1758) from the colony kept by JSR Genetics Limited at Thorpe Willoughby (Selby, Yorkshire, UK) for commercial artificial insemination; various breeds were represented. The semen samples were supplied (via Royal Mail Special Delivery, arriving the day after semen collection) already diluted in Beltsville thawing solution (BTS) extender, and were stored for no more than one more day.

Prior to experimentation, sperm were isolated from the diluted semen by sedimentation through a two-step Percoll gradient. 7-ml aliquants of diluted semen were layered over 2 ml 35% Percoll on 2 ml 70% Percoll; the Percoll suspensions were iso-osmotic, prepared according to the method of Vincent and Nadeau (Vincent and Nadeau, 1984) in a saline medium: 137 mmol l^{-1} NaCl, 10 mmol l^{-1} glucose, 2.5 mmol l^{-1} KOH,

20 mmol l^{-1} Hepes, pH 7.4 at 20°C. Centrifugation was performed for 15 min at 200 $\boldsymbol{g}_{max}$ followed by 900 $\boldsymbol{g}_{max}$. After centrifugation, the supernatant layers were removed by aspiration to leave about 0.4 ml of the 70% Percoll, in which the loose sperm pellets were resuspended. These preparations (final concentration approximately 4×10^8 sperm ml^{-1}, viability >90%) were kept at ambient temperature protected from draughts and used within 2 h of washing.

Media and reagents

A basal Tyrode's-based incubation medium (M solution) (Harrison et al., 1996b) consisted of 116 mmol l^{-1} NaCl, 3.1 mmol l^{-1} KCl, 0.4 mmol l^{-1} $MgSO_4$, 0.3 mmol l^{-1} NaH_2PO_4, 5 mmol l^{-1} glucose, 21.7 mmol l^{-1} sodium lactate, 1 mmol l^{-1} sodium pyruvate, 1 mmol l^{-1} EGTA, 20 mmol l^{-1} Hepes (adjusted with NaOH to pH 7.6 at 20°C), 3 mg BSA ml^{-1}, 100 μg kanamycin ml^{-1}, and 20 μg phenol red ml^{-1}; the final pH at 38°C was 7.4 and the osmolality 300 mOsmol kg^{-1}. Bicarbonate/CO_2 was added in the form of suitable aliquants of a 300 mmol l^{-1} aqueous solution of $NaHCO_3$ saturated with 100% CO_2 (a ratio of bicarbonate:CO_2 yielding, after dilution, pH 7.4 at 38°C; addition of this stock did not disturb the pH of the medium). Control incubations received similar aliquants of 300 mmol l^{-1} NaCl unless stated otherwise. To prevent loss of CO_2 during subsequent incubation, the bicarbonate-containing suspensions were maintained under a CO_2-containing atmosphere. 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) was prepared as a 1 mmol l^{-1} stock solution in M solution and added as appropriate to the experimental tubes (final concentrations 100 μmol l^{-1} or 600 μmol l^{-1}) prior to the addition of spermatozoa.

Measurement of intracellular pH

Measurements of intracellular pH (pH_i) were undertaken using the H^+-sensitive fluoroprobe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF) following the procedures described by Browning and Wilkins (Browning and Wilkins, 2004) and Santi et al. (Santi et al., 1998). Fluorescence ratios were measured (using a Gemini Spectramax microplate reader; Molecular Devices Ltd, Wokingham, UK; excitation wavelengths 490 nm and 440 nm; emission 535 nm) in triplicate, from 100 μl cell suspensions in 96-well plates. Calibration of the procedure was undertaken using a nigericin-high K^+ technique over the pH range 6.2–8.0. A linear calibration curve (pH *vs* R_{BCECF}) was constructed for each experiment; R^2 for acceptable calibration curves always exceeded 0.995.

Preparation of apical plasma membrane fractions

Oviducts were obtained from a local abattoir and placed in ice within 20 min of slaughter. These were transported to the laboratory in ice cold PBS and separated from the remainder of the reproductive tissue, washed five times in PBS and kept on ice for the duration of the procedure. As previous studies in this laboratory have shown that apical plasma membrane preparations (APM) and solubilised APM (sAPM) from isthmus and ampulla are equally effective in prolonging sperm viability *in vitro* (Fazeli et al., 2003), the two regions of oviductal tissue were pooled for this study. The methods used for preparing APM and sAPM have been described previously (Holt et al., 2005).

Experimental protocol for sperm motility analysis

Because each video recording of motility took about 3 min, and needed to be initiated at set intervals after the initiation of treatments, incubations were carried out one at a time. Six or eight incubations were normally carried out on a single day, from which direct comparisons (either between different ejaculates or between treatments) could be made.

Incubations were carried out as follows. M solution (1 ml), containing sAPM or DIDS if appropriate, was prewarmed to 38°C in a capped 15-ml polystyrene tube (Sterilin, Stone, Staffs, UK). An aliquant (3–5 μl) of washed sperm (final concentration approximately 2×10^6 cells ml^{-1}) was added, and the suspension incubated at 38°C for 10 min. A 60 μl sample was then removed for motility analysis. Next, half the remaining suspension was transferred to an empty prewarmed tube and to it was added the control treatment (NaCl as control for bicarbonate addition). 12 min after the initiation of incubation, the bicarbonate/CO_2 mixture ('activator') was added to the rest of the sperm suspension (in the first tube), achieving a final bicarbonate concentration of 15 mmol l^{-1}. Incubation of both tubes was continued, and further 60 μl samples were taken for motility analysis: from the 'experimental' tube at 5-min intervals up to a maximum of 32 min after addition of activator. A final sample was taken from the 'control' tube 5 min after the last activated sample.

Sperm motility was recorded by videomicroscopy as described by Holt and Harrison (Holt and Harrison, 2002). 60 μl samples were taken from the sperm suspensions and placed on electropositive glass slides; microscopy was performed using an Olympus BH-2 microscope with a ×10 negative–high phase contrast objective. Sperm video sequences were recorded on CDs using a VCD recorder (VDR-3000; Datavideo UK Ltd, Manchester, UK).

Two hundred individual sperm trajectories were analysed quantitatively for each of the treatment/time combinations, using a Hobson Sperm Tracker (Hobson Tracking Systems, Sheffield, UK) operating at 50 Hz within an IBM-compatible Pentium computer. The 'search radius' used was 5.9 μm, and the 'minimum track points' setting was 50 frames.

The measured descriptors of sperm motion are summarised in Table 1. Details regarding the use of the Hobson Sperm Tracker and discussion of these parameters may be found in earlier publications (Abaigar et al., 1999; Davis and Siemers, 1995).

Detailed exploration of individual sperm trajectories was undertaken using purpose-written software (GET XY) supplied by Prof. G. S. Hobson (Hobson Tracking Systems, Sheffield, UK). This software provides series of x,y coordinates of sequential sperm head positions at a resolution of 50 Hz from

Table 1. *Definitions of motility descriptors*

Descriptors	Units	Description
Curvilinear velocity (VCL)	$\mu m\ s^{-1}$	Velocity of progression along the entire trajectory
Average path velocity (VAP)	$\mu m\ s^{-1}$	Velocity of progression along the smoothed trajectory
Straight line velocity (VSL)	$\mu m\ s^{-1}$	Velocity of progression from first to last coordinates
Beat cross frequency (BCF)	Hz	Frequency that the sperm head crosses the smoothed trajectory
Amplitude of lateral head displacement (ALH)	μm	Mean lateral sperm head displacement along the smoothed trajectory
Linearity of track (LIN)	%	VSL/VCL×100
Straightness of track (STR)	%	VSL/VAP×100

video recordings; these can be used to reconstruct and plot sperm trajectories using auxiliary software such as Microsoft Excel or Statistica for Windows (Statsoft UK, Letchworth, UK).

Intracellular pH responses to the presence of sAPM or 100 $\mu mol\ l^{-1}$ DIDS (with and without bicarbonate addition) were tested directly in parallel with the sperm motility responses; this was achieved using split semen samples (N=9 boars). pH_i response profiles were recorded at 5-min intervals up to 60 min.

Preparation and use of protein-coated beads

sAPM proteins or bovine serum albumin were coupled to polymeric beads with derivatised (formyl groups), chemically active surface properties (Toyopearl AF-formyl 650M; Tosoh Corporation, Japan) using a carbodiimide amine coupling procedure following the manufacturer's protocol. Protein solutions (sAPM and bovine serum albumin; 0.125–2.00 $mg\ ml^{-1}$) were mixed with resin and $NaCNBH_3$ and agitated gently overnight at room temperature. Formyl groups were blocked using further $NaCNBH_3$ treatment for 1 h at 25°C. Efficiency of protein coupling was determined using a fluorescamine-based protein assay suitable for a fluorescence microplate reader (Lorenzen and Kennedy, 1993).

Percoll-washed semen samples were diluted to a concentration of $100–120\times10^6\ ml^{-1}$ in modified Tyrode's solution (TLP) containing 4 $mg\ ml^{-1}$ BSA. 0.5 ml of sperm suspension was applied to small columns of protein-coupled resin beads (0.25 ml packed volume prepared in 1 ml disposable syringes). The sperm samples were washed through the columns with a series of 16×0.5 ml aliquants of M medium. The columns were then washed with 0.4 ml of fixative (4% v/v glutaraldehyde, 4% w/v formaldehyde in 100 $mmol\ l^{-1}$ phosphate buffer). Finally, the column materials were extruded and aliquants of resin beads were applied to glass coverslips that had been precoated with 0.5% poly-L-lysine. The preparations were subsequently dehydrated and sputter-coated with gold for scanning electron microscopy.

Statistical analysis

Sperm trajectory data from the Hobson Sperm Tracker were downloaded into Statistica for Windows (Statsoft UK, Letchworth, UK) for analysis. For the preliminary analysis of summary statistics, mean parameter values were derived from each set of 200 sperm trajectories. These represented replicate values and were used in initial analyses of variance (ANOVAs) after log or arcsine transformation (Zar, 1984). Planned comparisons within the experimental designs were analysed using contrast analysis. More detailed analyses were also performed using multivariate pattern analysis software PATN (Abaigar et al., 1999).

Multivariate analyses of sperm motion parameters were carried out using the computer program PATN (Belbin, 1993). The program uses a series of procedures to analyse and compare the motility parameter values associated with each spermatozoon so as to identify sub-groups within the sperm population ('patterns'). The identification of the sub-groups and their hierarchical classification is carried out by the program independently of the investigator, who is simply required to judge to what degree subgroups may be combined to yield a sufficiently small number of groups to allow practical interpretation. In all of the experiments described here the PATN software identified three sperm subpopulations.

A more complete description and illustration of the use of PATN analysis to identify subpopulations within boar sperm samples is given by Abaigar et al. (Abaigar et al., 1999). However, a few key points are worth mentioning. The PATN analysis was performed using data from all individual spermatozoa within a single experiment and the data need not be normally distributed. Occasional trajectories where AREA>600 μm^2 (representing groups of spermatozoa) were excluded from the data; any zero values for straight line velocity ($\mu m\ s^{-1}$; VSL), beat cross frequency (Hz; BCF) and amplitude of lateral head displacement (ALH) were transformed to 0.1. Data sets for analysis were prepared by merging raw data files from every measured sperm sample. As each of these contains data from approximately 200 spermatozoa, the combined data sets contain many thousands of cases (sets of values for each sperm trajectory). Unlike many conventional cluster analysis software packages, PATN is not limited by the computer memory and such large amounts of data can be analysed on a conventional PC. Upon completion of the PATN analysis, each individual spermatozoon was categorised as belonging to one of the small number of groups, or subpopulations, described above. In this study the groups were distinguished on the basis of multivariate combinations of motion descriptors, and qualitative interpretation of the group structure was therefore based on the descriptive interpretation of the sperm motion behaviour that each group represents. Multivariate group centroids were calculated to

Table 2. *Summary of group mean (±s.d.) sperm motion parameters derived from PATN analysis of data from experiment 1: effects of sAPM*

PATN group	VCL (μm s^{-1})	VAP (μm s^{-1})	VSL (μm s^{-1})	BCF (Hz)	ALH (μm)	LIN (%)	STR (%)
1*	92.45±24.36	58.84±28.72	48.10±31.36	6.642±4.76	6.81±8.38	49.86±25.97	80.29±26.66
2	39.90±12.21	37.00±10.81	5.87±5.87	0.018±0.298	0.037±0.62	15.88±6.13	17.06±6.43
3	37.46±15.07	14.87±6.80	9.79±5.17	0.158±0.78	0.707±4.33	29.01±13.27	70.09±24.28

The analysis was performed on data from 12 012 individual tracks.
*Group 1 is considered to be the 'fast-linear' cohort.

assist with this interpretation. In some instances, treatment statistics relating to a single subpopulation are presented; this is possible where the subpopulations continue to show significant within-group variance after the PATN analysis.

Once the sub-populations had been identified, the relative frequencies of spermatozoa within each experimental sample, and belonging to each group, were compared by ANOVA using Statistica for Windows (Statsoft UK, Letchworth, UK). Replicated experiments were evaluated by combining frequency data (percentages) across replicates, then using ANOVAs for further analysis. Data expressed as percentages were subjected to arcsine transformation prior to ANOVA.

Summary tables showing PATN group motility characteristics derived for each experiment are shown in Tables 2–4.

Results

Effects of oviductal proteins on sperm subpopulations; characterisation of bicarbonate and sAPM responses

The first two experiments (experiments 1 and 2) were aimed at characterising the modulatory effects of oviductal proteins on the bicarbonate-induced motility response. In the first experiment the effects of a fixed concentration of sAPM

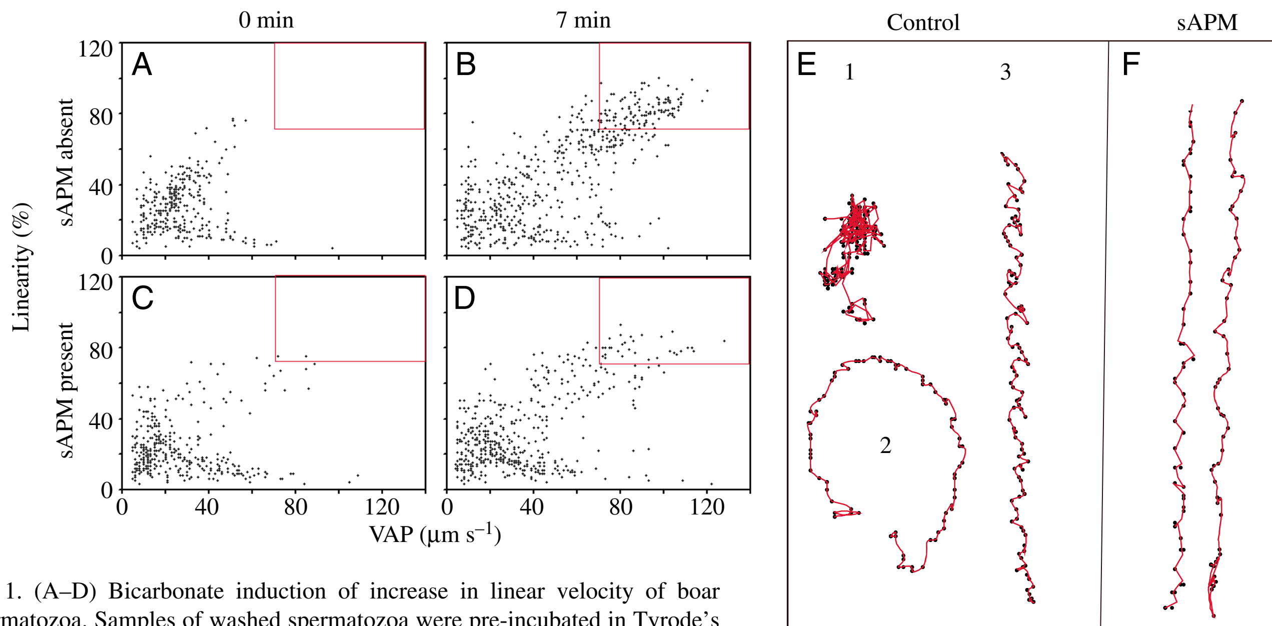

Fig. 1. (A–D) Bicarbonate induction of increase in linear velocity of boar spermatozoa. Samples of washed spermatozoa were pre-incubated in Tyrode's medium before the addition of 15 mmol l^{-1} bicarbonate. Subsamples were taken for video recording of motility shortly before ('zero time') and at intervals after bicarbonate addition. Motility parameter values were obtained by analysis of individual sperm tracks using the Hobson Sperm Tracker. These are presented as scatterplots (VAP *vs* LIN) (average path velocity *vs* linearity) of individual spermatozoa from a combination of two representative boars; each point represents a single sperm trajectory. In the absence of bicarbonate/CO_2 (A,C) most spermatozoa exhibit low VAP and LIN, although a small number of solubilised apical plasma membrane protein fraction (sAPM)-treated spermatozoa show high velocity (>60 μm s^{-1}) and straight tracks (LIN >60%); (highlighted in the box in the upper right corner). 7 min after the addition of bicarbonate/CO_2, a sizeable proportion of spermatozoa show activation (B,D); the boxes in the upper right of these panels also highlight spermatozoa showing high velocity (>60 μm s^{-1}) and straight tracks (LIN >60%). The density of spermatozoa within the upper right hand box is higher in the absence than in the presence of sAPM (compare B and D). (E) Representative trajectories of spermatozoa activated in the absence of sAPM. Track 3 represents the most activated trajectory (fast and linear), while tracks 1 and 2 represent subpopulations that would be classified as slow and/or non-linear. (F) Two representative fast-linear tracks activated in the presence of sAPM. These tracks show high linearity because there is relatively little deviation from the average path. Black dots represent *x–y* coordinates measured at 20-ms intervals; red lines are fitted spline curves.

[200 $\mu g\ ml^{-1}$; the concentration that promotes prolonged sperm survival *in vitro* (Elliott et al., 2001; Fazeli et al., 2003)] on bicarbonate-stimulated sperm motility was tested. Spermatozoa were pre-incubated in the presence or absence of sAPM for 10 min before adding bicarbonate/CO_2 (or NaCl as a control). Sperm motility was observed before bicarbonate/CO_2 or NaCl addition, at 2 min after bicarbonate/CO_2 addition and at 5 min intervals up to 17 min. A final observation of NaCl-treated control spermatozoa was performed at 22 min. The same approach was used in the second experiment, except that the dose–response effect of sAPM was examined (200, 100, 50, 25 and 0 $\mu g\ ml^{-1}$).

Experiment 1: data from 12,012 individual spermatozoa were obtained from eight replicates (boars); individual sperm motion parameters were analysed but the whole data set was also subjected to PATN analysis which classified them into three groups (Table 2). Group 1 was identified as a fast-linear subpopulation, whereas the other two groups were slow and non-linear.

Examination of the activation effects of bicarbonate/CO_2, and the attenuating effects of sAPM on individual spermatozoa, revealed that these were more complex than is apparent from simple statistical comparisons. Fig. 1A,C shows that prior to bicarbonate/CO_2 stimulation most spermatozoa move slowly along complex and nonlinear tracks (tracks 1 and 2 in Fig. 1E). The addition of bicarbonate/CO_2 in the presence or absence of sAPM stimulated a proportion of spermatozoa to higher velocity and track linearity (Fig. 1B,D; a cloud of new points is visible in the top right corner of B). Nevertheless, many spermatozoa failed to respond to bicarbonate/CO_2 activation, remaining in the bottom left hand corner of the scatterplots.

Fig. 2A,B illustrate the sperm velocity (VSL) and flagellar beat cross frequency (BCF) responses that occurred when Percoll-washed spermatozoa were exposed to 15 $mmol\ l^{-1}$ bicarbonate/CO_2. VSL and BCF both increased significantly ($P<0.01$) upon bicarbonate/CO_2 addition, reflecting the rapid and visible rise in sperm activity and straightening of trajectories. Spermatozoa activated in the absence of sAPM showed forward progression, but many lateral excursions of the sperm head were typical (track 3 in Fig. 1E).

After the initial rapid increase, VSL remained constant throughout the 17 min incubation period; VSL and BCF of the untreated control sample (addition of NaCl only and measured at 22 min) remained unchanged throughout incubation, emphasising the strict requirement for bicarbonate/CO_2 to induce the activation effect. Exposure to 200 $\mu g\ ml^{-1}$ sAPM for 10 min prior to bicarbonate/CO_2 addition significantly reduced, but did not abolish, the stimulation effect on both mean VSL ($P<0.01$) and mean BCF ($P=0.015$).

Inspection of the scatterplots suggested that sAPM reduced the proportion of spermatozoa undergoing bicarbonate-induced activation (compare Fig. 1B and D). This was confirmed when the relative frequencies of fast-linear spermatozoa (determined by PATN analysis) were compared (Fig. 2C,D).

Frequency variations of fast-linear spermatozoa along the experimental time course showed that, when sAPM was absent, bicarbonate/CO_2 addition caused rapid recruitment into the fast-linear group (Fig. 2C). This frequency reached a maximum at 12 min post-bicarbonate/CO_2 addition, followed by a significant decline. However, pre-exposure to sAPM induced a considerable change in this profile. About 40% of spermatozoa were classified as group 1 (fast-linear) at time 0 min; however, bicarbonate/CO_2 addition failed to induce a statistically significant ($P=0.248$) initial rise in group 1 frequency. There was nevertheless an increase that was immediately followed by a decline that could be seen consistently in each of the eight replicates (Fig. 2D). In addition to these statistical effects the sAPM induced qualitative increases in track linearity in typical sperm trajectories (Fig. 1F). Comparing these tracks with the control, non sAPM-treated spermatozoon (Fig. 1E, track 3) illustrates this increased linearity of forward progression, where fewer lateral excursions result in the convergence of VSL and curvilinear velocity ($\mu m\ s^{-1}$; VCL) and a ratio that approaches unity.

Oviductal proteins affect individual boars differentially

As with many animal experiments, replicate (individual boar) differences were significant within the analyses ($F_{7,76}=3.62$; $P=0.002$). More specific investigation of these differences revealed that they were systematic. In the absence of sAPM, there was a negative correlation ($r^2=-0.653$; $P=0.11$) between the relative sizes of the fast-linear sperm subpopulations before and after bicarbonate/CO_2 addition (times 0 and 7 min); however, this correlation became positive but weaker in the presence of sAPM ($r^2=0.432$; $P=0.285$; compare Fig. 3A and B). Not only was this reversal of slope observed, but when the individual boars were ranked by size of the post-activation cohort of active spermatozoa (sAPM absent), their respective rankings were changed by the presence of sAPM (Fig. 3C). Notably, the boars occupying the first and last rank positions reversed their ranks in the presence and absence of sAPM.

In experiment 2, the frequency of fast-linear spermatozoa within ejaculates was negatively correlated with sAPM concentration ($r^2=0.74$; $P<0.001$; Fig. 4A); the inhibitory effect was most noticeable with bicarbonate/CO_2-stimulated samples although it was even weakly apparent without stimulation. The overall stimulatory effect of bicarbonate/CO_2 nevertheless remained statistically significant ($F_{1,141}=38.3$, $P<0.0001$). Data were obtained from 28,273 individual spermatozoa (6 boars) and subjected to PATN analysis (Table 3).

Testing the dose–response relationship (0, 25, 50, 100, 200 $\mu g\ ml^{-1}$) and the suppression of bicarbonate/CO_2 activation with an orthogonal contrast vector (–2, –1, 0, +1, +2) confirmed that the dose response was directly proportional to the $\log_{(base\ 2)}$ of the sAPM concentration ($F_{1,141}=43.52$, $P<0.0001$). It is important to point out that this result represents quantal responses, i.e. fewer spermatozoa became

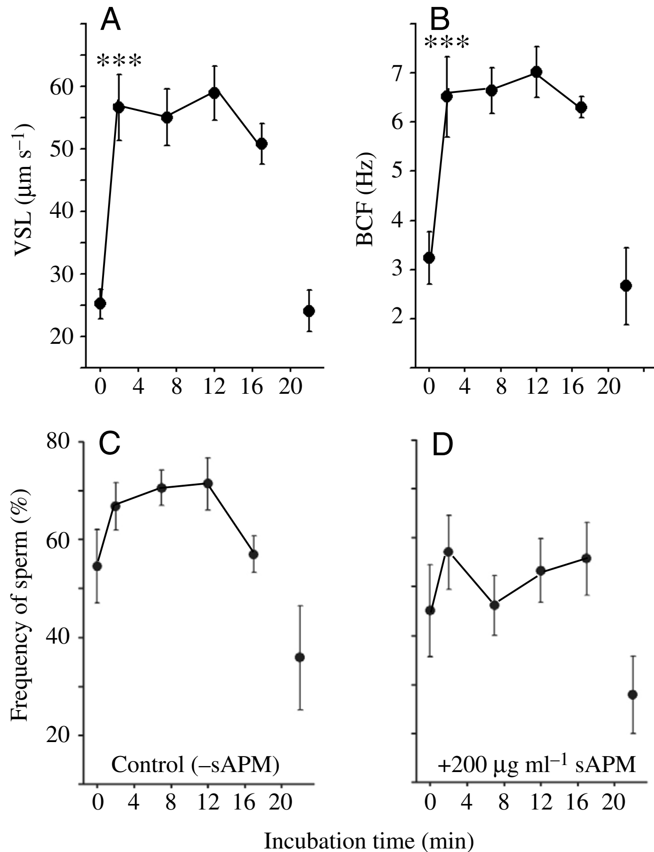

Fig. 2. (A) Straight line velocity (μm s^{-1} VSL) and (B) beat cross frequency (Hz; BCF) responses of boar spermatozoa to the addition of 15 mmol l^{-1} bicarbonate/CO_2. Data are means (± s.e.m.) calculated from treatment means for eight individual boars. The points at 22 min represent the NaCl-control samples, which had not been exposed to bicarbonate/CO_2. ***Bicarbonate/CO_2 *vs* no bicarbonate/CO_2 planned contrasts; VSL effect ($F_{1,87}$=61.9), P<0.001 and BCF effect ($F_{1,87}$=49.9), P<0.001. Changes in mean frequencies (± s.e.m. from eight individual boars) of group 1 (fast and linear) spermatozoa plotted against the time elapsed after bicarbonate/CO_2 addition. Points at 22 min represent the NaCl-control samples. (C,D) The difference between the absence and presence of sAPM, respectively.

activated in the presence of increasing doses of sAPM, but it is noteworthy that almost 40% of the available spermatozoa still became activated at the highest dose of sAPM (Fig. 4A). Most motion parameters of these progressive spermatozoa (e.g. VCL, VSL and BCF) changed relatively little in response to increasing sAPM concentration, but linearity (LIN) showed a positive dose–response relationship (Fig. 4B).

These results support the hypothesis that oviductal proteins act upon a particular subset of spermatozoa, and moreover, suggest that individual spermatozoa exhibit varying sensitivities to sAPM. The results of the previous experiment were confirmed, in that even at the highest concentration tested (200 μg ml^{-1}) approximately 30–40% of spermatozoa remained unaffected by the proteins. The strict dose relationship is consistent with the view that sAPM acts via a receptor–ligand mechanism in which individual spermatozoa are inactivated when they interact with different numbers of signalling molecules.

Oviductal proteins do not inhibit bicarbonate uptake

In searching for alternative explanations for the inhibitory effects of sAPM on spermatozoa we considered the possibility that some of the proteins in the sAPM fraction might be preventing the uptake of bicarbonate/CO_2 through anion channels. As spermatozoa are known to possess bicarbonate-exchange proteins (Darszon et al., 2001; Demarco et al., 2003; Holappa et al., 1999; Parkkila et al., 1993) that exchange with chloride or sodium, we used the specific bicarbonate transport inhibitor, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) (Zeng et al., 1996), to compare the effects of bicarbonate transport inhibition with the oviductal protein interactions.

In experiment 3 sperm motility responses to 50 μg ml^{-1} sAPM were compared with those to 100 μmol l^{-1} and 600 μmol l^{-1} DIDS, both in terms of motility responses and effects on intracellular pH_i. Motility effects were tested up to 32 min after bicarbonate/CO_2 addition, and a final control (NaCl treated) was examined at 35 min. Intracellular pH responses to the presence of sAPM or 100 μmol l^{-1} DIDS (with and without bicarbonate/CO_2 addition) were tested directly in parallel with the sperm motility responses; this was achieved using split semen samples.

Data were obtained from 39 989 spermatozoa and subjected to PATN analysis using the parameters VCL, average path velocity (μm s^{-1}; VAP), straightness (%; STR) and BCF. It became evident during the analysis that 600 μmol l^{-1} DIDS produced anomalous results (i.e. a considerable degree of sperm motility stimulation together with unstable and highly variable pH_i effects) and these results are not reported here.

Table 3. *Summary of group mean (±s.d.) sperm motion parameters derived from PATN analysis of data from experiment 2: sAPM dose response*

PATN group	VCL (μm s^{-1})	VAP (μm s^{-1})	VSL (μm s^{-1})	BCF (Hz)	ALH (μm)	LIN (%)	STR (%)
1	83.15±20.65	34.85±13.84	25.28±11.18	6.84±2.73	11.19±8.35	32.43±16.08	75.05±22.39
2*	123.07±20.71	77.63±20.78	67.76±24.53	10.67±3.23	8.61±4.99	56.54±21.20	85.40±16.75
3	52.07±18.98	26.39±18.06	8.84±4.48	0.03±0.42	0.08±1.06	18.78±9.61	51.61±32.64

The analysis was performed on data from 28 273 individual tracks.
*Group 2 is considered to be the ‘fast-linear’ cohort.

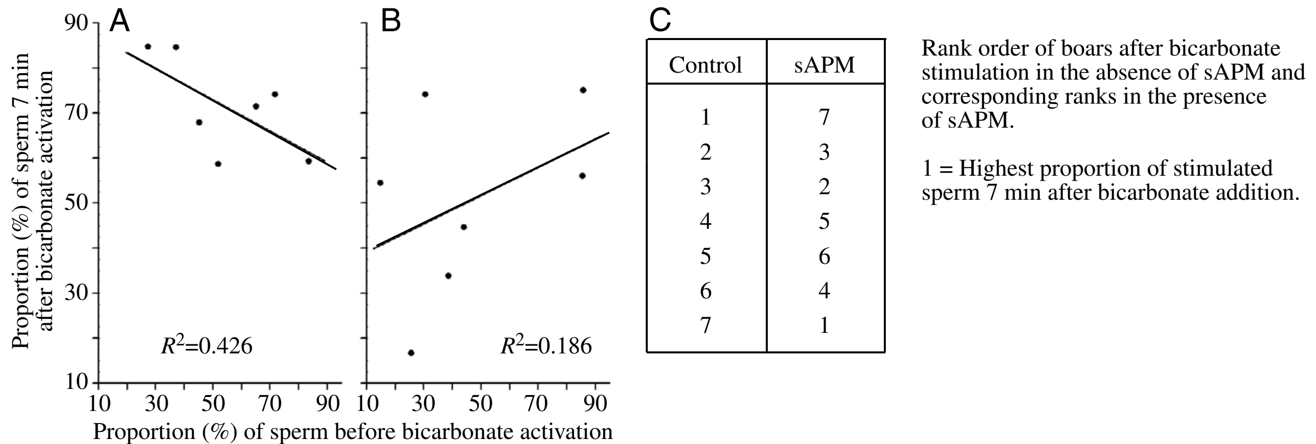

Fig. 3. Scatterplots showing relationships between the proportions of fast-linear spermatozoa present before (time 0 min) and after (time 7 min) bicarbonate/CO_2 activation in the absence (A) and presence (B) of solubilised apical plasma membrane protein fraction (sAPM). Each data point represents a separate boar ejaculate; N=7 in this graph because matched data was not available for one boar. (C) Ranking of boars in descending order of the proportion of fast-linear spermatozoa after 7 min in the absence of sAPM; control); the equivalent ranking is changed in the presence of sAPM.

However, PATN analysis for the classification of sperm subpopulations was nevertheless performed on the dataset that included these treatments. All sperm motion data was classified into three groups distinguished on the basis of their velocity and linearity (Table 4).

After bicarbonate/CO_2 addition, both 100 μmol l^{-1} DIDS and 50 μg ml^{-1} sAPM reduced the extent of bicarbonate-induced sperm stimulation (Fig. 5A; control *vs* sAPM and DIDS; $F_{1,161}$=6.12; P=0.014). However, in contrast to sAPM,

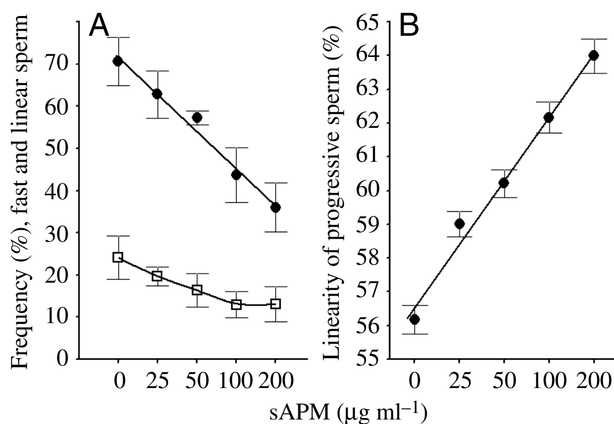

Fig. 4. (A) Solubilised apical plasma membrane protein fraction (sAPM) dose response of the fast-linear sperm subpopulation in the presence (filled circles) and absence (open squares) of bicarbonate/CO_2. The data summarise group sperm frequencies (%), calculated for each individual boar (N=6) and treatment (mean % ± s.e.m.). Data representing bicarbonate/CO_2-treated spermatozoa are from treatments 7 min after bicarbonate/CO_2 addition. Data from the 'bicarbonate-absent' groups are from the NaCl control samples, measured at 0 and 17 min. (B) Within the fast-linear sperm subpopulation, the mean (± s.e.m.) LIN (linearity) values increased in relation to the $\log_{(\mathrm{base}\ 2)}$ sAPM concentration.

100 μmol l^{-1} DIDS also significantly reduced the BCF of the activated sperm group (Fig. 5B,C; contrast within ANOVA on the fast-linear sperm subpopulation; $F_{1,24536}$=18.9; P<0.001). Although 100 μmol l^{-1} DIDS and sAPM both suppressed sperm activation, the pH_i measurements confirmed that sAPM did not act through the inhibition of bicarbonate uptake. Bicarbonate/CO_2 addition initiated a linear rise in pH_i in all treatments; but whereas sAPM enhanced the rate of pH_i elevation with respect to the control treatment ($F_{1,356}$=52.45; P<0.001; Fig. 6B *vs* C) the DIDS caused significant inhibition ($F_{1,252}$=93.32; P<0.001) (Fig. 6A *vs* B). After 60 min incubation in the presence of bicarbonate/CO_2 the sAPM-treated samples attained pH_i values around 8.5, while the DIDS-treated samples only reached about pH 7.6.

Interaction of spermatozoa with sAPM-coated beads

To see whether spermatozoa interacted with oviductal proteins on a solid surface in a manner that was similar to the situation within the oviduct, sAPM proteins were covalently bound to the surface of chemically reactive polymeric beads. Qualitative analyses of the interactions revealed that whereas a significant proportion of the sperm population failed to bind (Fig. 7A), 10–20 spermatozoa did indeed become bound to each bead. The binding interaction consistently occurred *via* the anterior acrosomal region of the sperm heads (Fig. 7B,C) whereas their flagellae protruded away from the bead surface. Control beads, prepared using BSA, bound spermatozoa indiscriminately *via* their heads and tails (not shown).

Discussion

The bicarbonate-induced activation of boar sperm motility provided an excellent test system for evaluating both the differential immediate responses of sperm subpopulations and the way in which the presence of sAPM modulates these

Table 4. *Summary of group means (±s.d.) sperm motion parameters derived from PATN analysis of data from experiment 3: sAPM and DIDS*

PATN group	VCL (μm s^{-1})	VAP (μm s^{-1})	VSL (μm s^{-1})	BCF (Hz)	ALH (μm)	LIN (%)	STR (%)
1*	84.22±31.72	51.19±30.17	43.46±30.28	5.93±4.57	7.14±8.81	49.43±25.53	82.42±21.10
2	47.60±17.89	44.52±15.86	5.75±3.20	0.184±1.09	0.35±2.19	13.17±6.15	13.99±6.55
3	18.90±5.02	18.39±4.53	4.39±1.52	0.00±0.00	0.00±0.00	25.22±8.28	25.73±8.13

The analysis was performed on data from 39 989 individual tracks.
*Group 1 is considered to be the 'fast-linear' cohort.

effects. Boar spermatozoa exhibit considerable sensitivity to their bicarbonate/CO_2 environment and thus maximal stimulation might be expected within the oviduct where the concentration is more than twofold higher than that used in the present series of experiments. The formation of a sperm reservoir in the oviductal isthmus, where many spermatozoa become attached to oviductal epithelial cells suggests, however, that in some spermatozoa the motility activation response is suppressed by additional signal transduction pathways. However, some spermatozoa do not bind to the oviductal epithelium and may reach the site of fertilisation relatively quickly (<1 h) when insemination and ovulation are closely synchronised. Such basic observations suggest that two functionally distinct sperm subpopulations have the ability to reach and enter the oviduct. Entry to the oviduct itself is a highly selective process in its own right, being dependent on the intrinsic motility of each individual spermatozoon (Olds-Clarke, 1996). The present data implies that this response is both sperm specific and boar specific, thereby providing considerable opportunity for the operation of selective mechanisms.

The sAPM protein fraction used in these experiments has previously been evaluated for its ability to maintain sperm viability over an extended period at body temperature *in vitro* (Elliott et al., 2001; Fazeli et al., 2003). sAPM consists of a subset of peripheral membrane proteins that can be removed in soluble form from the apical plasma membranes of oviductal epithelial cells by treatment with 1 mol l^{-1} NaCl (Holt et al., 2005). Prior to these observations, Smith and Nothnick (Smith and Nothnick, 1997) had demonstrated that apical plasma membrane fractions from the rabbit oviductal epithelium possess the ability to enhance rabbit sperm survival *in vitro.* These observations lend support to the view that the sAPM effects seen here are analogous to *in vivo* interactions between spermatozoa and the oviductal epithelial cells.

Before bicarbonate/CO_2 stimulation, but in the presence of sAPM, approximately 40% of the spermatozoa were classified into the fast-linear group; this was only about 10% lower than the corresponding value measured in the absence of sAPM. The sAPM therefore initially affected only about 10% of the total sperm population, which implies that the 40% of unsuppressed spermatozoa are responsive to neither bicarbonate/CO_2 stimulation nor suppression by oviductal protein(s). The origin of such diversity is unclear but is partly

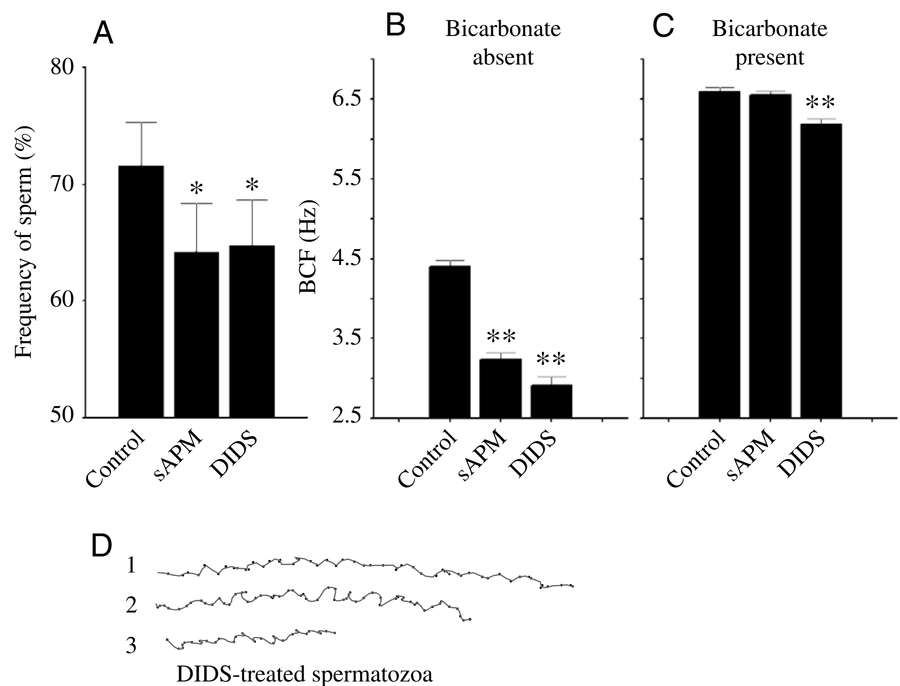

Fig. 5. (A) Bar chart showing the mean (±s.e.m.) frequency (%) of the fast-linear sperm population present in control, solubilised apical plasma membrane protein fraction (sAPM) and 100 μmol l^{-1} 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) treatments. Both treatments caused reduction of bicarbonate-induced activation (P<0.05). (B,C) In comparison to the control treatment, both sAPM and 100 μM DIDS significantly reduced beat cross frequency (Hz; BCF) in the fast-linear sperm group if bicarbonate/CO_2 was absent. In the presence of bicarbonate/CO_2, there was no inhibitory effect of sAPM on BCF, but DIDS still induced significant reduction of flagellar beat frequency ($F_{1,24536}$=18.9; P<0.001). Data are mean ± s.e.m; N=5 replicates). (D) Three representative trajectories of spermatozoa exposed to DIDS show high linearity because there is relatively little deviation from the average path. Black dots represent x-y coordinates measured at 20 ms intervals; grey lines are fitted spline curves.

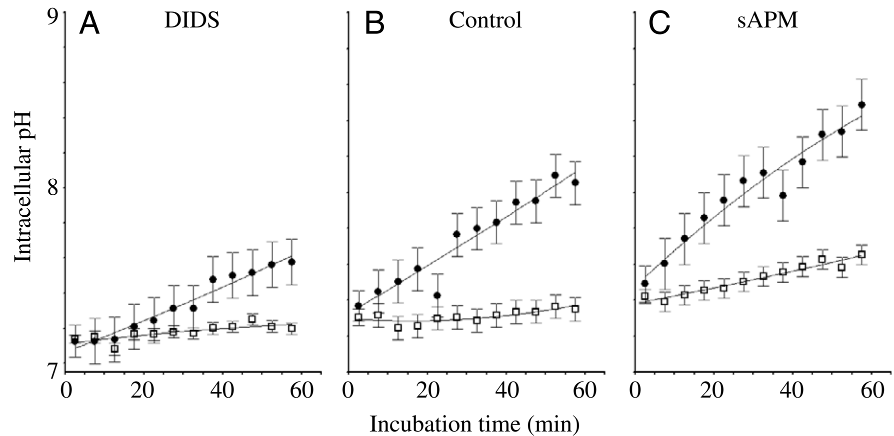

Fig. 6. Effects of 100 μmol l^{-1} 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) (A), control treatment with neither DIDS nor solubilised apical plasma membrane protein fraction (sAPM) (B) and 50 μg ml^{-1} sAPM (C) on the intracellular pH of boar spermatozoa incubated for 1 h in the presence (filled circles) and absence (open squares) of bicarbonate/CO_2. Whereas bicarbonate/CO_2 induced increased pH_i in all treatments, DIDS significantly inhibited ($F_{1,252}$=93.3; P<0.001) and sAPM significantly enhanced ($F_{1,356}$=52.45; P<0.001) the pH increase with respect to the control. (Data are means ± s.e.m.; N=6

attributable to differences in sperm maturation status as a result of mixing within the epididymis (Flesch et al., 2001). Alternatively, signalling responses within individual spermatozoa may be dependent on its intrinsic genetic characteristics. Such variation, at least in mice carrying *t*-locus mutations, is known to depend on the regulation of the intracellular sperm-specific motility kinase (Smok) (Herrmann et al., 1999) which, if over-expressed and not corrected by other regulatory mechanisms, causes flagellar beat patterns that are incompatible with entry to the oviduct and fertilisation. As the *t*-locus mutations are also sex linked, this inappropriate motility is responsible for sex ratio skewing of litters (Olds-Clarke, 1991).

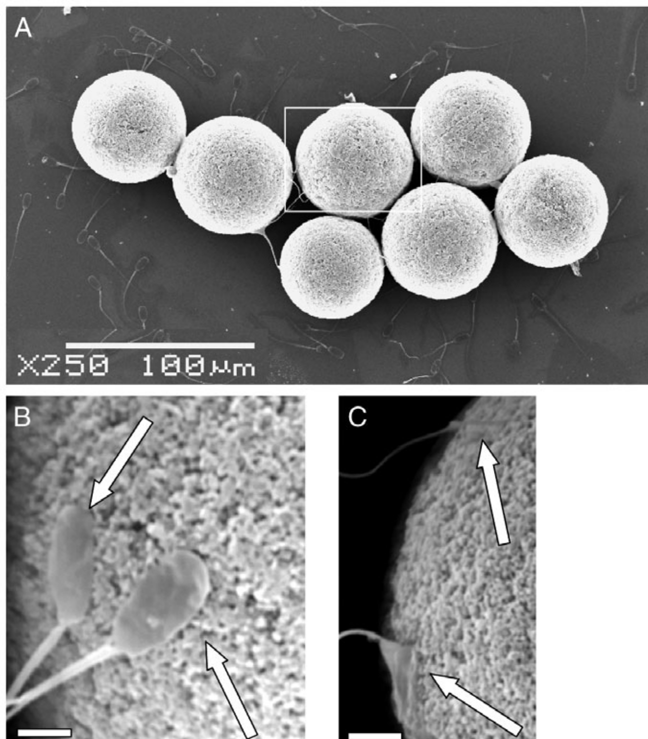

Fig. 7. Sperm binding to solubilised apical plasma membrane protein fraction (sAPM)-coated beads. (A) Low magnification view of the sAPM-coated beads showing that although some spermatozoa are bound to the bead surfaces many unbound spermatozoa are also present. Bar, 100 μm. (B,C) Spermatozoa interact with the bead surface *via* the anterior acrosomal region (arrows) while the flagellae are unbound. Bars, 5 μm.

The presence of sAPM suppressed further recruitment into the fast-linear group, despite the initial transient increase. If this occurs within the oviductal environment it would allow both oviductal reservoir formation and the continued progression of an unaffected subpopulation. The observation that spermatozoa became attached to sAPM-coated beads provides further support for this model. The sperm-bead interaction bears striking resemblance to published micrographs of spermatozoa bound to oviductal cells *in vitro* and *in vivo* (Green et al., 2001; Hunter, 1981). Although it may seem surprising that flagellar activity is suppressed by a binding mechanism that principally involves the sperm head, recent studies of flagellar wave modulation have suggested that the calcium storage sites at the sperm neck region are crucially important in determining the directionality of sperm progression (Ho and Suarez, 2003). In this context it is of interest that the unsuppressed spermatozoa responded to sAPM by increasing their track linearity. This may mean that, within the oviduct, some spermatozoa are assisted to reach the site of fertilisation as rapidly as possible.

The dose-responsive nature of the sperm–sAPM interaction supports the belief that the effects are mediated via receptor-ligand interactions. As the analysis was based on population frequencies, the results show that increasing concentrations of sAPM lead to the suppression of ever more individual spermatozoa. At the same time the spermatozoa that remain active appear to exhibit an increasingly linear pattern of movement. This is consistent with *in vivo* observations of boar spermatozoa in the oviduct (Suarez et al., 1992) whose flagellae demonstrate higher radius of curvature, i.e. are straighter, and whose movements were more linear than equivalent free-swimming spermatozoa. This complex subpopulation response differs from responses obtained by exposing spermatozoa to glycolytic or mitochondrial inhibitors, which typically cause global reductions in sperm

activity. In the oviductal environment such a global response would be inappropriate because it would synchronously prevent the activity of the entire sperm population.

Although the stimulatory pathway is now well documented (Chen et al., 2000; Holt and Harrison, 2002; Okamura et al., 1985; Tajima et al., 1987), the existence of an opposing pathway mediated by oviductal components has yet to be elucidated. The observation that sAPM does not cause motility suppression by blocking the uptake of the bicarbonate anion supports the belief that spermatozoa are controlled by a specific sperm-oviduct signal transduction pathway. In fact, the present data showed enhancement of intracellular pH elevation. Such an effect would be consistent with the hypothesis that when spermatozoa become bound to the oviduct epithelium they undergo accelerated capacitation (Fazeli et al., 1999), a process that is accompanied by increased pH_i (Murphy and Yanagimachi, 1984; Zeng et al., 1996). The existence of sperm–oviduct signal transduction pathways that cause *de novo* gene transcription in the oviduct upon the arrival of spermatozoa have been confirmed recently (Fazeli et al., 2004).

These results may have profound implications for the operation of sperm selection, or self-selection, and sperm competition mechanisms in the oviduct. They show that the stimulatory effects of bicarbonate are exerted differentially at both the individual sperm and individual boar levels. More importantly, the oviductal proteins also differentially suppressed the activation of individual spermatozoa from the different boars, and transformed the way in which boars were ranked after sperm activation. In fact, the data showed that in some boars the proportion of fast-linear sperm in a sperm sample was actually diminished by the addition of bicarbonate/CO_2 when oviductal proteins were present. If these boar-specific effects are a reflection of the processes that take place *in vivo*, the skewed results obtained from heterospermic insemination experiments (Berger et al., 1996; Popwell and Flowers, 2004; Stahlberg et al., 2000) might readily be explained. The presence of a universally conserved bicarbonate receptor in spermatozoa of many species (Chen et al., 2000; Livera et al., 2005; Pastor-Soler et al., 2003) and specific demonstrations that bicarbonate activates motility in human (Luconi et al., 2004; Luconi et al., 2005) and mouse (Wennemuth et al., 2003) spermatozoa, imply, moreover, that the oviductal effects may be of universal significance for sperm selection and sperm competition.

Our data suggest that if spermatozoa successfully enter the oviduct two outcomes are possible; they either bind to the epithelial surface and form the isthmic reservoir, or progress immediately along the oviduct towards the ampulla. At present it is impossible to predict, in the absence of oviductal proteins, the relative extent to which these outcomes are favoured. However, if spermatozoa from different boars bind unequally to the oviductal epithelium, then the relative conception rates are likely to depend largely on the synchrony of insemination with ovulation, and the relative dynamics of capacitation and sperm release. Spermatozoa that do not bind to the oviduct would have a greater chance of fertilisation if oocytes are present around the time of insemination, while spermatozoa that were stored, and then released some hours later, would be able to fertilise oocytes if ovulation were delayed until some time after insemination.

We therefore propose a working hypothesis as a basis for future experiments in sperm selection and sperm competition. Our data confirm that boar spermatozoa are exquisitely sensitive to the presence of bicarbonate/CO_2; this is consistent with the molecular identification of soluble adenylyl cyclase as a bicarbonate sensor in spermatozoa (Chen et al., 2000). A crucial aspect of our hypothesis is the observation that exposure to bicarbonate affects individual spermatozoa differentially. Some spermatozoa are actively progressive in the absence of bicarbonate while others are quiescent until activated. Together, these two groups typically make up about 50–80% of all spermatozoa in an ejaculate, the remainder apparently being rather insensitive to their environment. We propose that the inter-boar differences in relative proportions of these sperm subpopulations are likely to be responsible for between-boar differential fertility. More specifically, we propose that differences in the relative numbers of spermatozoa physically enabled to enter the oviduct would differ between boars, even if the inseminate contained equal numbers of spermatozoa from different boars.

List of abbreviations

ALH	amplitude of lateral head displacement
ANOVA	analysis of variance
APM	apical plasma membrane preparation
BCECF	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
BCF	beat cross frequency (Hz)
BTS	Beltsville thawing solution
DIDS	4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid
LIN	linearity (%)
PATN	name of pattern analysis software
STR	straightness (%)
TLP	modified Tyrode's solution
sAPM	solubilised apical plasma membrane protein fraction
VAP	average path velocity ($\mu m\ s^{-1}$)
VCL	curvilinear velocity ($\mu m\ s^{-1}$)
VSL	straight line velocity ($\mu m\ s^{-1}$)

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References

Abaigar, T., Holt, W. V., Harrison, R. A. P. and del Barrio, G. (1999). Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol. Reprod.* **60**, 32-41.

Beatty, R. A., Bennett, G. H., Hall, J. G., Hancock, J. L. and Stewart, D. L. (1969). An experiment with heterospermic insemination in cattle. *J. Reprod. Fertil.* **19**, 491-502.

Beatty, R. A., Stewart, D. L., Spooner, R. L. and Hancock, J. L. (1976). Evaluation by the heterospermic insemination technique of the differential effect of freezing at –196°C on the fertility of individual bull semen. *J. Reprod. Fertil.* **47**, 377-379.

Bedford, J. M. and Breed, W. G. (1994). Regulated storage and subsequent transformation of spermatozoa in the fallopian tubes of an Australian marsupial, *Sminthopsis crassicaudata*. *Biol. Reprod.* **50**, 845-854.

Belbin, L. (1993). PATN, pattern analysis package. Canberra, Australia: Division of Wildlife and Ecology, CSIRO.

Berger, T., Anderson, D. L. and Penedo, M. C. T. (1996). Porcine sperm fertilizing potential in relationship to sperm functional capacities. *Anim. Reprod. Sci.* **44**, 231-239.

Birkhead, T. R. (1995). Sperm competition: evolutionary causes and consequences. *Reprod. Fertil. Dev.* **7**, 755-775.

Birkhead, T. R. and Moller, A. P. (1993). Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. *Biol. J. Linn. Soc. Lond.* **50**, 295-311.

Browning, J. A. and Wilkins, R. J. (2004). Mechanisms contributing to intracellular pH homeostasis in an immortalised human chondrocyte cell line. *Comp. Biochem. Physiol.* **137A**, 409-418.

Burkman, L. J., Overstreet, J. W. and Katz, D. F. (1984). A possible role for potassium and pyruvate in the modulation of sperm motility in the rabbit oviducal isthmus. *J. Reprod. Fertil.* **71**, 367-376.

Chen, Y. Q., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R. and Buck, J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289**, 625-628.

Creech, M. M., Arnold, E. V., Boyle, B., Muzinich, M. C., Montville, C., Bohle, D. S. and Atherton, R. W. (1998). Sperm motility enhancement by nitric oxide produced by the oocytes of fathead minnows, *Pimephelas promelas*. *J. Androl.* **19**, 667-674.

Darszon, A., Beltran, C., Felix, R., Nishigaki, T. and Trevino, C. L. (2001). Ion transport in sperm signaling. *Dev. Biol.* **240**, 1-14.

Davis, R. O. and Siemers, R. J. (1995). Derivation and reliability of kinematic measures of sperm motion. *Reprod. Fertil. Dev.* **7**, 857-869.

Demarco, I. A., Espinosa, F., Edwards, J., Sosnik, J., De La Vega-Beltran, J. L., Hockensmith, J. W., Kopf, G. S., Darszon, A. and Visconti, P. E. (2003). Involvement of a Na+/HCO_3 cotransporter in mouse sperm capacitation. *J. Biol. Chem.* **278**, 7001-7009.

Ellington, J. E., Evenson, D. P., Wright, R. W., Jr, Jones, A. E., Schneider, C. S., Hiss, G. A. and Brisbois, R. S. (1999). Higher-quality human sperm in a sample selectively attach to oviduct (fallopian tube) epithelial cells in vitro. *Fertil. Steril.* **71**, 924-929.

Elliott, R. M. A., Duncan, A., Watson, P. F., Holt, W. V. and Fazeli, A. R. (2001). Peripheral bound membrane proteins are involved in the maintenance of boar sperm viability by oviductal apical plasma membrane preparations in vitro. *Mol. Biol. Cell Suppl.* **12**, 117a.

Esponda, P. and Moreno, M. (1998). Acrosomal status of mouse spermatozoa in the oviductal isthmus. *J. Exp. Zool.* **282**, 360-366.

Fazeli, A., Duncan, A. E., Watson, P. F. and Holt, W. V. (1999). Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol. Reprod.* **60**, 879-886.

Fazeli, A., Watson, P. F. and Holt, W. V. (2000). Isthmic apical plasma membrane preparations maintain boar sperm viability in vitro in a dose dependent manner. *Biol. Reprod.* **62**, 388.

Fazeli, A., Elliott, R. M., Duncan, A. E., Moore, A., Watson, P. F. and Holt, W. V. (2003). In vitro maintenance of boar sperm viability by a soluble fraction obtained from oviductal apical plasma membrane preparations. *Reproduction* **125**, 509-517.

Fazeli, A., Affara, N. A., Hubank, M. and Holt, W. V. (2004). Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biol. Reprod.* **71**, 60-65.

Flesch, F. M., Brouwers, J. F. H. M., Nievelstein, P. F. E. M., Verkleij, A. J., van Golde, L. M. G., Colenbrander, B. and Gadella, B. M. (2001). Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *J. Cell Sci.* **114**, 3543-3555.

Gage, M. J. G., Macfarlane, C. P., Yeates, S., Ward, R. G., Searle, J. B. and Parker, G. A. (2004). Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. *Curr. Biol.* **14**, 44-47.

Green, C. E., Bredl, J., Holt, W. V., Watson, P. F. and Fazeli, A. (2001). Carbohydrate mediation of boar sperm binding to oviductal epithelial cells *in vitro*. *Reproduction* **122**, 305-315.

Gualtieri, R. and Talevi, R. (2003). Selection of highly fertilization-competent bovine spermatozoa through adhesion to the fallopian tube epithelium in vitro. *Reproduction* **125**, 251-258.

Harrison, R. A. and Miller, N. G. (2000). cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Mol. Reprod. Dev.* **55**, 220-228.

Harrison, R. A., Ashworth, P. J. and Miller, N. G. (1996a). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* **45**, 378-391.

Harrison, R. A. P. (2004). Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol. Reprod. Dev.* **67**, 337-352.

Harrison, R. A. P., Ashworth, P. J. C. and Miller, N. G. A. (1996b). Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membrane. *Mol. Reprod. Dev.* **45**, 378-391.

Herrmann, B. G., Koschorz, B., Wertz, K., McLaughlin, K. J. and Kispert, A. (1999). A protein kinase encoded by the *t*-complex responder gene causes non-Mendelian inheritance. *Nature* **402**, 141-146.

Ho, H. C. and Suarez, S. S. (2003). Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biol. Reprod.* **68**, 1590-1596.

Holappa, K., Mustonen, M., Parvinen, M., Vihko, P., Rajaniemi, H. and Kellokumpu, S. (1999). Primary structure of a sperm cell anion exchanger and its messenger ribonucleic acid expression during spermatogenesis. *Biol. Reprod.* **61**, 981-986.

Holt, W. V. and Harrison, R. A. (2002). Bicarbonate stimulation of boar sperm motility via a protein kinase A-dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. *J. Androl.* **23**, 557-565.

Holt, W. V., Elliott, R. M. A., Fazeli, A., Satake, N. and Watson, P. F. (2005). Validation of an experimental strategy for studying surface-exposed proteins involved in porcine sperm-oviduct contact interactions. *Reprod. Fertil. Dev.* **17**, 683-692.

Hunter, R. H. F. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil.* **63**, 109-117.

Livera, G., Xie, F., Garcia, M. A., Jaiswal, B., Chen, J., Law, E., Storm, D. R. and Conti, M. (2005). Inactivation of the mouse adenylyl cyclase 3 gene disrupts male fertility and spermatozoon function. *Mol. Endocrinol.* **19**, 1277-1290.

Lorenzen, A. and Kennedy, S. W. (1993). A fluorescence-based protein assay for use with a microplate reader. *Anal. Biochem.* **214**, 346-348.

Luconi, M., Carloni, V., Marra, F., Ferruzzi, P., Forti, G. and Baldi, E. (2004). Increased phosphorylation of AKAP by inhibition of phosphatidylinositol 3-kinase enhances human sperm motility through tail recruitment of protein kinase A. *J. Cell Sci.* **117**, 1235-1246.

Luconi, M., Porazzi, I., Ferruzzi, P., Marchiani, S., Forti, G. and Baldi, E. (2005). Tyrosine phosphorylation of the A kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate. *Biol. Reprod.* **72**, 22-32.

McNutt, T. L., Olds-Clarke, P., Way, A. L., Suarez, S. S. and Killian, G. J. (1994). Effect of follicular or oviductal fluids on movement characteristics of bovine sperm during capacitation *in-vitro*. *J. Androl.* **15**, 328-336.

Murphy, S. J. and Yanagimachi, R. (1984). The pH dependence of motility and the acrosome reaction of guinea pig spermatozoa. *Gamete Res.* **10**, 1-8.

Nakanishi, T., Isotani, A., Yamaguchi, R., Ikawa, M., Baba, T., Suarez, S. S. and Okabe, M. (2004). Selective passage through the uterotubal junction of sperm from a mixed population produced by chimeras of calmegin-knockout and wild-type male mice. *Biol. Reprod.* **71**, 959-965.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H. and Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J. Biol. Chem.* **260**, 9699-9705.

Olds-Clarke, P. (1986). Motility characteristics of sperm from the uterus and oviducts of female mice after mating to congenic males differing in sperm transport and fertility. *Biol. Reprod.* **34**, 453-467.

Olds-Clarke, P. (1991). The genetics of sperm function in fertilization. *Ann. N.Y. Acad. Sci.* **637**, 474-485.

Olds-Clarke, P. (1996). How does poor motility alter sperm fertilizing ability. *J. Androl.* **17**, 183-186.

Overstreet, J. W. and Cooper, G. W. (1975). Reduced sperm motility in the isthmus of the rabbit oviduct. *Nature* **258**, 718-719.

Overstreet, J. W., Katz, D. F. and Johnson, L. L. (1980). Motility of rabbit spermatozoa in the secretions of the oviduct. *Biol. Reprod.* **22**, 1083-1088.

Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**, 525-567.

Parkkila, S., Rajaniemi, H. and Kellokumpu, S. (1993). Polarized expression of a band 3-related protein in mammalian sperm cells. *Biol. Reprod.* **49**, 326-331.

Pastor-Soler, N., Beaulieu, V., Litvin, T. N., Da Silva, N., Chen, Y., Brown, D., Buck, J., Levin, L. R. and Breton, S. (2003). Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling. *J. Biol. Chem.* **278**, 49523-49529.

Popwell, J. M. and Flowers, W. L. (2004). Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. *Anim. Reprod. Sci.* **81**, 97-113.

Ralt, D., Manor, M., Cohendayag, A., Turkaspa, I., Benshlomo, I., Makler, A., Yuli, I., Dor, J., Blumberg, S., Mashiach, S. et al. (1994). Chemotaxis and chemokinesis of human spermatozoa to follicular factors. *Biol. Reprod.* **50**, 774-785.

Rodriguez-Martinez, H., Saravia, F., Wallgren, M., Tienthai, P., Johannisson, A., Vazquez, J. M., Martinez, E., Roca, J., Sanz, L. and Calvete, J. J. (2005). Boar spermatozoa in the oviduct. *Theriogenology* **63**, 514-535.

Santi, C. M., Santos, T., Hernandez-Cruz, A. and Darszon, A. (1998). Properties of a novel pH-dependent Ca^{2+} permeation pathway present in male germ cells with possible roles in spermatogenesis and mature sperm function. *J. Gen. Physiol.* **112**, 33-53.

Smith, T. T. and Nothnick, W. B. (1997). Role of direct contact between spermatozoa and oviductal epithelial cells in maintaining rabbit sperm viability. *Biol. Reprod.* **56**, 83-89.

Stahlberg, R., Harlizius, B., Weitze, K. F. and Waberski, D. (2000). Identification of embryo paternity using polymorphic DNA markers to assess fertilizing capacity of spermatozoa after heterospermic insemination in boars. *Theriogenology* **53**, 1365-1373.

Stewart, D. L., Spooner, R. L., Bennett, G. H., Beatty, R. A. and Hancock, J. L. (1974). A second experiment with heterospermic insemination in cattle. *J. Reprod. Fertil.* **36**, 107-116.

Suarez, S. S. (1998). The oviductal sperm reservoir in mammals; mechanisms of formation. *Biol. Reprod.* **58**, 1105-1107.

Suarez, S. S., Dai, X. B., DeMott, R. P., Redfern, K. and Mirando, M. A. (1992). Movement characteristics of boar sperm obtained from the oviduct or hyperactivated *in vitro*. *J. Androl.* **13**, 75-80.

Sun, F., Bahat, A., Gakamsky, A., Girsh, E., Katz, N., Giojalas, L. C., Tur-Kaspa, I. and Eisenbach, M. (2005). Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. *Hum. Reprod.* **20**, 761-767.

Tajima, Y., Okamura, N. and Sugita, Y. (1987). The activating effects of bicarbonate on sperm motility and respiration at ejaculation. *Biochim. Biophys. Acta* **924**, 519-529.

Vicente, J. S., Viudes de Castro, M. P., Lavara, R. and Moce, E. (2004). Study of fertilising capacity of spermatozoa after heterospermic insemination in rabbit using DNA markers. *Theriogenology* **61**, 1357-1365.

Vincent, R. and Nadeau, D. (1984). Adjustment of the osmolality of Percoll for the isopycnic separation of cells and cell organelles. *Anal. Biochem.* **141**, 322-328.

Wennemuth, G., Carlson, A. E., Harper, A. J. and Babcock, D. F. (2003). Bicarbonate actions on flagellar and Ca^{2+}-channel responses: initial events in sperm activation. *Development* **130**, 1317-1326.

Xiang, X. Y., Burnett, L., Rawls, A., Bieber, A. and Chandler, D. (2004). The sperm chemoattractant "allurin" is expressed and secreted from the Xenopus oviduct in a hormone-regulated manner. *Dev. Biol.* **275**, 343-355.

Xiang, X. Y., Kittelson, A., Olson, J., Bieber, A. and Chandler, D. (2005). Allurin, A 21 kD sperm chemoattractant, is rapidly released from the outermost jelly layer of the Xenopus egg by diffusion and medium convection. *Mol. Reprod. Dev.* **70**, 344-360.

Zar, J. H. (1984). *Biostatistical Analysis.* New Jersey: Prentice Hall.

Zeng, Y., Oberdorf, J. A. and Florman, H. M. (1996). pH regulation in mouse sperm: Identification of Na^{+}-, Cl^{-}, and pH-dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Dev. Biol.* **173**, 510-520.