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Theriogenology xx (2010) xxx

Theriogenology

[www.theriojournal.com](http://www.theriojournal.com)

## Effects of porcine pre-ovulatory oviductal fluid on boar sperm function

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Received 5 December 2009; received in revised form 5 February 2010; accepted 7 March 2010

### Abstract

Sperm storage within the oviductal isthmus prior to ovulation typically involves binding to oviductal epithelial cells, which are thought to modulate sperm functions including internal calcium concentration, membrane fluidity, and motility. Around the time of ovulation the spermatozoa are gradually released so that they eventually encounter the oocytes within the oviductal ampulla. Previous studies have shown that the oviductal epithelial cells selectively sequester high quality spermatozoa, but the role of oviductal fluid as a selective modulator of sperm function has been investigated to a lesser extent. Here we address the hypothesis that oviductal fluid is also likely to modulate sperm function. Using samples of porcine oviductal fluid collected in the follicular phase of the estrus cycle, we show that short exposure (20 min to 50  $\mu\text{g}/\text{mL}$  of oviductal fluid proteins) to either of two separate proteins fractions ( $>$  or  $<$  100 kDa) promotes boar sperm viability and acrosomal integrity, decreases sperm plasma membrane fluidity (measured using merocyanine S540), and increases zona binding and polyspermy during *in vitro* fertilization. Exposure to the lower molecular fraction significantly inhibited, but did not abolish, the bicarbonate-induced stimulation of motility. The results show that subpopulations of spermatozoa respond differentially to oviductal fluid, and suggest that exposure to oviductal fluid *in vivo* could exert a further level of functional sperm selection.

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**Keywords:** Sperm function; Oviduct; Oviductal fluid; Sperm motility; *In vitro* fertilization

### 1. Introduction

When a spermatozoon eventually meets an oocyte within the oviduct prior to natural fertilization, it is one of the relatively small and highly selected subset of

spermatozoa that have managed to overcome the physical and physiological obstacles imposed by the female reproductive tract. The final stages of sperm transport towards the oocyte are both arduous and selective; the spermatozoa enter the oviduct via the utero-tubal junction (UTJ) and interact with the oviductal epithelium to form a sperm reservoir before eventually being released and progressing towards the oocyte [1–3]. Recent studies have shown that the arrival of spermatozoa initiates a mutual sperm-epithelial signalling dialogue; the sper-

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matozoa cause the oviductal cells to modify their patterns of gene expression and protein synthesis [4,5] while sperm motility within the oviduct is reduced [6,7] and events associated with capacitation begin to take place.

As the sperm-epithelial signalling dialogue takes place within the oviduct, the spermatozoa are also exposed to a further potentially important source of signal transduction stimuli present within oviductal fluid. This fluid, which contains a complex mixture of organic and inorganic molecules, represents the physiological milieu in which fertilization itself occurs. While considerable attention has been paid to the formation of the sperm reservoir in pigs by sperm-oviduct epithelial binding [8], fewer studies exist on the way in which sperm function is affected by oviductal fluid (OF). This is due in part to the difficulty of obtaining samples of oviductal fluid under physiological conditions [9]. Collecting oviductal fluid from slaughtered animals at defined phases of interest during the estrus cycle and pooling the samples from different animals to avoid individual variations should give us an approximate idea about the situation in the oviduct during this phase and the effect of the fluid on sperm function. As it is before ovulation when mating or artificial insemination results in arrest and adhesion of spermatozoa in the caudal portion of the isthmus [1], the oviductal fluid from the follicular phase of the cycle, when medium to large follicles can be found in the ovary and estrogen levels are high [10], represents a sample of suitable interest for studies on sperm function.

Here we report some initial studies in which we aimed to evaluate the effects of porcine oviductal fluid (pOF), collected during the preovulatory phase of the estrus cycle, on specific aspects of boar sperm function, namely plasma membrane integrity, their ability to undergo bicarbonate-induced motility stimulation, plasma membrane fluidity and acrosomal integrity. We have also evaluated the direct functional influence of oviductal fluid on the performance of spermatozoa within an *in vitro* fertilization system. The bicarbonate-induced sperm motility stimulation is of specific interest because when spermatozoa enter the female reproductive tract, and the oviduct in particular, they are exposed to increased environmental concentrations of bicarbonate (33–40 mM [11]), which acts as a signalling molecule promoting capacitation. At these concentrations, bicarbonate stimulates boar sperm motility activation *in vitro*; however, this would seem incompatible with the *in vivo* formation of a sperm reservoir within the oviductal isthmus, and suggests that the oviductal cells or

oviductal fluids may produce or secrete molecules that oppose the motility stimulation response through antagonistic signal mechanisms.

In a preliminary attempt to determine whether the outcomes of this study were caused by large or small macromolecules, we performed these experiments using high and low molecular weight fractions of oviductal fluids (> or <100 kDa). This level of discrimination permitted some differential effects to be identified. Interestingly, while the motility effects were restricted to the lower molecular weight fraction, this was not the case for the other attributes.

## 2. Materials and methods

### 2.1. Collection of oviductal fluid

Genital tracts from 7 mo old Landrace/LargeWhite gilts (N = 16) weighing approximately 95 kg were obtained at the abattoir immediately after slaughter and transported to the laboratory on ice within 30 min. Once in the laboratory, oviducts (N = 32) were classified on the basis of their ovarian morphology on both ovaries from the same female. Only oviducts obtained from gilts with follicular development indicating high levels of blood estradiol were used. The criteria were as follows: ovaries showing more than 5 follicles with a diameter larger than 5 mm. According to Yen et al., this would correspond, approximately, to day 17–18 of the estrus cycle [10], and the concentrations of estradiol-17 $\beta$  (mean  $\pm$  SE, ng/mL) in follicular fluid are likely to range between  $150.9 \pm 49.8$  and  $275.9 \pm 51.5$ . Bearing in mind that the follicular phase of the cycle in the gilt lasts approximately 7 d and that the follicular size at the ovulation time reaches 10 mm in diameter, the oviductal fluid collected from the oviduct of these animals was considered as belonging to the 48 h previous to ovulation.

Oviducts were separated from the tracts and quickly washed once with 0.4% v/v cetrimide (alkyltrimethylammonium bromide) solution and two times with Dulbecco's PBS and transferred to Petri dishes on ice and dissected. Once dissected, the oviducts were not opened lengthwise but the porcine OF from whole oviduct was collected by aspiration with an automatic pipette by introducing the tip into the ampulla for a maximum 200  $\mu$ L volume and making a manual ascendent pressure from the isthmus to the ampulla as described previously [12]. Once aspirated, the fluid was centrifuged at  $7000 \times g$  for 10 min at 4°C to remove cellular debris. Then the supernatant was immediately stored at -80°C until use. This method of oviductal fluid collection has

been shown to keep the enzyme and protein activity in previous studies [12,13]. Two fractions (<100 kDa and >100 kDa) of the pooled pOF samples were obtained by three successive ultracentrifugations at  $7200 \times g$  for 20 min in centrifugal filter devices (Amicon ultra, Millipore, Billerica, MA, USA). PAGE gels of the proteins in each fraction revealed that, although not perfect, fractionation provided a majority of >100 kDa proteins in the high molecular weight fraction and a majority of <100 kDa proteins in the low molecular weight fraction. Protein concentration in every fraction was determined by the bicinchoninic acid assay (BCA method; [14] following manufacturer's instructions (Pierce, Rockford, Illinois, USA). Bovine serum albumin was used as the standard for the protein assays. Three measurements of protein were run in each pOF sample with 12, 8, and 4  $\mu\text{L}$  of pOF. For each sample, concentration of protein was the mean among these three measurements. The fractions were lyophilized and stored at  $-80^\circ\text{C}$  until used.

## 2.2. Semen collection and sperm washing

Sperm-rich fractions of semen were collected from fertile boars at different AI centres. The semen samples were diluted in Beltsville thawing solution (BTS) extender [15] and were stored for no more than 1 d. Before experimentation, spermatozoa were isolated from the diluted semen by sedimentation through a two-step Percoll (Pharmacia, Uppsala, Sweden) gradient [7]. After centrifugation, the supernatant layers were removed by aspiration and the resultant sperm pellets (final concentration approximately  $4 \times 10^8$  spermatozoa/mL, viability >90%) were kept at ambient temperature protected from draughts.

## 2.3. Experiment 1. Effects of high and low molecular weight fractions of porcine oviductal fluid on sperm motility

For the sperm motility assays, the protocol described by Satake et al. was followed [7]. Samples from 2 groups of 6 boars were washed using Percoll, as described above, and used for experimentation within 2 h of washing. For motility experiments 3  $\mu\text{L}$  of sperm suspension in 70% (w/v) Percoll were added to 1 mL of suspending media; thus the Percoll was diluted to a final concentration of 0.2 % w/v.

Incubations were carried out as follows. One mL of a modified Hepes-buffered medium, (M solution; [16], containing 50  $\mu\text{g}/\text{mL}$  oviductal fluid fraction (>100 kDa or <100 kDa) ("test samples") or 50  $\mu\text{g}/\text{mL}$  bovine serum albumin Fraction V (Sigma-Aldrich, Poole,

UK) ("control samples"), was pre-warmed to  $38^\circ\text{C}$  in a capped 6-mL polystyrene tube (Sterilin, Stone, Staffs, UK). It should be noted that the "M" medium contained BSA at a concentration of 3 mg/mL, and that the additional 50  $\mu\text{g}/\text{mL}$  was added to control samples for balancing the protein concentrations between treatments. The 50  $\mu\text{g}/\text{mL}$  concentration of oviductal protein was chosen according to previous dose response studies following the same protocol in which it was shown that a soluble apical plasma membrane fraction derived from the oviduct at this protein concentration caused an effect on spermatozoa [7]. An aliquant (3–5  $\mu\text{L}$ ) of washed spermatozoa (final concentration approximately  $2 \times 10^6$  cells/mL) was added to 1 mL of M medium, and the suspension incubated at  $38^\circ\text{C}$  for 10 min. A 60  $\mu\text{L}$  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 (300 mM NaCl as a control for 15 mM bicarbonate addition; this addition ensured that osmotic conditions were the same between the control and bicarbonate treatments). Thirteen min after the initiation of incubation, a 300 mM solution of  $\text{NaHCO}_3$  saturated with 100%  $\text{CO}_2$  (bicarbonate/ $\text{CO}_2$  mixture or "activator") was added to the rest of the sperm suspension (in the first tube), achieving a final bicarbonate concentration of 15 mM but without affecting pH. Incubation of both tubes was continued, and further 60  $\mu\text{L}$  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 [17]. Sixty  $\mu\text{L}$  samples were taken from the sperm suspensions and placed on electropositive glass slides (Fisher Scientific UK Ltd, Loughborough, UK); microscopy was performed using an Olympus BH-2 microscope with a  $\times 10$  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  $\mu\text{m}$ , and the "minimum track points" setting was 50 frames.

The measured descriptors of sperm motion are summarised in Table 1 and details regarding the use of the

Table 1  
Definitions of sperm motility descriptors.

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

Hobson Sperm Tracker and discussion of these parameters may be found in an earlier publication [18].

#### 2.4. Experiment 2. Effect of different fractions of porcine oviductal fluid on sperm viability, membrane fluidity, acrosome reaction and *in vitro* fertilizing capacity

The remaining experiments were performed by incubating the sperm samples for 20 min in TALP medium containing either BSA (control group), porcine oviductal fluid >100 kDa fraction, or porcine oviductal fluid <100 kDa fraction, with a final 50  $\mu\text{g/mL}$  protein concentration in the three media. After this incubation the sperm samples were used for the evaluation of the different parameters (assessment of viability, plasma membrane fluidity, acrosome reaction and *in vitro* fertilization (IVF).

For the flow cytometry analysis and IVF assays, protocols were as described previously [19,20]. Aliquots of the semen samples (0.5 mL) were centrifuged ( $700 \times g$ , 30 min) through a discontinuous Percoll gradient and the resultant sperm pellets were diluted in TALP medium and centrifuged again for 10 min at  $100 \times g$ . Finally, the pellet was diluted in TALP containing different pOF protein supplements depending on the experimental groups, and later used for the analysis of viability, plasma membrane fluidity, acrosomal integrity or IVF assays.

#### 2.5. Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc, Miami, Fla). A 15-mW argon ion laser operating at 488 nm was used for excitation of the fluorophores. Data from 10,000 events per sample were collected in list

mode, and four measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

#### 2.6. Assessment of plasma membrane fluidity

To detect an increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 [16]. Stock solutions of M540 (1 mM) and Yo-Pro 1 (25  $\mu\text{M}$ , Molecular Probes, Eugene, OR) in DMSO, were prepared. For each 1 mL diluted semen sample (containing  $5\text{--}10 \times 10^6$  cells), 2.7  $\mu\text{L}$  M540 stock solution (final concentration 2.7  $\mu\text{M}$ ) and 1  $\mu\text{L}$  of Yo-Pro (25 nM final concentration) were added. M540 fluorescence was collected with a FL2 sensor using a 575 nm band-pass filter and Yo-Pro 1 with a FL1 sensor using a 525 nm band-pass filter. Cells were classified in three categories: low merocyanine fluorescence (viable, low fluidity), high merocyanine fluorescence (viable, high fluidity) or Yo-Pro-1 positive (dead).

#### 2.7. Assessment of the acrosome integrity

Each 1 mL diluted semen sample (containing  $5\text{--}10 \times 10^6$  cells) was incubated with 2  $\mu\text{L}$  of fluorescein-labelled lectin from the peanut plant, *Arachis hypogaea* (FITC-PNA, 100 mg/mL) and 5  $\mu\text{L}$  of propidium iodide (PI) stock solution (500 mg/mL), at room temperature for 10 min. Fluorescence was measured by flow cytometry using a FL-1 sensor, a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575 nm band-pass filter to detect PI. Three sperm subsets were detected: live acrosome intact, live acrosome damaged and dead spermatozoa (with and without acrosome intact).

#### 2.8. Experimental protocols for IVF experiments

Unless otherwise indicated, all chemicals and reagents for the IVF were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). The medium used for pig oocyte maturation was NCSU-37 [21] supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5  $\mu\text{g/mL}$  insulin, 50  $\mu\text{M}$   $\beta$ -mercaptoethanol, 10 IU/mL eCG (Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/mL hCG (Veterin corion, Divasa Farmavic, Barcelona, Spain), and 10% porcine follicular fluid (v/v).

Within 30 min of slaughter, ovaries from Landrace  $\times$  Large-white prepubertal gilts were transported to the laboratory in saline containing 100  $\mu\text{g/mL}$  kana-

mycin sulphate at 38°C, washed once in 0.04% cetrimide solution and twice in saline. Oocyte-cumulus cell complexes (COCs) were collected from antral follicles (3–6 mm diameter), washed twice with Dulbecco's PBS supplemented with 1 mg/mL polyvinyl alcohol (PVA), and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO<sub>2</sub> in air. Only COCs with complete and dense *cumuli oophori* were used for the experiments. Groups of 50 COCs were cultured in 500 µL maturation medium for 22 h at 38.5°C under 5% CO<sub>2</sub> in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20–22 h.

The basic medium used for IVF, designated as TALP medium, consisted of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-Lactate 5H<sub>2</sub>O, 0.5 mM MgCl<sub>2</sub> 6H<sub>2</sub>O, 0.35 mM NaH<sub>2</sub>PO<sub>4</sub>, 25.07 mM NaHCO<sub>3</sub>, 10 mM Na lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/mL BSA-FAF (A-6003), 1 mg/mL PVA, and 0.17 mM kanamycin sulfate [20].

COCs cultured for a total of 44 h in maturation medium were washed three times with TALP medium. Groups of 30–35 oocytes were transferred into each well of a 4-multiwell containing 250 µL of TALP medium previously equilibrated at 38.5°C under 5% CO<sub>2</sub> and inseminated with 250 µL of the sperm suspension (pre-incubated for 20 min with 50 µg/mL pOF or BSA proteins), giving a final concentration of  $2 \times 10^5$  cells/mL [19] and 25 µg/mL pOF or BSA.

At 15 min post-insemination (hpi) oocytes were washed twice with fresh TALP by gentle aspiration through a glass pipette and allowed to continue in culture at 38.5°C under 5% CO<sub>2</sub> until fixation. At 18–20 hpi, putative zygotes were fixed for 30 min (0.5% glutaraldehyde in PBS), stained for 15 min (1% Hoechst 33342 in PBS), washed in PBS containing 1 mg/mL polyvinylpyrrolidone, mounted on glass slides and examined under an epifluorescence microscope at 400X magnification for evidence of sperm penetration and pronuclear formation. Penetration, number of spermatozoa per penetrated oocyte, pronuclear formation and spermatozoa bound to zona pellucida were assessed for each oocyte.

## 2.9. 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. Planned comparisons within the experimental designs were analysed using contrast analysis. More detailed analyses were also performed using multivariate pattern analysis software PATN [18]. Multivariate analyses of sperm motion parameters were carried out using the computer program PATN. 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 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. [18]. 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 (µm/s 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. 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 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.

Data from the IVF experiments were expressed as mean  $\pm$  SEM and analysed by ANOVA, considering the specific sperm treatment as the main variable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison *post hoc* test (Tukey). Differences were considered statistically significant at  $P < 0.05$ . In vitro penetration rates and percentages of spermatozoa were modelled according to the binomial model of parameters by arcsin transformation of the data and were analysed by ANOVA.

### 3. Results

#### 3.1. Experiment 1. Effects of high and low molecular weight fractions of porcine oviductal fluid on sperm motility

Summary motion parameter values were calculated separately for the low and high pOF datasets and used in one way ANOVAs to examine the effects of oviductal fluid fractions on sperm motility activation. Values were averaged across all incubation times (5 min intervals up to a maximum of 32 min after addition of activator) involving bicarbonate stimulation (i.e.,  $>2$  min) and the 12 individual boars (2 groups of 6) were used as replicates. Mean values for all motion parameters measured (VCL, VAP, VSL, BCF, ALH, and LIN) were significantly ( $P < 0.001$ ) lower for incubations with the low molecular weight pOF than the corresponding controls (pOF absent) (Fig. 1A). By contrast, there were no differences when the same analysis was performed for the high molecular weight pOF treatment data ( $P > 0.5$ ).

As demonstrated previously [18], such mean and standard deviation values do not accurately reflect the distribution of data points within the samples. To illustrate this point, Figures 1B and 1C represent a comparison (VAP  $\times$  LIN scatterplots) between two single corresponding samples from the same boar (7 min after bicarbonate addition), where one sample (Fig. 1C) has been pre-treated for 10 min with low molecular weight pOF. The cloud of data points distributed towards the top right corner represents spermatozoa showing fast-linear motion, while spermatozoa towards the bottom

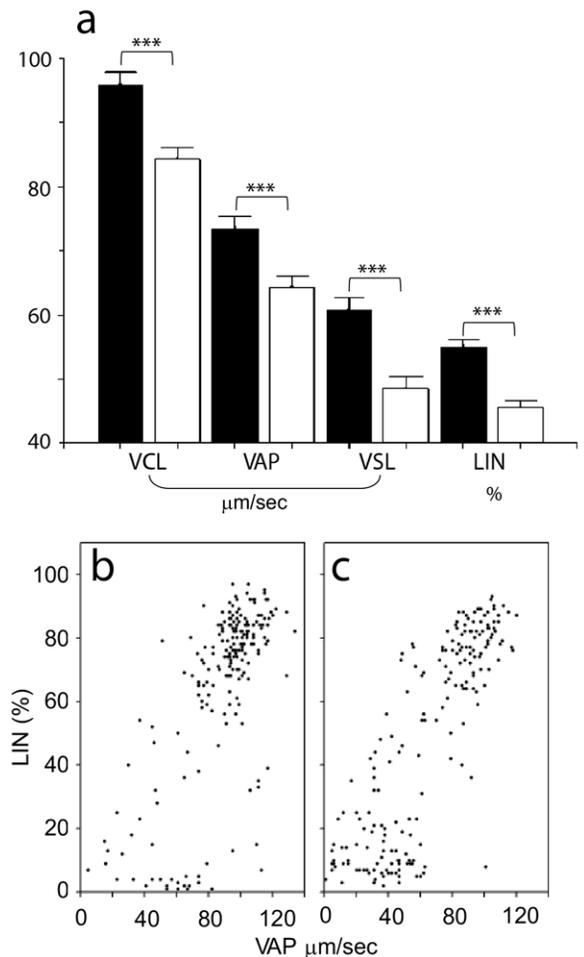


Fig. 1. (A) This figure shows a comparison of the motion parameter values for spermatozoa incubated in the presence (open bars) and absence (black bars) of 50  $\mu\text{g}/\text{mL}$  pOF ( $<100$  kDa). Values are mean ( $\pm$ SEM) of individual boar results at 2, 7 and 12 min after bicarbonate addition. Figure 1 (B) and 1 (C) show VAP v LIN scatterplots for two individual sperm samples 7 min after bicarbonate addition. Individual dots represent individual sperm data measured in the absence (Fig 1B) and presence (Fig 1C) of 50  $\mu\text{g}/\text{mL}$  pOF ( $<100$  kDa).

left corner represent slow moving and non-linear spermatozoa.

To investigate the data in more detail we therefore used PATN analysis, which aims to classify the entire set of individual spermatozoa into functionally meaningful subsets, so that the data can be interpreted more easily. All spermatozoa ( $N = 26,986$  spermatozoa; 12 boars (replicates); incubation times = 0 (no bicarbonate), 2, 7, 12, 17, and 35 min (repeat of the no bicarbonate tube); motility parameters VAP, VSL, BCF, and ALH) were classified by PATN into one of three groups (Table 2). PATN group 1 represented slow-non linear

Table 2

Summary of group mean ( $\pm$ SD) sperm motion parameters derived from PATN analysis of data of Experiment 1.

PATN group	(%)	VAP ( $\mu$ m/s)	VSL ( $\mu$ m/s)	BCF (Hz)	ALH ( $\mu$ m)
1	22.74	22.05 $\pm$ 4.86	7.05 $\pm$ 5.95	0.10 $\pm$ 0.01	0.10 $\pm$ 0.01
2	24.56	61.63 $\pm$ 18.82	5.07 $\pm$ 1.32	0.35 $\pm$ 0.30	0.34 $\pm$ 0.25
3	52.70	58.96 $\pm$ 30.33	54.37 $\pm$ 30.79	7.78 $\pm$ 2.61	9.19 $\pm$ 4.23

spermatozoa; PATN group 2 represented fast-non linear spermatozoa and PATN group 3 represented the fast-linear sperm population. Treatment effects were then examined by expressing the proportions of each PATN group in every individual sample  $\times$  treatment  $\times$  boar  $\times$  time combination by the use of frequency analysis. The proportions were then used in ANOVAs to examine effects of boar and treatments (presence of OF; presence of bicarbonate, and incubation time).

There were no significant effects of the higher molecular weight pOF fraction on motility in the presence of bicarbonate, but the presence of bicarbonate nevertheless resulted in significant motility stimulation ( $F_{1/56} = 23.87$ ;  $P < 0.001$ ; data not shown). The low molecular weight pOF fraction did not affect sperm motility if bicarbonate was absent, but caused significant depression of the bicarbonate stimulation response (bicarbonate  $\times$  treatment interaction;  $F_{1/40} = 37.18$ ;  $P < 0.001$ ; Figure 2). Significant differences in the way that sperm samples from individual boars responded to bicarbonate were observed ( $F_{5/48} = 5.3$ ;  $P < 0.001$ ), but there was no significant overall boar  $\times$  treatment interaction ( $P > 0.75$ ).

### 3.2. Experiment 2.1. Effect of high and low molecular weight fractions of porcine oviductal fluid on sperm viability, plasma membrane fluidity, and acrosomal integrity

After 20 min incubation in fractions of porcine oviductal fluid, sperm viability and acrosomal integrity were approximately 20% higher ( $P < 0.01$ ) than in the corresponding control samples. (Tables 3 and 4). The lower molecular weight fraction (pOF  $< 100$  kDa) supported sperm viability and significantly reduced the percentage of spermatozoa showing high membrane fluidity (capacitated). The high molecular weight fraction (pOF  $> 100$  kDa) also maintained sperm viability and reduced the percentage showing high membrane fluidity, but to a lesser extent than in the low molecular weight fraction. The presence of oviductal proteins slightly increased (less than 1%) the percentage of acrosome reacted spermatozoa compared to the control group (Table 4).

### 3.3. Experiment 2.2. Effect of the high and low molecular weight fractions of porcine oviductal fluid on IVF results

Incubation of sperm samples for 20 min with pOF (50  $\mu$ g/mL, high molecular weight fraction,  $> 100$  kDa) before IVF resulted in a significant decrease in the percentage of monospermy ( $P < 0.001$ , Table 5) and a significant increase ( $P < 0.001$ ) in the mean number of spermatozoa per penetrated oocyte. This increase appeared to be a direct consequence of the higher number of sperm bound to the ZP ( $P < 0.001$ ) of those oocytes (Significant Pearson correlation for these two variables,  $P < 0.01$ ).

When the experiment was repeated with the low molecular weight fraction ( $< 100$  kDa) of porcine oviductal fluid, the results were similar to those reported for the high molecular weight fraction (Table 6).

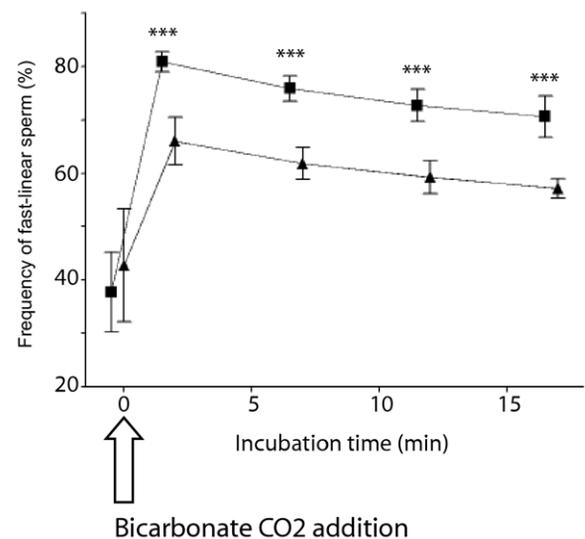


Fig. 2. Graph showing how the proportion of fast-linear spermatozoa (PATN group 3) increased rapidly after the introduction of bicarbonate/CO<sub>2</sub>, irrespective of whether pOF ( $< 100$  kDa) was present (▲) or absent (■). This plot also shows that the pOF significantly suppressed the initial stimulation response and that the suppression was maintained throughout the period of the observation. Data are shown as means ( $\pm$ SEM). N = 6 boars.

Table 3

Flow cytometric evaluation of membrane fluidity (lipid packing disorder) and viability in boar sperm subpopulations estimated using merocyanine 540 and Yo-Pro 1.

	Viable low lipid disorder (%)	Viable high lipid disorder (%)	Viability (%)	Viable high lipid disorder/viable (%)
Control	48.38 ± 6.18 <sup>a</sup>	3.83 ± 0.56 <sup>a</sup>	52.22 ± 6.66 <sup>a</sup>	7.28 ± 0.58a
pOF (>100 kDa)	68.54 ± 5.41 <sup>b</sup>	2.23 ± 0.45 <sup>b</sup>	70.77 ± 5.19 <sup>b</sup>	3.72 ± 0.97 <sup>b</sup>
pOF (<100 kDa)	76.11 ± 3.03 <sup>b</sup>	1.04 ± 0.10 <sup>b</sup>	77.15 ± 3.12 <sup>b</sup>	1.33 ± 0.09 <sup>c</sup>
P values	<0.01	<0.01	<0.01	0.03

Boar spermatozoa were incubated for 20 min in TALP medium in the presence and absence of fractions of porcine oviductal fluid (pOF).

a,b,c: Numbers within columns with different superscripts differ ( $P < 0.05$ ).

#### 4. Discussion

This study has revealed that porcine oviductal fluid from the follicular phase of the estrus cycle induces biochemical, biophysical, and functional alterations in spermatozoa after a relatively short exposure time. Sperm viability was significantly protected, together with improved maintenance of acrosomal integrity, and the overall effect could therefore be viewed as the prevention of premature capacitation. While these general effects of oviductal fluid were induced by both high and low molecular weight fractions, the lower molecular weight fraction was specifically able to suppress the extent to which sperm motility could be stimulated by exposure to bicarbonate. As the suppressive effect was evident at a pOF concentration of 50  $\mu\text{g/mL}$ , and in the presence of 3 mg/mL BSA, we regard this outcome as being directly caused by component(s) present in the pOF but not present in BSA. While all of these effects might have been predicted to cause lower fertilization performance in IVF experiments, the opposite was true. Fertilization rate itself was elevated, although not enough to be statistically significant, but the increased incidence of both polyspermy and zona binding after exposure to oviductal fluid indicated that physiological sperm function appeared to be enhanced.

Table 4

Acrosomal integrity evaluated using flow cytometry in subpopulations of spermatozoa after staining with FITC-PNA and PI.<sup>c</sup>

	Intact acrosome and viable (%)	Acrosome reacted and viable (%)	Viability (%)
Control	51.63 ± 4.19 <sup>a</sup>	0.56 ± 0.07 <sup>a</sup>	52.19 ± 4.18 <sup>a</sup>
pOF (>100 kDa)	65.67 ± 7.54 <sup>a,b</sup>	0.60 ± 0.11 <sup>a</sup>	66.27 ± 7.57 <sup>a,b</sup>
pOF (<100 kDa)	75.89 ± 3.09 <sup>b</sup>	1.37 ± 0.17 <sup>b</sup>	77.26 ± 3.19 <sup>b</sup>
P values	< 0.01	< 0.01	< 0.01

Boar spermatozoa were incubated for 20 min in TALP medium in the presence and absence of fractions of porcine oviductal fluid (pOF).

a,b,c: Numbers within columns with different superscripts differ ( $P < 0.05$ ).

This result provides an interesting contrast to the results of previous experiments in which the oocytes, but not the spermatozoa, were exposed to the same oviductal fluid. In that instance the oviductal fluid caused a reduction of polyspermy, probably associated with the significant zona hardening induced at the same time [13]. The effects observed here on sperm viability, membrane fluidity and IVF success were apparent after only 20 min pre-exposure to pOF and in reality may have been induced more rapidly. These outcomes are therefore difficult to compare with other studies in which bull spermatozoa were pre-exposed to OF for longer periods, up to 4 h [22], or in which OF was added directly to the IVF medium and incubated for 6 h. Evidence of motility modulation has been found with bull spermatozoa and bovine oviductal fluid, suppressive effects on curvilinear velocity being noted after 4 h incubation at a concentration of 60% v/v [23].

Making precise comparisons between the present results and those reported by McNutt et al. [23] is difficult because of differences between protocols; however, as these authors reported their data as a series of histograms, it is apparent that they also found differential sperm subpopulation effects, as reported here, and that despite a major subpopulation experiencing motility suppression, a smaller group of spermatozoa remained largely unaffected.

The present results have to be interpreted in the context of several different aspects of oviductal function. Detailed observations have shown that when boar spermatozoa enter the oviduct they become segregated into two major subpopulations [24,25]. The minority, which are found within the oviductal lumen, typically show evidence of poor viability and degeneration, while those which interact with the epithelial cell surfaces possess intact acrosomes (in bovine) [26], are uncapacitated (in porcine) [27], and can be considered as having been selected for their high quality (in bovine) [28]. The sperm-oviduct interaction stimulates the transcription of oviductal genes and the *de novo* syn-

Table 5

Effect of incubation of sperm with 50  $\mu\text{g}/\text{mL}$  of high molecular weight fraction ( $>100$  kDa) of porcine oviductal fluid on IVF results.

Treatment	PEN (N)	MON	MPN	SPZ/O	SPZ/ZP
Control	69.28 $\pm$ 3.59 (166)	44.34 $\pm$ 4.63 <sup>a</sup>	97.39 $\pm$ 1.49	2.17 $\pm$ 0.13 <sup>a</sup>	9.52 $\pm$ 0.74 <sup>a</sup>
pOF ( $>100$ kDa)	77.51 $\pm$ 3.22 (169)	25.95 $\pm$ 3.82 <sup>b</sup>	98.47 $\pm$ 1.07	2.99 $\pm$ 0.16 <sup>b</sup>	14.63 $\pm$ 0.99 <sup>b</sup>

N: Number of inseminated oocytes; PEN: Penetration percentage from N; MON: Monospermy percentage calculated from penetrated oocytes; MPN: Percentage of male pronuclear formation; SPZ/O: Mean number of penetrated sperm per oocyte; SPZ/ZP: Mean number of sperm bound to zona pellucida.

a,b: Different letters in the same column indicate significant differences ( $P < 0.001$ ).

Table 6

Effect of incubation of sperm with 50  $\mu\text{g}/\text{mL}$  of low molecular weight fraction ( $<100$  kDa) of porcine oviductal fluid on IVF results.

Treatment	PEN (N)	MON	MPN	SPZ/O	SPZ/ZP
Control	84.62 $\pm$ 3.35 (117)	41.41 $\pm$ 4.97 <sup>a</sup>	97.98 $\pm$ 1.42	2.56 $\pm$ 0.17 <sup>a</sup>	12.17 $\pm$ 0.93 <sup>a</sup>
pOF ( $<100$ kD)	91.3 $\pm$ 2.22 (161)	17.69 $\pm$ 3.15 <sup>b</sup>	100	4.93 $\pm$ 0.33 <sup>b</sup>	22.53 $\pm$ 0.88 <sup>b</sup>

N: Number of inseminated oocytes; PEN: Penetration percentage from N; MON: Monospermy percentage calculated from penetrated oocytes; MPN: Percentage of male pronuclear formation; SPZ/O: Mean number of penetrated sperm per oocyte; SPZ/ZP: Mean number of sperm bound to zona pellucida.

a,b: Different letters in the same column indicate significant differences ( $P < 0.001$ ).

thesis of proteins [4]. While these spermatozoa remain bound to epithelial cells there would be little requirement for maximally active flagellar beating. In Experiment 1 we were trying to see whether oviductal fluid has any physiological signalling capacity that could reduce (or enhance) the sperm motility. Adding the pOF before the bicarbonate meant that we could separate the enhancement effect of bicarbonate from the suppressive effect of OF. By this approach we found that the pOF interfered with the motility stimulation response, and interpret this as showing that it must be acting via extracellular receptors that form part of a signal transduction pathway.

The observation that oviductal fluid tends to suppress sperm motility, even in the presence of bicarbonate at concentrations (15 mM) below those found in isthmus oviductal fluid (33–35 mM [11,29]), therefore seems quite logical. Furthermore it suggests that since bicarbonate stimulates motility via a signal transduction pathway that involves the activation of adenylyl cyclase, and subsequent protein phosphorylation [17,30,31], oviductal fluid must contain components that are capable of suppressing this pathway in a sizeable proportion of the spermatozoa. The present data suggests strongly that the molecules responsible for this motility suppression effect are smaller than 100 kDa, as the higher molecular weight pOF fraction did not affect motility activation. In contrast, the present study showed that membrane lipid disorder (fluidity) was reduced, and plasma membrane integrity protected, by both of the pOF fractions, leading to the conclusion that

the motility suppression effect and the membrane effects are not necessarily caused by the same molecules.

These results are similar to those reported previously [7], where it was found that solubilised apical plasma membrane proteins (sAPM) from oviductal epithelial cells inhibited bicarbonate-induced motility activation in a dose-dependent manner, but did not abolish it. Satake et al. [7] did not establish the exact identity of the molecular components responsible for this inhibition, but were able to show that the entry of bicarbonate into the cells was not inhibited. In fact, exposure of spermatozoa to sAPM was accompanied by enhancement of the intracellular pH increase that accompanies capacitation. Exploration of pig sAPM-sperm binding interactions eventually led to the proposal that one of the most significant sAPM components able to promote sperm viability is heat shock 70 kDa protein 8 (HSPA8) [32]. This protein has been detected within the OF preparation used here (Mondéjar I, Saavedra MD, Avilés M and Coy P; unpublished data), and has also been reported by others as a component of the oviductal epithelial proteome [4,32,33]. In addition, the same protein has been shown to promote the survival of ram [34] and bull [35] spermatozoa during prolonged *in vitro* incubation at body temperature. The potential involvement of heat shock proteins as a modulator of membrane fluidity in spermatozoa is supported by observations that sperm plasma membrane sulphogalactolipids, or seminolipids [36,37], interact with a specific binding protein (SLIP-1, (for review, see [38]) as a prelude to capacitation and can modulate lipid packing

structures. SLIP-1, a 68 kDa protein, is regarded as being closely related to the heat shock 70 kDa protein family [39], and indeed HSPA8 itself is known to have sulphogalactolipid binding abilities [40,41]. The modulatory properties of oviductal fluid proteins on sperm plasma membrane fluidity are therefore entirely plausible.

Although this discussion has focused upon chaperone proteins, we recognize that the oviductal fluid is a complex mixture of proteins. Notable among the high molecular weight molecules of oviductal fluid are oviduct-specific glycoprotein (OVGP1) [42], glycosidases such as  $\alpha$ -L-fucosidase,  $\beta$ -D-galactosidase or D-mannosidase [12,43], together with sulphated and non-sulphated glycosaminoglycans [44,45]. Low molecular weight components of the oviductal fluid with demonstrated effects on sperm function include glycodefins, which modulate capacitation by controlling plasma membrane fluidity [46–49] and could be responsible for some of the observations reported here. Moreover, these proteins are unlikely to function in isolation and it is probable that some groups of proteins, such as the chaperones, might form multiprotein complexes involving sperm plasma membrane proteins in order to fulfil their functions [50].

#### Disclosure statement

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

#### Acknowledgments

P.C.'s stay in London was supported by the Spanish Ministry of Education, Grant PR2006-0506. This work was supported by MEC and FEDER, Ref. AGL2006-03495 and AGL2009-12512-C02-01.

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