Sperm binding to oviduct epithelial spheroids varies among males and ejaculates but not among females in pigs

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Original Research Article

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ABSTRACT

The elimination of ejaculates and males with low fertility despite good sperm motility and morphology is crucial to maintain high pregnancy rates after artificial insemination (AI) in farm animals. The ability of sperm to survive in the female tract is particularly crucial in pigs due to the large variation in the timing between AI and ovulation and the high number of oocytes to fertilise. The objective of this study was to characterise a new *in vitro* model of oviduct sperm reservoir using porcine oviduct epithelial spheroids (OES) and to assess the variability in sperm binding to OES among gilts, boars and their ejaculates. Isthmic mucosa fragments were collected from gilt oviducts at a slaughterhouse, and after 48 h of culture, the OES that had spontaneously formed were sorted according to their vesicle shape and size (150–200 μm in diameter) for characterisation and sperm binding assays. The OES contained viable, cytokeratin-positive and vimentin-negative cells, of which 36.4 ± 2.0% were multiciliated. The average proportion of multiciliated cells per OES did not change among culture replicates. After co-incubation with boar fresh semen, only sperm of normal morphology were found to bind, by their head, to cilia of OES. The density of sperm bound to the OES surface increased linearly with sperm concentration. The bound sperm density on OES was used to assess the binding capacity of fresh ejaculates collected from Pietrain boars. For a given ejaculate, the bound sperm density did not vary among pools of OES female donors. The analysis of five successive ejaculates from nine boars indicated significant differences in bound sperm densities on the OES among individual boars and their ejaculates ($P < 0.01$). There was no correlation between the sperm bound density and sperm parameters measured by computer-assisted sperm analysis or the initial dilution of the ejaculate. In conclusion, the OES characterised in this study offered physiological conditions to study sperm binding to the isthmic reservoir and evidenced that sperm from different ejaculates and different boars vary in their ability to bind to these oviduct spheroids despite homogeneous motility and morphology.

1. Introduction

Artificial insemination (AI) is the most widely used reproductive technology to spread the genetic value of males selected for breeding traits in farm animal populations. Ejaculates from boars used for AI are typically collected twice a week, with one ejaculate providing usually 20 to 40 AI doses that are shipped at temperatures between 16 and 19 °C to production farms to be used within 4–5 days. The traditional methods used to evaluate boars and ejaculates in AI centres include the systematic assessment of sperm concentration, motility and morphological abnormalities. However, some subfertile ejaculates pass through these quality controls, and fertility drops attributable to the AI doses are recorded [1]. Therefore, it is crucial to rapidly eliminate boars and ejaculates with low fertility to increase the productivity and sustainability of pork production.

The ability of sperm to survive in the female tract after AI is particularly crucial in pigs due to the large variation in the timing from the onset of oestrus to ovulations and the high number of oocytes to fertilise [2,3]. A major site of sperm survival in the female tract is the caudal part of the oviduct, namely the isthmus, where a subpopulation of spermatozoa firmly binds to multiciliated oviduct epithelial cells (OEC) [4–6]. Sperm binding to the isthmic epithelium is assumed to prolong sperm

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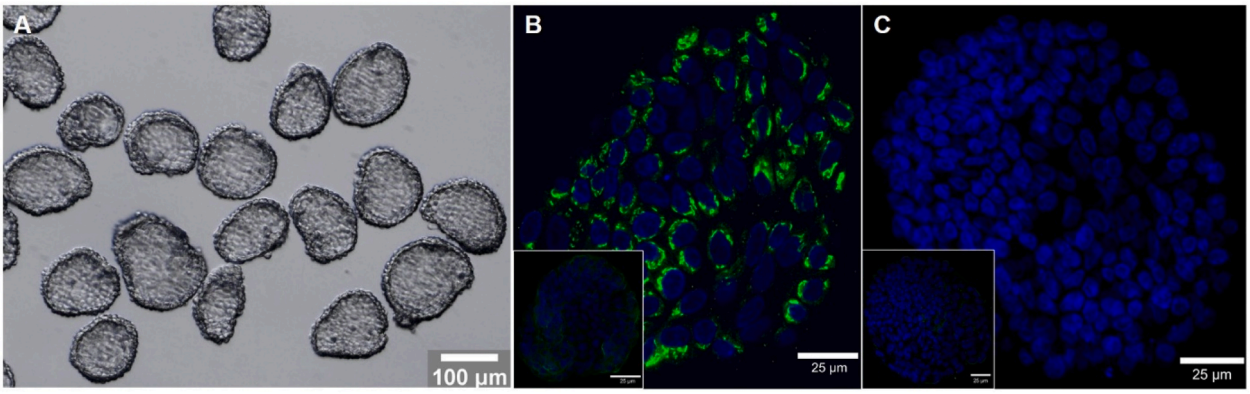

Fig. 1. Porcine oviduct epithelial spheroids (OES) selected for sperm binding assays. (A) Spheroids sorted according to shape and size before being used for sperm binding assays; (B) Representative picture after immunostaining of spheroids for the epithelial cell marker pan-cytokeratin; (C) Representative picture after immunostaining of spheroids for the mesenchymal cell marker vimentin. Cell nuclei appear in blue. Respective negative controls incubated with the immunoglobulin isotype of the primary antibody are shown in inserts. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lifespan and increase the fertilisation time window through the progressive release of alive sperm towards the ampulla, where oocytes are expelled at the time of ovulation [4–7]. Therefore, in addition to traditional methods of semen analysis, assays on sperm binding to oviductal epithelial explants or specific glycans that mimic the oviduct lumen have been proposed as an attempt to better predict male fertility in pigs [8–13]. Previous studies in pigs evidenced differences in the number of boar sperm bound to oviduct explants among individual boars [8,9,11–13], with little or no influence regarding the reproductive status of the female cell donor (sow vs. gilt), oviductal region (ampulla vs. isthmus) or stage of the oestrous cycle [8,10,13]. However, these data on sperm binding capacity have been reported as the average performance of several ejaculates from individual boars [9,10,13] or of single ejaculates [11,12], precluding any evaluation of the variation among ejaculates from the same boar.

Furthermore, most studies exploring sperm-oviduct interactions in mammals used oviductal explants or OEC monolayers [8–10,13,14]. Oviduct explants offer the advantage of being rapidly produced from oviduct mucosa sheets but are typically heterogeneous in shape and size and difficult to standardise among replicates [7,13]. Indeed, oviduct explants usually contain many folds due to the anatomy of the oviduct mucosa, leading to a rough estimate of the binding surface and number of bound sperm. Furthermore, due to the high variability in shape, total number of cells and proportions of ciliated cells among explants, the total number of sperm binding sites may considerably vary between replicates. In contrast, the OEC monolayers offer a standardised number of cells at confluence but require a long time to establish (minimum 5–7 days) and dedifferentiate during mitosis when grown on plastic [14]. One study in pigs reported the use of oviductal vesicles cultured in suspension, but these vesicles were not properly characterised [15].

In this context, the objectives of this study were 1) to characterise an *in vitro* model of porcine oviduct epithelial spheroids (OES) obtained in suspension culture and offering a standardised surface for sperm binding assays; and 2) to assess the variability of the sperm binding capacity to OES among females, males and their ejaculates.

2. Materials and methods

Otherwise stated, chemicals were purchased from Sigma-Merck (Darmstadt, Germany).

2.1. Boars and semen collection

Fresh commercial ejaculates from 43 Pietrain boars, provided by the AI-centre YXIA (Saint-Gilles, France), were used. Boar age varied from 6 to 24 months. Boars were housed in individual barns of 6 m^2, and semen was routinely collected twice a weak using a semi-automated system (CollectOr, Ecopor, France). After motility and morphology assessment, semen was diluted in the KobiDil + extender (YXIA) to reach a final concentration of 25 to 35.10^6 spermatozoa/mL and dispensed into AI doses of 80 mL. All doses were cooled and shipped at 16–19 °C to the INRAE laboratory within 8 h after collection, where they were stored at 17 °C before use for further motility assessment and binding assays. The number of boars per AI dose used varied among the experiments (see Experimental design). The fertility data of boars were not available as the commercial AI doses used were mixes of ejaculates from several boars (polyspermic doses).

2.2. Hoechst staining and motility assessment of semen

Sperm motility and morphology were assessed before co-incubation with oviduct epithelial spheroids (OES), i.e., around 60 h after semen collection. Preliminary experiments showed that sperm nuclei staining with bisbenzimide H 33342 trihydrochloride (Hoechst) simplified the quantification of bound sperm on spheroids without altering sperm motility (data not shown). Briefly, 4 mL of fresh semen was incubated for 30 min with 1 µg/mL Hoechst in the dark in a water bath at 38 °C and subsequently washed twice in non-capacitating Tyrode's-based medium [16] containing 100 mM Nacl, 3.1 mM KCl, 0.3 mM NaH_2PO_4., 2.1 mM $CaCl_2$., 0.4 mM $MgCl_2$., 1 mM sodium pyruvate, 27.4 mM sodium lactate, 10 mM HEPES and 1 mg/mL polyvinyl alcohol; pH and osmolarity were 7.4 and 300 mOsm, respectively (TALP medium). After centrifugation (500 g, 5 min, 25 °C), the sperm pellet was resuspended in 1 mL TALP medium and assessed for sperm concentration, motility and morphological abnormalities using computer-assisted sperm analysis (CASA, IVOS II, IMV Technologies, L'Aigle, France). For that, 3 µL of sperm aliquots were placed in pre-warmed (37 °C) glass counting chambers (CellVision 20-µm CASA 4 chambers). A minimum of 2 chambers and 3 fields per chamber were analysed and the following sperm parameters were evaluated: total motile sperm (%), progressively motile sperm (%), sperm with a proximal droplet (%), sperm with a distal droplet (%), sperm with a bent tail (%), sperm with a coiled tail (%), and the total % of morphologically abnormal sperm. When sperm presented an average path velocity (VAP) lower than 15 µm/s, they were classified as non-motile, and when they exhibited a VAP higher than 45 µm/s and a straightness index (STR) higher than 45%, they were considered as progressively motile. The proportion of motile and normal sperm typically exceeded 60% and 65%, respectively, prior to incubation with OES.

2.3. Collection of oviducts and obtention of epithelial spheroids

Pairs of oviducts and ovaries were collected from 6-month-old gilts of various genetic origins at a local commercial slaughterhouse (Vendôme, France), immediately placed on ice and processed in the laboratory within 2 h after animal death. The ovaries were inspected for the presence of follicles and corpora lutea, and only the oviducts of prepubertal gilts (ovaries showing only small follicles) were collected. Ten pairs of oviducts were dissected from the surrounding vessels and tissues and cut at the utero-tubal and isthmus-ampulla junctions to isolate the isthmus (approximately 5 cm in length). The isthmic sections were 2 s washed in 70% ethanol and rinsed in 0.9% NaCl; subsequently, the mucosa folds were expelled with a sterile glass slide in a 15-mL Falcon tube containing 10 mL of washing medium (Medium 199, Earle's salts) supplemented with 10% FCS, 50 μg/mL gentamycin and 25 mM HEPES. The mix was incubated at 38.8 °C for 10 min for cell sedimentation, and the supernatant was discarded. After a second dilution in 10 mL washing medium and sedimentation, the pellet was diluted 10 times in culture medium (Medium 199 with Earle's salts, L-glutamine and sodium bicarbonate) supplemented with 10% FCS and 50 μg/mL gentamycin. Subsequently, 3.3 mL of the resulting mixture was added to 46.7 mL of culture medium in a 50-mL Falcon tube containing (final cell dilution of 1:150) then distributed in 60-mm Petri dishes (Falcon, 353004) and cultured for 48 h at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air. After 48 h, vesicle-shape OES of 150–200 μm in diameter, homogeneous in form with no surface folding and vigorous ciliary beating outward, were selected for all experiments (Fig. 1).

2.4. Cell viability and movement analysis of oviduct epithelial spheroids

The cell viability in OES was evaluated after incubation in 500 μL of culture medium containing ethidium homodimer-1 (10 μM; ThermoFisher Scientific, Invitrogen) and Hoechst 33342 (2 μg/mL) for 30 min at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air. Then, the OES were washed in M199, mounted on SuperFrost Plus slides (ThermoFisher Scientif) and immediately observed under a confocal microscope (Zeiss LSM 700 Carl Zeiss, Oberkochen, Germany). Cells with a blue nucleus (due to Hoechst staining only) were considered as viable, whereas cells with a purple nucleus (due to both Hoechst and ethidium homodimer-1 staining) were considered as non-viable. The ciliary activity of the OES was evaluated through the analysis of their movement and straight-line velocity. Groups of 20–25 OES in the suspension were gently placed at the centre of the dish and left there for 15 s for stabilisation; subsequently, 15 successive pictures were taken at 2-sec intervals at 40 × magnification, allowing to calculate the proportion of moving OES and track the movement of individual OES. The straight-line track of moving OES (in pixel) was calculated from the first and last pictures at a 30-sec interval, using the TrackMate plugin in ImageJ software (version 1.54f), and expressed in μm/sec. The automated tracking of each OES was manually corrected using the TrackScheme function of the TrackMate plugin. Then, the mean straight-line velocity (in μm/sec) of moving OES per condition was calculated.

2.5. Immunostaining of cytokeratin, vimentin and acetylated alpha-tubulin in oviduct epithelial spheroids

For immunostaining, groups of 10–20 OES were fixed with 4% paraformaldehyde in PBS supplemented with 0.25% Triton X-100 and 1% bovine serum albumin (PBS-BSA) for 60 min at 37 °C, washed three times in PBS-BSA and incubated for 30 min in a blocking solution (10% goat serum in PBS-BSA, 25 °C) before being incubated overnight at 4 °C with mouse monoclonal anti-pan-cytokeratin (clone C11; C2931; 1:400), anti-vimentin (V9 clone; V6630; 1:100) or anti-acetylated tubulin (clone 6-11B-1; T7451; 1:1000) antibodies. Control OES were incubated with the isotype IgG1 (M9269) at the same concentration as that used for the corresponding primary antibody. After washing in PBS-BSA, the OES were incubated with the secondary antibody (goat anti-mouse IgG, AP124) coupled with Alexa Fluor 488 (A11001, Invitrogen, Massachusetts, USA; final concentration of 2 μg/mL) for 3 h at 25 °C under agitation. After washing twice in PBS-BSA, the OES nuclei were stained with Hoechst (1 μg/mL) and observed under a confocal microscope. Groups of 20 OES from 3 to 5 replicates were evaluated for each marker.

2.6. Sperm-spheroid binding assays and quantification of bound sperm density

Two hours before co-incubation with sperm, OES of 150–200 μm in diameter were washed 5 min in TALP medium then transferred to 70 μL of TALP medium in a sterile 96-well culture plate at 38.8 °C. Groups of 20 OES were co-incubated with spermatozoa in a final volume of 80 μL of TALP medium at 38.8 °C. The sperm concentration and time of incubation varied among experiments (see Experimental design). After co-incubation, the sperm-OES complexes were washed once in 500 μL of TALP medium to eliminate slightly attached spermatozoa and immediately fixed in 2.5% glutaraldehyde diluted in 0.1 M cacodylate for 30 min in the dark at 37 °C. After three washes in PBS-BSA, sperm-OES complexes were stored at 4 °C in the dark. To evaluate the bound sperm density, sperm-OES complexes were placed on a glass slide coated with poly-L-lysine, mounted with a drop of Vectashield medium and covered with a coverslip for observation under a confocal microscope (Zeiss LSM 700, Germany). Bound sperm density per OES unit was quantified as previously described [17]. Briefly, pictures of both sides of each sperm-OES complex were saved with white light (no fluorescence) and under a 405-nm excitation wavelength to observe the sperm nuclei. The total number of bound sperm per OES was quantified on the Image J software, and this number was divided by the total OES area to obtain the bound sperm density per OES surface unit (in sperm/mm^2). Each group of 20 OES-sperm complexes was considered as one biological unit for statistical analysis.

2.7. Scanning electron microscopy (SEM)

Sperm-OES interactions were examined according to a method previously described [17]. Sperm-OES complexes were immersed in a fixative solution (2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4) and stored at 4 °C until processing. The fixative was removed, and samples were rinsed in the sodium cacodylate solution (pH 7.4) then deposited on sterile cover-glasses discs (Marienfeld, VWR, France). The samples underwent progressive dehydration by soaking in a graded series of ethanol (50–100%) before critical point drying under CO_2. Samples were mounted on aluminum SEM sample stubs (15 mm diameter x 6 mm M4, Micro to Nano, Haarlem, Netherlands) with carbon adhesive discs (Agar Scientific, Oxford Instruments SAS, Gometz-la-Ville, France) and sputter coated with Gold–Palladium (Polaron SC7640, Milexia, Verrières-le-buisson, France) for 220 s at 10 mA. Samples were visualized by field emission gun scanning electron microscopy. They were viewed as secondary electron (2 kV) with a Hitachi S4500 instrument (Milexia, Verrières-le-buisson, France).

2.8. Experimental design

For all experiments, fresh OES sorted 48 h after the culture start of isthmic mucosa fragments were used.

Experiment 1 evaluated the cell viability, expression of epithelial cell markers and proportion of multiciliated cells in OES. The experiment was repeated 5 times with groups of OES from different gilts.

Experiment 2 evaluated the density of bound sperm on OES according to sperm concentration. Groups of 20 OES were incubated with spermatozoa at a final concentration of 1.6, 3.2, 6.4 or 12.8.10^5

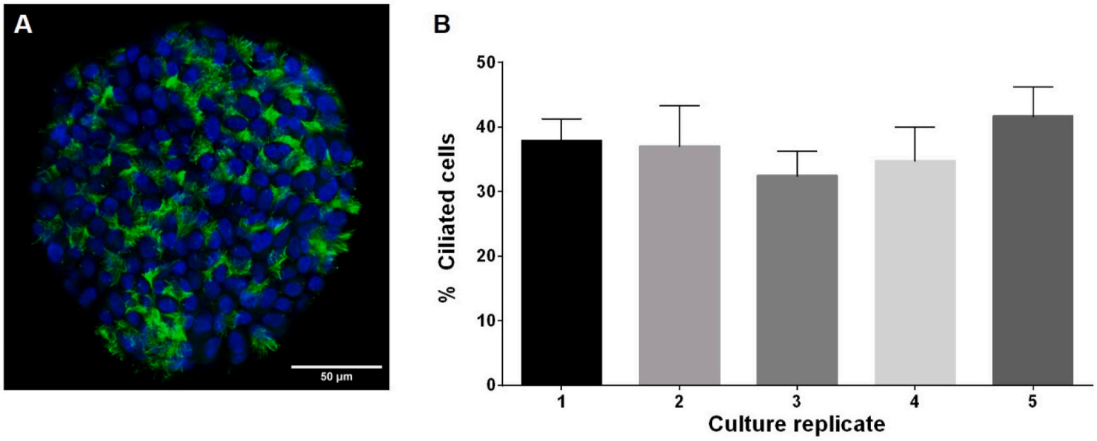

Fig. 2. Proportions of multiciliated cells in porcine oviduct epithelial spheroids among culture replicates. (A) The proportion of multiciliated cells was quantified by confocal microscopy after immunodetection of acetylated alpha-tubulin (in green) and nuclei staining with Hoechst (in blue); (B) Spheroids contained on average 37% multiciliated cells with no variation between five successive cultures of isthmic mucosa fragments (Experiment 1). Means ± SEM of 9–23 spheroids per replicate (total of 5 replicates). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

per mL. The experiment was repeated 3 times with groups of OES from different gilts and different mixes of five ejaculates.

Experiment 3 evaluated the kinetics of sperm binding on OES. Groups of 20 OES were incubated with spermatozoa at a final concentration of $1.6.10^5$ per mL for 15, 30, 60, 120 or 240 min. The experiment was repeated 3 times with OES from different gilts and different mixes of five ejaculates. Sperm-OES complexes were observed by confocal and scanning electron microscopy after 30 min to evaluate sperm interaction with OES cilia.

Experiment 4 evaluated if sperm binding density on OES varied according to the female origin of OES. Groups of 20 OES selected from three different pools of five gilts (total of 15 gilts per experiment) were incubated for 30 min with spermatozoa from one ejaculate at a final concentration of $1.6.10^5$ per mL. The experiment was repeated 4 times with different pools of gilts and ejaculates.

Experiment 5 evaluated if sperm binding density on OES varied according to the ejaculate and boar. Nine boars were collected over 2 years: boars A, B, C and D from January to June 2022, and boars E, F, G, H, I from February to March 2023. In each experiment, groups of 20 OES were incubated for 30 min with spermatozoa at a final concentration of $1.6.10^5$ per mL. The experiment was repeated with 5 successive ejaculates per boar using OES from different pools of 10 gilts.

2.9. Statistical analysis

All analyses were performed using the GraphPad Prism software (version 8.0.1). Data normality was determined using the Shapiro–Wilk test. The effects of the culture replicates on the proportion of multiciliated cells and those of sperm concentration and time of incubation on sperm binding density on OES were analysed by one-way analysis of variance (ANOVA). The effects of females and boar on sperm binding density on OES were determined via two-way ANOVA followed by Tukey's post-tests for multiple comparisons. The effects of boars and their ejaculates on sperm binding density on OES were analysed by two-way ANOVA considering the ejaculates of each boar as repeated measures, followed by Tuckey's post-tests. To determine the relationships between bound sperm density and sperm parameters, Pearson's correlation coefficients and the non-parametric Spearman correlation coefficients (for data that did not follow normality) were used. A P-value <0.05 was considered statistically significant. The results are presented as means ± SEM.

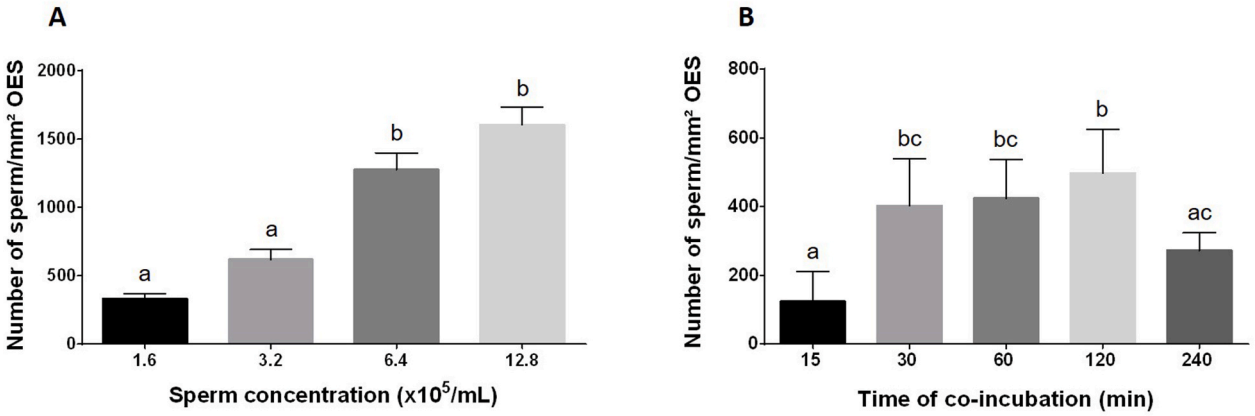

Fig. 3. Sperm binding density according to sperm concentration and time of co-incubation. (A) Groups of 20 spheroids were incubated with increasing concentrations of fresh boar spermatozoa for 30 min (Experiment 2) or (B) with sperm at a final concentration of $1.6.10^5$/mL throughout the incubation (Experiment 3). Bound sperm densities per spheroid surface unit are expressed as means ± SEM of three replicates for each experiment. Different letters indicate significant differences with a P-value <0.05.

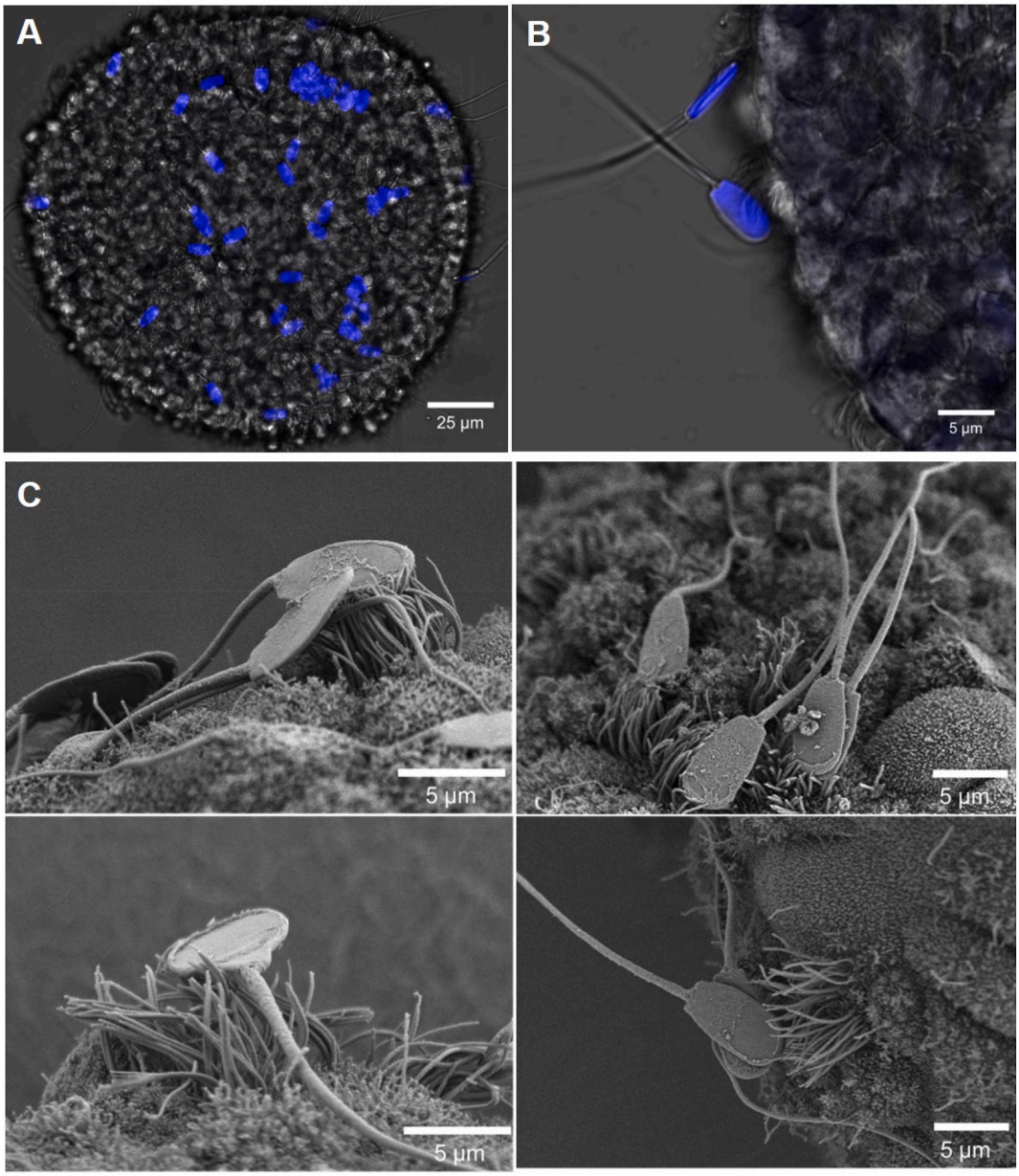

Fig. 4. Interactions of boar sperm with oviduct epithelial spheroids. (A) Representative pictures of sperm-OES complex observed under confocal microscopy (sperm nuclei are stained with Hoechst and appear in blue); (B) Higher magnification showing sperm head bound to cilia; (C) Sperm-cilia interactions observed under scanning electron microscopy (SEM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Characterisation of oviduct epithelial spheroids selected for sperm binding assays (Experiment 1)

The OES started to form after 24 h of culture of isthmic mucosa fragments from peripubertal gilts. After 48 h, OES homogeneous in shape (round vesicle) and size (150–200 µm in diameter), and displaying outward ciliary beating, were selected for sperm co-incubation (Fig. 1A). The OES contained on average 489 ± 45 cells, of which 99.7 ± 0.2% were viable (N = 59 OES from three replicates). All cells in the OES displayed a positive signal for the epithelial marker pan-cytokeratin (Fig. 1B). By contrast, no clear signal for the mesenchymal marker vimentin was evidenced (Fig. 1C). Due to ciliary beating, 100% of the OES moved in the suspension, and their average straight-line velocity was 6.6 ± 0.9 µm/s (N = 52 OES from three replicates). Immunostaining for acetylated alpha-tubulin revealed that the OES contained on average 36.4 ± 2.0% multiciliated cells (Fig. 2A; N = 85 OES from five replicates). This proportion of multiciliated cells did not vary between culture replicates among five collections of oviducts (Fig. 2B).

3.2. Sperm binding densities according to sperm concentration and incubation time (experiments 2 and 3)

The density of bound sperm per OES increased linearly with sperm concentration (333.0 ± 13.5 to 1576 ± 294 sperm/mm^2; $P < 0.002$; Fig. 3A). At the highest concentrations, clumps of sperm formed at the surface of OES. To avoid a saturation of sperm binding sites on OES and allow reliable bound sperm counting, a final concentration of 1.6.10^5 sperm/mL was chosen for the following experiments. As shown in Fig. 3B, the mean density of bound sperm per OES increased from 15 to 30 min of co-incubation and then reached a plateau after 120 min, before decreasing again at 240 min after incubation start. In the following experiments, the bound sperm density on the OES was

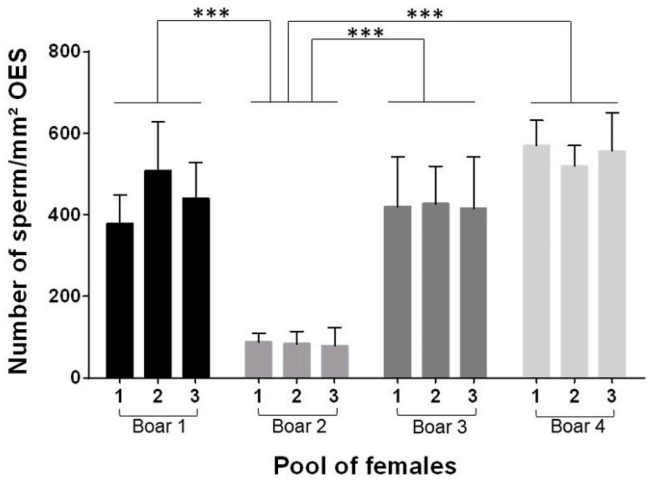

Fig. 5. Sperm binding density according to the female origin of OES. Groups of 20 spheroids selected from three different pools of gilts (five gilts/pool) were incubated with $1.6.10^5$/mL spermatozoa from the same ejaculate during 30 min (Experiment 4). Bound sperm densities per spheroid surface unit are expressed as mean ± SEM of 12–19 spheroids per replicate. Bars indicate significant differences among boars (***P < 0.001).

quantified after 30 min of co-incubation. Observation of sperm-OES complexes by confocal microscopy (Fig. 4A–B) and scanning electron microscopy (Fig. 4C) after 30 min of incubation revealed that only sperm of normal morphology bound by their head to cilia.

3.3. Sperm binding densities among pools of gilts (Experiment 4)

To test if the density of bound sperm varied with the female origin of OES, the same ejaculate was co-incubated with three different pools of OES (five gilts/pool). The experiment was repeated with four different boars. As shown in Fig. 5, for a given boar and ejaculate, the bound sperm density did not vary among pools of OES female donors. However, differences in sperm densities were observed among boars (P < 0.001).

3.4. Sperm binding densities among boars and ejaculates (Experiment 5)

The sperm parameters of the ejaculates used for this experiment (mean of five ejaculates from nine boars) are indicated in Table 1. Statistical analysis of bound sperm densities on OES revealed a strong effect of the individual boar and ejaculate (P < 0.01 and P < 0.0001, respectively), with a significant interaction between these two factors (P < 0.0001; Fig. 6). Except for boar H, sperm bound densities on OES varied significantly among ejaculates for a given boar. There was a weak correlation between the bound sperm density on OES and sperm motility (r = 0.32; p = 0.024) and no significant correlation with other sperm parameters or the dilution factor of the ejaculate with the extender (Fig. 7 and Table 2).

4. Discussion

The rapid and reliable elimination of subfertile boars and ejaculates is critical for pig AI centres as one boar ejaculate provides up to 50 AI fresh doses of semen spread over dozens of production farms. In this study, the use of porcine OES provided a standardisable tool for boar sperm binding assays, irrespective of the pools of females used for cell culture. However, the density of bound sperm per OES varied among ejaculates for eight out of the nine boars tested. Furthermore, the ability of sperm to bind to OES was not correlated with the sperm morphological parameters or initial dilution of the ejaculate.

This study provides the characterisation of an OES model useable for further studies on sperm–oviduct interactions in pigs and other mammals. Previous *in vitro* models for sperm binding assays in mammals included oviduct explants of various sizes (150 μm–1 mm) [9–13,18–20] and monolayers of OEC [21,22]. Oviduct explants are useable the day of oviduct collection and similar to the *in vivo* epithelium [14]. However, their variability in shape and size complicates standardisation. Furthermore, oviduct explants contain mucosa folds and edges, leading to difficulties in the reliable evaluation of their aera and bound sperm density at their surface (personal observation). In contrast, OEC monolayers require a preliminary culture of at least 5 days before their use in sperm binding assays. The main drawback of this *in vitro* model is that OEC lose their differentiation and cilia during culture on plastic dishes [14], leading to unspecific sperm binding. An alternative method to reproduce a high proposition of multiciliated cells consists in the primary culture of OECs in an air-liquid interface system [21,23]. However, this model requires at least 3 weeks of culture and hormonal stimulation and is therefore not adapted to a commercial use. The OES model offers several benefits: OES are easy to obtain after only 48 h of culture and sortable according to their diameter and shape, offering a normalised and repeatable binding surface to a given sperm population. Furthermore, OES are composed of fully differentiated epithelial multiciliated cells with the cilia oriented outside, *i.e.*, accessible to spermatozoa, and vigorous beating comparable to the *in vivo* condition. Although depending on oviducts from the slaughterhouse, OES are likely a good model to test the ability of sperm to bind to the oviduct reservoir *in vivo* because the cells kept their polarity, and the multiciliated cells are well-differentiated.

The average proportion of multiciliated cells in OES was 36.4%, which is similar to previous observations in the isthmus of sexually mature sows (34.0%–38.7%) [24,25], although prepubertal 6-month-old gilts were used in the present study. Previous *in vivo* studies reported important variations in the morphology of the oviduct epithelium and the proportions of multiciliated cells across the oestrous cycle in mammals, including pigs [24–28]. However, drastic changes in cell height and proportion of cilia across the cycle were seen exclusively in

Table 1
Sperm parameters of the nine Pietrain boars tested for sperm binding on oviduct epithelial spheroids in Experiment 5. Means ± SEM of five ejaculates for each boar. Sperm parameters were assessed 60 h after semen collection. The semen dilution factor indicates the extent of the initial dilution of the ejaculate with the extender.

Boar	Total motile sperm (%)	Progressively motile sperm (%)	Sperm with proximal droplet (%)	Sperm with distal droplet (%)	Sperm with bent tail (%)	Sperm with coiled tail (%)	Total morphologically abnormal (%)	Semen dilution factor (min-max)
A	81.8 ± 2.2	53.9 ± 3.7	3.8 ± 0.5	8.1 ± 3.3	0.6 ± 0.5	0.4 ± 0.3	19.3 ± 1.5	8–14
B	67.8 ± 3.8	47.9 ± 4.3	4.6 ± 0.4	7.2 ± 1.4	1.0 ± 0.9	0.2 ± 0.1	21.2 ± 2.1	12.8–23.5
C	68.9 ± 2.9	39.3 ± 5.8	3.8 ± 0.3	10.2 ± 1.5	0.4 ± 0.2	0.2 ± 0.1	35.3 ± 3.1	7.9–16.9
D	63.0 ± 2.9	44.3 ± 4.7	4.5 ± 0.8	7.5 ± 0.8	0.9 ± 0.5	0.4 ± 0.2	24.8 ± 2.5	11.5–16.5
E	73.5 ± 1.8	52.0 ± 4.5	3.9 ± 0.8	6.2 ± 0.9	2.2 ± 0.9	0.1 ± 0.1	9.0 ± 3.1	12.9–17.5
F	78.5 ± 7.8	39.4 ± 9.1	9.4 ± 0.4	7.0 ± 0.8	4.0 ± 0.8	0.1 ± 0.0	6.8 ± 2.0	9.7–20.4
G	62.7 ± 4.1	30.4 ± 4.7	11.7 ± 1.5	11.3 ± 1.5	9.6 ± 1.6	0.5 ± 0.1	9.0 ± 1.8	11.6–13.8
H	76.8 ± 4.6	43.2 ± 4.4	8.1 ± 0.7	10.1 ± 0.8	3.7 ± 0.6	0.2 ± 0.1	7.0 ± 0.5	10.5–16.9
I	76.6 ± 2.1	50.2 ± 2.8	9.0 ± 1.3	8.4 ± 0.8	6.1 ± 1.5	0.2 ± 0.0	6.3 ± 1.3	15.3–18.5

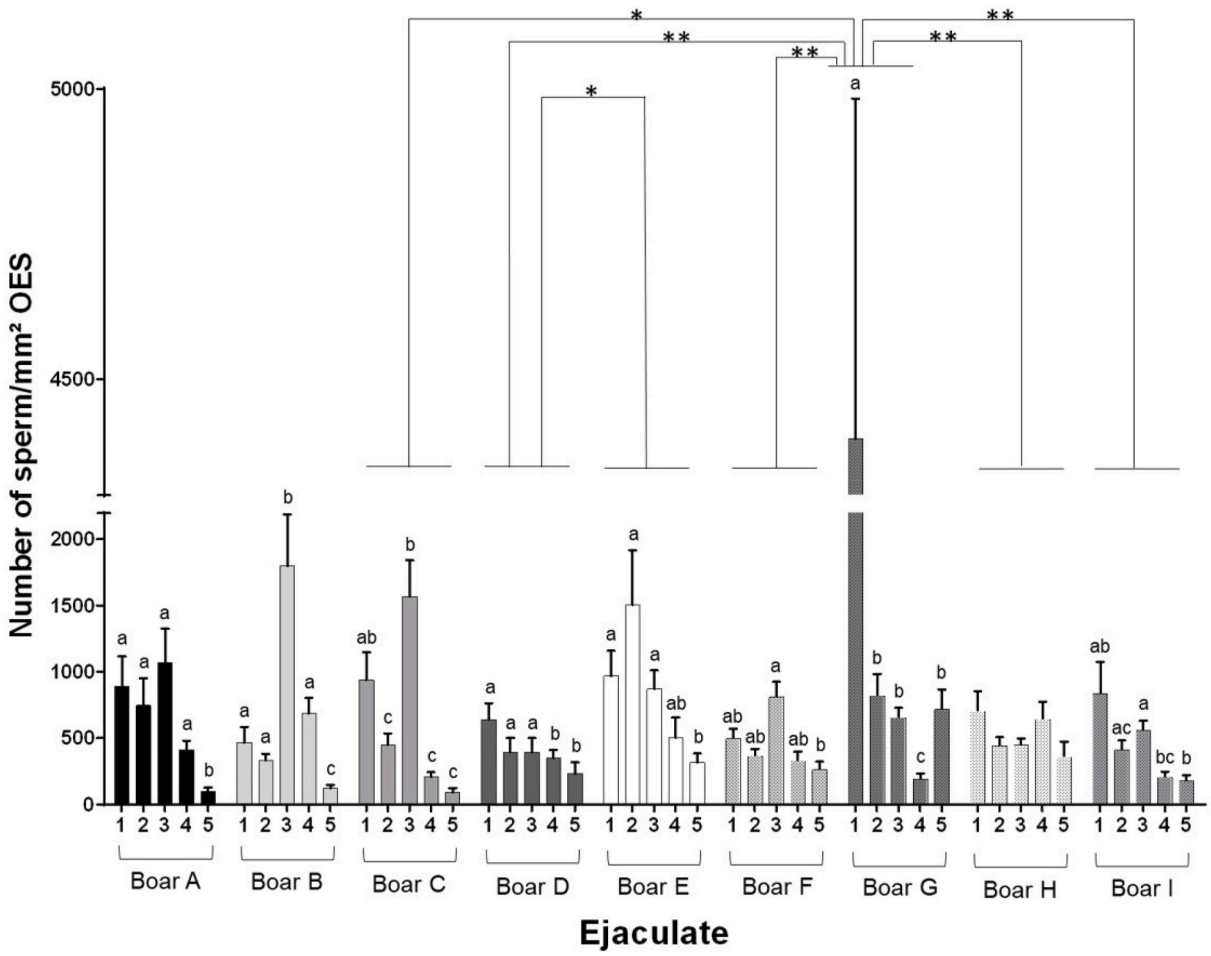

Fig. 6. Sperm binding density according to the boar and successive ejaculates (Experiment 5). Groups of 20 OES selected from pools of 10 gilts were incubated for 30 min with $1.6.10^5$/mL spermatozoa from five successive ejaculates (one per week) per boar before the analysis of bound sperm density per spheroid surface unit. Mean ± SEM of 15–20 spheroids per replicate. Different letters indicate significant differences with a P-value <0.05.

the fimbriae and ampulla and not in the isthmic part of the porcine oviduct [24,25], suggesting a more stable cell population in this region of the sperm reservoir. Furthermore, sperm bound mostly by their head to the cilia of OES, in accordance to previous observations *in vivo* [29,30] and *in vitro* [7,31]. One previous predictive model that included boar sperm binding capacity and quality parameters was able to predict pregnancy rates after AI using oviduct explants but not soluble oviduct glycans [9], showing the importance of sperm interacting with intact cells and cilia. Here, the functionality of multiciliated cells in OES was assessed through their capacity to move in the suspension and the measurement of their straight-line velocity. The ciliary beating of OECs has been previously evaluated by technically challenging methods such as fluorescent beads coupled with image analysis [32] or a digital videomicroscopic system [33].

One prerequisite for the evaluation of male fertility using OEC-derived binding assays is the repeatability in numbers of sperm binding sites among replicates. The proportion of multiciliated cells per OES did not vary among culture replicates, as previously shown by our group for OES derived from bovine oviducts [17]. A linear relationship between bound sperm densities and sperm concentrations between $1.6.10^5$ and $12.8.10^5$ per mL was observed. A similiar linear relationship was reported by Petrunkina et al. [13] using oviduct explants and sperm concentrations between $2.5.10^4$ and 4.10^5 per mL. It is likely that the saturation of binding sites on OES would be observed using higher sperm concentrations. However, given that the highest concentration tested led to difficulties for accurate sperm counting and that the saturation of binding sites on OES may hidden differences between males and ejaculates, a sperm concentration of $1.6.10^5$ per mL was chosen for all experiments. Using the same boar ejaculate, the mean density of sperm bound per OES did not change according to female donors of OES, indicating comparable numbers of sperm binding sites on OES irrespective of the pools of gilts and the culture replicates. In line with this result, previous studies failed to evidence any effect of individual sows [10] or pools of sows *vs.* gilts [13] on boar sperm binding to oviduct explants.

Despite the stable proportion of multiciliated cells in OES and the absence of female effects on sperm binding among replicates, differences among boars were evidenced, in accordance with previous studies that used oviduct explants co-incubated with either fresh [8,9] or frozen-thawed [11] boar semen. Furthermore, a high heterogeneity in sperm binding capacity was observed among ejaculates from the same boar. To our knowledge, this is the first report of the effect of the ejaculate on sperm binding to OECs as previous studies on pigs reported data from single ejaculates [11,12,34,35] or used the average binding capacity of three or more ejaculates [9,10,13]. The present data were obtained over 5–12 weeks to analyse five successive ejaculates per boar and a total of 45 ejaculates. Fresh semen was used because it is standard practice in porcine insemination, leading to an assay of each ejaculate on a different day. Although the surface of OES and proportion of ciliated cells did not vary among culture replicates, the day-to-day variability is a limitation of the present study. Nevertheless, the variability in sperm binding density was likely due to differences in sperm properties among ejaculates. Thus, despite a homogeneous sperm population in terms of motility and morphology, a high variability in sperm binding capacity to

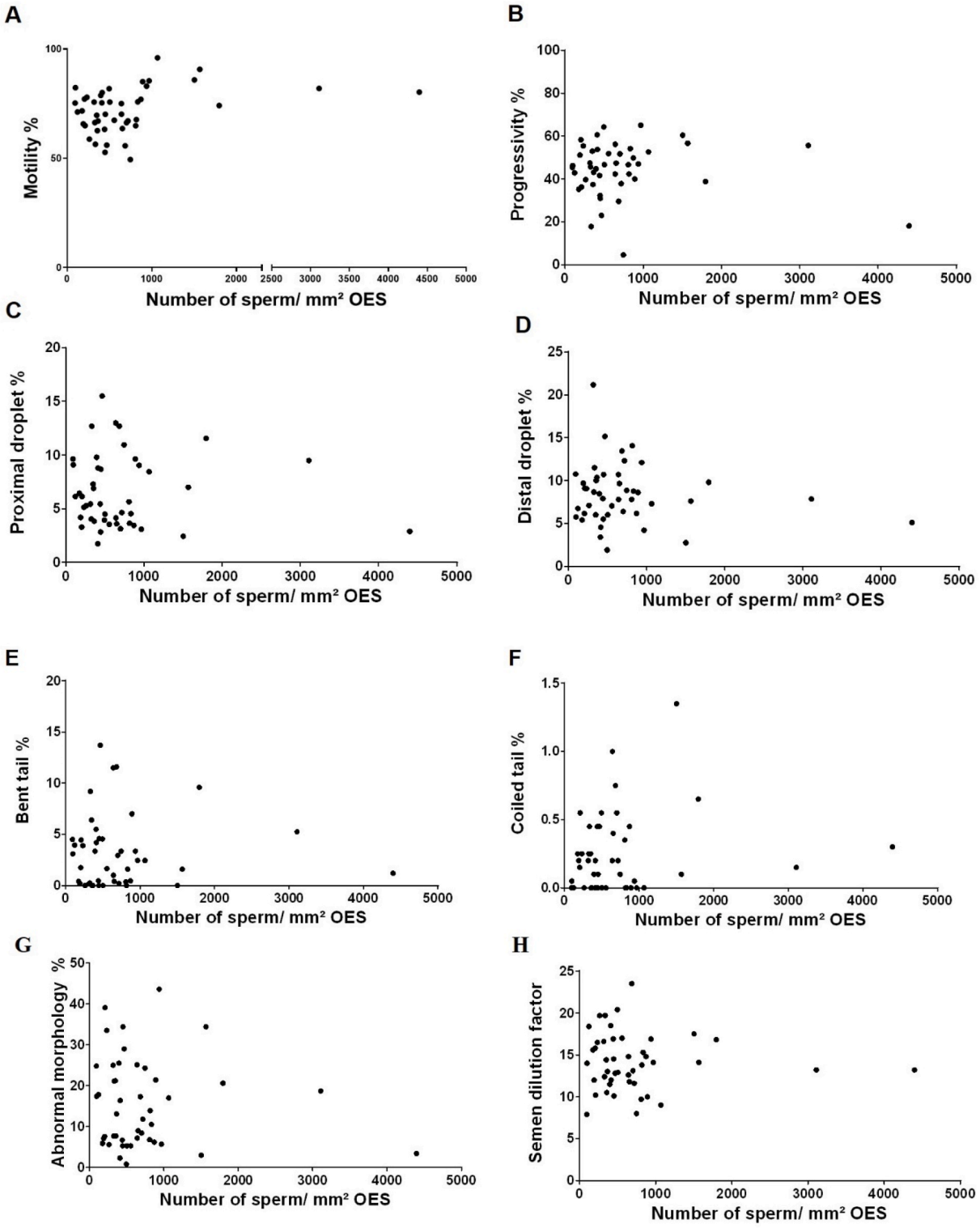

Fig. 7. Scatterplots of sperm binding densities on OES with sperm parameters. Scatterplots of the density of bound sperm per spheroid surface unit *versus* the proportions of motile, progressive sperm, of sperm with distal droplets, bent tails, coiled tails, of morphologically abnormal sperm and the dilution factor of the ejaculate with the extender (N = 45 ejaculates from nine boars, five ejaculates/boar).

OES among ejaculates was observed. In accordance, a considerable variation in the number of spermatozoa recovered from oviduct sections of gilts was reported after AI using several ejaculates from one single boar (37–3250 in the isthmus) [36], questioning the variability in sperm ability to survive in the female tract after AI.

No relationship between the sperm parameters assessed by CASA and the bound sperm density on OES could be identified, except a poor correlation (r = 0.32) with the total percentage of motile sperm, which was mostly due to two ejaculates with high binding densities. In accordance, previous studies showed no significant correlation between sperm binding to oviduct explants and sperm motility [9,10,12,13,35] or viability [10]. A discrepancy was reported concerning sperm

Table 2
Pearson correlations between bound sperm density on oviduct epithelial spheroids and sperm parameters measured by CASA or the dilution factor with the extender (Experiment 5).

Sperm parameter	r	P-value
Motility (%)	0.3239	0.0240
Progressivity (%)	0.1342	0.5075
Proximal droplet (%)	−0.0738	0.6300
Distal droplet (%)	−0.0535	0.3224
Bent tail (%)	0.0278	0.8562
Coiled tail (%)	0.1097	0.4732
Abnormal morphology (%)	−0.0813	0.5954
Semence dilution factor	−0.0547	0.7773

morphology as some [10,13,35] but not all [9] *in vitro* studies found a negative correlation between the proportion of morphologically altered spermatozoa and their ability to bind to oviduct explants. It should be noted that the ejaculates used in this study were commercial AI doses passing the traditional quality controls, and as such, they constituted a homogenous sperm population with less than 35% abnormal sperm. As only sperm of normal morphology were observed among sperm bound to OES, it is likely that a higher heterogeneity among the ejaculates used may have led to a negative relationship between sperm morphology and bound sperm density on OES.

The ejaculates used in this study were diluted 8.0 to 23.5 times in a commercial extender before being shipped to the laboratory in order to reach a standardized sperm concentration in AI doses. Although the complete mechanism by which sperm bind to isthmic OEC remain to be elucidated, there is evidence that the seminal plasma proteins coating the sperm at ejaculation, including the spermadhesin AQN1, play important roles in sperm binding to OECs [37,38]. In a previous study, epididymal spermatozoa displayed a much lower ability to bind to oviduct explants than ejaculated spermatozoa from the same boars [13]. We hypothesised that the initial dilution of the ejaculate may decrease the abundance of spermadhesins on the sperm head and alter their ability to bind to OES. However, there was no relationship between the bound sperm density on OES and the dilution factor of the ejaculates. Further studies are therefore necessary to evaluate the abundance of spermadhesins among bound and unbound sperm populations.

Sperm oviduct-binding assays have been proposed to predict individual boar fertility [8,9,11]. The AI centre that provided us with the ejaculates routinely uses heterospermic AI doses to avoid any fertility drops due to one single ejaculate. Therefore, the exact fertility data of the boars and ejaculates used were not available. From a practical point of view, the variability among ejaculates makes it impossible to predict male fertility using data from one ejaculate and questions the relevance of using oviduct binding assays to qualify each ejaculate. Indeed, beyond the economic issue, the accurate evaluation of each ejaculate would require the recording of pregnancy rates and litter size from dozens of sows at different farms, which is limited by the number of AI doses per ejaculate (usually 20 to 40). However, assays on the binding to OES may be proposed to predict the outcome of *in vitro* fertilisation (IVF) using fresh or frozen-thawed semen as one ejaculate may provide sufficient spermatozoa to fertilise hundreds of oocytes, thereby eliminating the ejaculate source variability. A previous study reported a positive linear relationship between sperm binding capacity to oviduct explants and polyspermic fertilisation using frozen-thawed semen [12]. The OES may also be valuable tools to evaluate sperm plasma membrane integrity after cryopreservation or sex sorting.

The OES can easily and rapidly be obtained from commercial slaughterhouse material and present interesting properties of homogeneity, differentiation status stability over time and functional repeatability in sperm binding function. These characteristics make them excellent *in vitro* models for the study of sperm binding functions and the maternal regulation of fertilisation. In pigs, oviduct fluid addition to the IVF medium decreased the rate of polyspermy, which is a major issue in pig IVF [39]. Furthermore, the oviduct extracellular vesicles (oEVs) had the same effect [40]. Since oEVs bind to both sperm and oocytes, their effect on polyspermy could be expressed through the modulation of sperm biology or the modification of oocyte properties (i.e., zona hardening). The use of OES may help to improve IVF in pigs and decipher the underlying mechanisms. In this study, we propose a new and valuable *in vitro* model for the study of important early mechanisms of reproduction occurring in the oviduct.

In conclusion, the OES model characterised in this study offers physiological conditions to study sperm binding to the isthmic reservoir and constitutes a potential tool to evaluate the quality of semen and study early reproductive events in pigs. Despite a homogeneous sperm population in terms of motility and morphology, a high variability in sperm binding capacity to OES among boars and ejaculates was observed, highlighting the potential variability in sperm capacity to survive in the female tract after AI.

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CRediT authorship contribution statement

Lorraine Schmaltz: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Théo Prudhomme:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Guillaume Tsikis:** Validation, Methodology, Investigation, Formal analysis. **Karine Reynaud:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Isabelle Mérour:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Pascal Mermillod:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Marie Saint-Dizier:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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