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1 **Title:** Progesterone induces sperm release from oviductal epithelial cells by modifying
2 sperm proteomics, lipidomics and membrane fluidity

3

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16 **Highlights:**

- 17 • Spermatozoa released by P4 showed a decreased abundance of Binder of
- 18 Sperm Proteins (BSP)-3 and -5
- 19 • Spermatozoa released by P4 from oviductal cells displayed an increased
- 20 membrane fluidity
- 21 • P4-induced release from oviductal cells modified the sperm lipidomic and
- 22 proteomic profiles
- 23 • A number of interesting proteins and lipids are found as potential sperm
- 24 fertility biomarkers

25 **Abstract**

26 The sperm reservoir is formed after insemination in mammals, allowing sperm
27 storage in the oviduct until their release. We previously showed that physiological
28 concentrations of progesterone (P4) trigger in vitro the sperm release from bovine
29 oviductal epithelial cells (BOECs), selecting a subpopulation of spermatozoa with a
30 higher fertilizing competence. Here, by using Western-Blot, confocal microscopy and
31 Intact Cell MALDI-TOF-Mass Spectrometry strategies, we elucidated the changes
32 derived by the P4-induced release on sperm cells (BOEC-P4 spz). Our findings show
33 that, compared to controls, BOEC-P4 spz presented a decrease in the abundance of
34 Binder of Sperm Proteins (BSP) -3 and -5, suggesting one mechanism by which
35 spermatozoa may detach from BOECs, and thus triggering the membrane remodeling
36 with an increase of the sperm membrane fluidity. Furthermore, an interesting number of
37 membrane lipids and proteins were differentially abundant in BOEC-P4 spz compared
38 with controls.

39

40 **Keywords:** progesterone, sperm capacitation, Binder of Sperm Proteins, oviduct,
41 proteomics, lipidomics

42 **1. Introduction**

43 In mammals, after mating or artificial insemination, only a few spermatozoa
44 reach the oviduct, where they bind to the luminal epithelial cells for hours to days
45 forming the so called “functional sperm reservoir” (Hunter and Wilmut, 1984). During
46 this storage, the interactions between the oviductal epithelial cells (OEC) and
47 spermatozoa are believed to play an important role in sperm selection (Mburu et al.,
48 1997; Tienthai, 2015) maintenance of sperm viability (Ellington et al., 1999) and
49 prevention of premature capacitation (Murray and Smith, 1997) before ovulation. Then,
50 around the time of ovulation, spermatozoa are released from the OEC and move towards
51 the fertilization site (Coy et al., 2012). The exact mechanism by which sperm detach
52 from the oviductal epithelium and become able to fertilize the oocyte *in vivo* is not fully
53 understood. However, the ovarian steroid hormone progesterone (P4) has been proposed
54 as a major regulator of sperm release in birds (Ito et al., 2011) and pigs (Hunter, 2012).
55 We reported a consistent increase in intra-oviductal P4 concentrations (on average from
56 6 ng/mL before ovulation to 57 ng/mL after ovulation) in bovine oviducts ipsilateral to
57 the side of ovulation (Lamy et al., 2016). Previous *in vivo* studies in pigs demonstrated
58 that an injection of P4 into the oviductal wall or directly into the sperm reservoir
59 induced the sperm release together with an increase in the polyspermy rates (Hunter,
60 2008). Thanks to an *in vitro* model consisting of monolayers of bovine OECs (BOECs)
61 from the whole oviduct, we showed in an earlier study that P4 at concentrations of 10
62 and 100 ng/mL was an inductor of sperm release from BOECs (Lamy et al., 2017), a
63 result recently confirmed by other researchers (Romero-Aguirregomezcorta et al.,
64 2019). Furthermore, spermatozoa bound to BOECs and then released from BOECs by
65 P4 action showed higher fertilizing ability compared with the control group (Lamy et
66 al., 2017). However, the exact changes induced by the binding to BOECs and

67 subsequent P4-induced release from these cells on sperm physiology remain to be
68 elucidated.

69 P4 was found to stimulate mammalian sperm capacitation, hyperactivated
70 motility, acrosome reaction and chemoattraction at various doses (Gimeno-Martos et al.,
71 2017; Teves et al., 2006). In addition, three proteins from the seminal plasma that bind
72 to the bovine sperm surface at the time of ejaculation have been identified as involved
73 in sperm binding to OECs: Binder of Sperm Protein 1 (BSP-1, also called PDC-109 or
74 BSP-A1/A2), BSP-3 (or BSP-A3), and BSP-5 (or BSP-30KDa) (Gwathmey et al., 2006,
75 2003). BSPs have been reported to play important roles along the female genital tract in
76 sperm membrane stabilization and prevention of premature capacitation (Plante et al.,
77 2016a). Furthermore, the removal of BSPs from the sperm surface by oviductal
78 components is considered as the initial step promoting cholesterol and phospholipids
79 efflux, leading to an increase in membrane fluidity, sperm capacitation and eventually
80 acrosome reaction (Thérien et al., 1999, 1998).

81 Thus, the aim of this study was to investigate the mechanisms involved in the
82 release of spermatozoa from the sperm reservoir using our in vitro model. Based on the
83 key role played by BSPs on sperm-BOECs binding, we first evaluated the abundance of
84 BSPs on spermatozoa bound to BOECs and then released by P4 compared with
85 unbound spermatozoa or control spermatozoa treated or not with P4. The changes in
86 membrane fluidity, a marker of sperm capacitation, was then investigated in the various
87 experimental groups. Last, changes induced by P4 in sperm proteins and lipids during
88 the releasing process were explored using mass spectrometry on intact sperm cells.

89 2. Materials and methods

90 Unless otherwise stated, all chemicals were purchased from Merck-Sigma-
91 Aldrich (Saint Quentin-Fallavier, France). Bovine oviducts were obtained from a
92 commercial slaughterhouse and bovine semen from a breeding cooperative; no
93 experiments on live animals were performed. The experiments performed are in
94 accordance with the EU Directive 2010/63/UE.

95 **2.1. Co-incubation of bovine spermatozoa with BOECs**

96 A pool of frozen semen from three bulls (*Bos Taurus*, 0.25-mL straws,
97 approximately 20×10^6 spermatozoa/straw) was used in all the experiments. Straws were
98 thawed in a water bath at 37°C for one minute and then motile spermatozoa were
99 selected through a Percoll (GE Healthcare Life Sciences, Velizy-Villacoublay, France)
100 density gradient (90-45%). The sperm pellet was rinsed in 10 mL of STL-medium
101 (Tyrode medium supplemented with 25 mM Bicarbonate, 10 mM Lactate, 100 IU/mL
102 Penicillin, 100 µg/mL Streptomycin and 2.4 mg/mL HEPES) and then centrifuged at
103 100 g for 10 min. Sperm motility was visually estimated by light microscopy before
104 each experiment and only samples with a sperm motility >90% were considered in
105 further analyses.

106 Oviductal epithelial cells were collected from scratching the whole oviduct inner
107 surface and used as confluent monolayers, as previously described (Lamy et al., 2017)
108 with slight modifications. Preliminary experiments showed no difference between fresh
109 and frozen-thawed BOECs in terms of viability, time to reach confluency, morphology,
110 sperm binding and release under P4 action (data not shown). For the present study,
111 different pools of frozen-thawed BOECs were used in the various experiments. Briefly,
112 both oviducts from 5-7 adult cows at peri-ovulatory stages were collected at a
113 slaughterhouse and transported to the laboratory within 2 hours after the death of the

114 animals. Only oviducts in the pre-ovulatory (presence of a pre-ovulatory follicle and
115 one corpus albicans) and post-ovulatory (red recently ovulated corpus luteum, no
116 follicle >10 mm in diameter) stages were used, as previously described (Lamy et al.,
117 2016). The process of primary oviductal cell culture used in the present study has been
118 described and validated by our group (Mermillod et al., 1993; Van Langendonck et al.,
119 1995). After dissection, BOECs were gathered by scratching the whole oviducts
120 (ampulla and isthmus tracts), pooled and then rinsed three times in a washing medium
121 (TCM 199 supplemented with Gentamicin 10 mg/mL and BSA 0.2%). BOECs were
122 then diluted 1:10 in a freezing medium (TCM 199 supplemented with DMSO 10%,
123 Gentamicin 80 µg/mL and FBS 10%), aliquoted in cryotubes before being placed
124 overnight at – 80°C and then stored in liquid nitrogen. For each experiment, an aliquot
125 of BOECs was thawed in a water bath at 34°C, then transferred into a thawing-washing
126 medium (TCM 199 with FBS 10% and Gentamicin 80 µg/mL), washed twice and
127 cultured in 12 well plates (TCM 199 with FBS 10% and Gentamicin 80 µg/mL). Once
128 reached the confluence (after 6-7 days of culture), BOECs were washed twice with IVF
129 medium (Tyrode medium supplemented with 25 mM bicarbonate, 10 mM lactate, 1 mM
130 pyruvate, 6 mg/mL fatty acid free BSA, 100 IU/mL penicillin and 100 µg/mL
131 streptomycin) and bovine spermatozoa were added to the cells at a final concentration
132 of 4×10^6 sperm cells/mL in the same IVF medium and under culture conditions
133 (humidified atmosphere, 5% CO₂, 38.8 °C). After 30 min of co-incubation with BOECs,
134 unbound and slightly attached spermatozoa (BOEC group) were collected by aspiration
135 of the supernatant and two additional washings with IVF medium. The release of bound
136 spermatozoa from BOECs was then induced by adding P4 (100 ng/mL) to the culture
137 medium for 1 h. According to P4 concentrations previously measured in the bovine
138 oviductal fluid at the postovulatory period (Lamy et al., 2016), a P4 concentration of

139 100 ng/mL was considered to be physiological. The P4 solution was diluted in absolute
140 ethanol and added at a final concentration of 0.5% ethanol in the culture medium.
141 Preliminary experiments in our laboratory showed that vehicle controls containing an
142 equivalent amount of ethanol had no effect on the time course of sperm binding and
143 release (data not shown). Furthermore, ethanol at low concentrations (0.1-1%) was
144 shown to have no detectable effect on sperm motility, viability, capacitation, cholesterol
145 efflux and acrosome reaction during up to 6 h of incubation (Lukacova et al., 2015;
146 Therien and Manjunath, 2003). Spermatozoa released from BOECs by P4 action
147 (BOEC-P4 group) were collected by washing three times with IVF medium. Two
148 control groups of spermatozoa at the same sperm concentration were run in parallel in
149 each experiment. Control groups were incubated in the IVF medium, centrifuged the
150 same time and manipulated the same way (including pipetting) than the treated BOEC-
151 P4 group. One control group was similarly manipulated but not incubated with BOECs
152 nor P4 (CTRL group). Another group of spermatozoa was incubated without BOECs for
153 30 min in the IVF medium and then P4 (100 ng/mL) was added to the culture medium
154 for 1 h (P4 group), i.e., at the same dose and incubation time than the BOEC-P4 group.
155 It was not possible to constitute a control group in which P4 was replaced by vehicle
156 after BOEC incubation and elimination of unbound sperm because 1h of incubation
157 with ethanol at 0.5% did not induce sperm release from BOEC.

158 **2.2. Evaluation by Western blot of BSP abundance in spermatozoa**

159 Previous studies using the same in vitro system showed that only sperm heads
160 with intact acrosome did bind to the surface of BOECs (Lamy et al., 2017). In order to
161 reject the hypothesis whereby the release of bound spermatozoa by P4 was due to the
162 loss of their acrosome, the integrity of acrosomes in the BOEC-P4 and CTRL groups
163 was evaluated using a double staining with PNA (Peanut Agglutinin lectin) and Hoechst

164 33342 followed by examination under confocal microscopy. Acrosomes were found to
165 be intact in more than 90% of spermatozoa in both groups (data not shown). For
166 Western-Blot analyses, spermatozoa from all the experimental groups were collected
167 and immediately washed twice in PBS and centrifuged at 2000 g for 3 min before
168 protein extraction. Samples were then diluted in lysis buffer (2% SDS in 10 mM Tris,
169 pH 6.8) with a protease inhibitor cocktail and centrifuged (15,000 g for 10 min, 4°C) to
170 separate the protein-rich supernatant from the cellular debris. The concentration in
171 proteins was assessed in sperm supernatants using the Uptima BC Assay kit (Interchim,
172 Montluçon, France) before dilution in loading buffer (Laemmli buffer 5×) and heating
173 (90°C for 5 min). Sperm samples extracts were migrated (10 µg of proteins per lane) on
174 a SDS-PAGE 4-15% gradient gel (Mini-PROTEAN® TGX™ Precast Protein Gels,
175 BioRad) and blotted on a nitrocellulose membrane using the Trans-Blot® Turbo™
176 Transfer System (BioRad, Marnes-la-Coquette, France). The membranes were stained
177 with Ponceau S solution (5 min at room temperature, gentle shaking) and scanned with
178 Image Scanner (Amersham Biosciences, GE Healthcare Life Sciences) to check the
179 homogeneous loading among lanes and for normalization (see below). Membranes were
180 blocked in 5% (w/v) milk powder diluted in TBS-T (Tris-buffered saline with 1% (v/v)
181 Tween 20) for 1 h and then incubated with the primary antibody diluted at 1:1000
182 (gentle shaking, 4°C, overnight). Anti-sera against purified bovine BSP-1, BSP-3 and
183 BSP-5 proteins were kindly provided by Dr. Manjunath (Department of Biochemistry
184 and Medicine, University of Montreal) and antibodies were purified with the Melon Gel
185 IgG spin purification kit (Thermo Fisher Scientific, City, Country), following the
186 supplier's instructions. Blots were finally incubated with fluorescent secondary
187 antibody IRDye® 800CW anti-Rabbit IgG (gently shaking, 37°C, darkness, 45 min)
188 diluted at 1:10,000 before revelation with infrared scanner Odyssey® CLx (LI-COR

189 Biotechnology, Lincoln, USA). Protein signals were analyzed by Image Studio™
190 software (LI-COR Biotechnology, Lincoln, USA). The bands were quantified
191 afterwards using ImageQuantTL (GE Healthcare LifeSciences). Three biological
192 replicates were performed for each antibody and each condition. To normalize the data,
193 Ponceau S staining was used, as previously described (Romero-Calvo et al., 2010).
194 Briefly, the whole lanes were quantified by densitometry using the TotalLab Quant
195 software (version 11.4, TotalLab, Newcastle upon Tyne, UK). Then the experimental
196 groups (BOEC, BOEC-P4 and P4) were normalized with the CTRL group.

197 **2.3. Evaluation of sperm membrane fluidity by fluorescence recovery after** 198 **photobleaching (FRAP) analysis**

199 Due to the need of analyzing live spermatozoa in a very short length of time,
200 only CTRL-spz and BOEC-P4 groups, which displayed the most contrasted profiles in
201 BSP abundance, were analysed. FRAP experiments were performed as previously
202 described (Bernabò et al., 2017) with some modifications. Briefly, the lipophilic
203 fluorescent molecule DilC12(3) perchlorate (ENZ-52206, Enzo Life Sciences, USA)
204 was added (1:1000) for the last 15 min of the sperm-BOECs co-incubations.
205 Spermatozoa were then collected and FRAP was carried out within 60 min after
206 collection with a laser-scanning confocal microscope LSM780 (Zeiss, Oberkochen,
207 Germany) with the following acquisition parameters: Plan Apo 63X oil objective,
208 numerical aperture 1.4; zoom 4.2; 1 airy unit; 1 picture every 0.230 sec; fluorescence
209 bleaching and recovery performed at $\lambda_{exc} = 561$ nm and $\lambda_{em} = 595$ nm with one scan
210 for basal fluorescence record at 2.4% of the maximum laser power, one scan at 100%
211 laser power for bleaching, and 25 scans for monitoring recovery at 2.4% of the
212 maximum laser power. Recovery curves were obtained and analysed using the
213 simFRAP plug-in for Fiji ImageJ (<https://imagej.nih.gov/ij/plugins/sim-frap/index.html>),

214 01/27/2019) (Blumenthal et al., 2015). The parameters set were the following: pixel size
215 0.109 μm ; acquisition time per frame 0.095 sec. The results are expressed as diffusion
216 coefficient (cm^2/sec). Six independent experiments were carried out, with an average of
217 15 spermatozoa analysed per condition and per replicate.

218 **2.4. Statistical analysis of Western-blot and FRAP data**

219 For statistical analysis, GraphPad Prism 6 Software (La Jolla, CA, USA) was
220 used. All data were first subjected to normality test (Agostino-Pearson and Shapiro-
221 Wilcoxon tests). As Western-blot and FRAP data did not follow a normal distribution,
222 differences between the groups were analyzed by Kruskal-Wallis' tests followed by
223 Dunn's multiple comparisons tests for Western-blot and Mann-Whitney U test for
224 FRAP analysis. Differences were considered statistically significant when $p < 0.05$.

225 **2.5. Sperm proteomic and lipidomic profiling by Intact Cell MALDI-TOF Mass** 226 **Spectrometry (ICM-MS)**

227 For proteomic and lipidomic analyses, all groups of spermatozoa were washed
228 three times in Tris-Sucrose Buffer (TSB, 20 mM Tris-HCl, 260 mM sucrose, pH 6.8) to
229 remove the culture media and salts. For proteomic profiling, 0.5 μL of saturated CHCA
230 (α -cyano-4-hydroxycinnamic acid) matrix dissolved in 100% ethanol was spotted on a
231 MALDI plate (MTP 384 polished steel) and dried (30 min, room temperature) before
232 adding 10^5 spermatozoa (determined using a Thoma cell counting chamber) in one μL ,
233 then 2.5 μL of saturated CHCA (α -cyano-4-hydroxycinnamic acid) matrix dissolved in
234 50% acetonitrile/50% water (v/v) in presence of 0.3% TFA (trifluoroacetic acid) was
235 added. For lipidomic profiling, 2×10^5 spermatozoa in 0.5 μL were spotted and overlaid
236 with 2 μL of DHAP (2,5-dihydroxyacetophenone) matrix at 20 mg/mL solubilized in
237 90% methanol/10% in presence of 2% TFA. For each condition, three biological

238 replicates were performed and for each biological replicate, twenty technical replicates
239 were spotted.

240 Spectra were acquired using a Bruker UltrafleXtreme MALDI-TOF instrument (Bruker
241 Daltonics, Bremen, Germany) equipped with a Smartbeam laser at 2 kHz laser
242 repetition rate following an automated method controlled by FlexControl 3.0 software
243 (Bruker Daltonics, Bremen, Germany). Spectra were obtained in positive linear ion
244 mode in the 1,000–30,000 m/z (mass/charge) range for proteomics and 100-1,800 m/z
245 range for lipidomics. Each spot was analyzed in triplicate. After external calibration,
246 each spectrum was collected as a sum of 1,000 laser shots in five shot steps (total of
247 5,000 spectra) with a laser parameter set at medium. To increase mass accuracy (mass
248 error <0.05%), an internal calibration was performed on a mix of cells and calibrant
249 solution (for proteomic, 1 μ L of calibrant solution containing Glu1-fibrinopeptide B,
250 ACTH (fragment 18-39), insulin and ubiquitin, cytochrome C, myoglobin and
251 trypsinogen, while for lipidomic, 1 μ L of calibrant solution containing Caffein, MRFA
252 peptide, Leu-Enkephalin, Bradykinine 2–9, Glu1-fibrinopeptide B; reserpine;
253 Bradykinine; Angiotensine I). A lock mass correction was applied to one peak with high
254 peak intensity in all spectra using flexAnalysis 4.0 software (Bruker). For proteomics,
255 the peak at m/z 6821.46 was selected, while for lipidomics, the calibration was achieved
256 with the mass corresponding to the phosphatidylcholine 34:1 (PC 34:1; [M+H]⁺ :
257 760.5856 m/z).

258 **2.6. Quantification and statistical analysis of ICM-MS data**

259 Spectral processing and analysis were performed with ClinProTools v3.0
260 software (Bruker Daltonics, Bremen, Germany). The data analysis began with an
261 automated raw data pre-treatment workflow, comprising baseline subtraction (Top Hat,

262 10% minimum baseline width) and two smoothings using the Savitzky-Golay
263 algorithm. The spectra realignment was performed using prominent peaks (maximal
264 peak shift 2000 ppm, 30% of peaks matching most prominent peaks, exclusion of
265 spectra that could not be recalibrated). Normalization of peak intensity was performed
266 using the Total Ionic Count (TIC) in order to display and compare all spectra on the
267 same scale. Automatic peak detection was applied to the total average spectrum with a
268 signal/background noise greater than 2.

269 The intra- and inter-experiment variability in measurements were evaluated by a
270 coefficient of variation (CV). For CTRL, BOEC, BOEC-P4 and P4 groups, mean CV
271 values did not exceed 18.4%, 21.2%, 23.1% and 20% for proteomics, and 31.3%,
272 34.5%, 34.5% and 36.1% for lipidomics, respectively. Differential analyses between
273 groups (N = 180-200 MALDI spectra per group) were performed using the non-
274 parametric Kruskal-Wallis and Wilcoxon tests for multiple and paired comparisons,
275 respectively. Fold Change (FC) was calculated as the ratio between the mean
276 normalized intensity values. Masses were considered statistically differential between
277 groups if the p-value was < 0.01 with a $FC > 1.5$ or < 0.67 . Receiver operating
278 characteristic (ROC) curves were generated and only masses with areas under the curve
279 (AUCs) > 0.8 were retained. Principal component analysis (PCA) and hierarchical
280 clustering were performed on differential masses using the RStudio Software (RStudio,
281 Boston, MA, USA) (installed packages: *readr*, *robustbase*, *caTools*, *RColorBrewer*,
282 *MALDIquantForeign*, *FactoMineR*, *gplots*).

283 **2.7. ICM-MS data processing for lipid and protein identification**

284 In order to identify the lipids corresponding to the differential peaks obtained by ICM-
285 MS profiling, the masses observed were confronted to a local database created from

286 previous analyses of bovine follicular cells and biological fluids. This database is a
287 merged list of lipids identified by high resolution mass spectrometry (HRMS) using
288 liquid chromatography coupled with mass spectrometry (LC-MS) and direct infusion
289 for MS/MS structural analyses, as recently described (Bertevello et al., 2018). The
290 comparison of the masses observed by ICM-MS and the monoisotopic masses identified
291 by HRMS was performed with a max mass tolerance of 0.1 Da for lipids.

292 In order to identify the proteins corresponding to the differential peaks obtained by
293 ICM-MS profiling, a local database from a previous analysis of ovine spermatozoa by
294 HRMS coupled to μ LC was used. In this analysis, 1 mg of the peptides/proteins was
295 subjected to various fractionations through reversed phase and gel filtration
296 chromatographic separations, as previously described (Soler et al., 2016). All the data
297 acquired by μ LC-MS/MS were automatically processed by the ProSight PC software
298 v4.0 (Thermo Fisher, San Jose, California, USA) (Soler et al., 2016). All the data files
299 (*.raw) were processed using the cRAWler application. Molecular weights of precursor
300 and product ions were determined using the xtract algorithm. Automated searches were
301 performed on PUF files using the “Biomarker” search option against a database made
302 from the UniprotKB Swiss-Prot Ovis aries release
303 ovis_aries_2017_07_top_down_complex (28256 sequences, 512072 proteoforms)
304 downloaded from <http://proteinaceous.net/database-warehouse/>. Iterative search tree
305 was designed for monoisotopic precursors and average precursors at 25 ppm and 2 Da
306 mass tolerance, respectively, and both at 15 ppm for fragment ion level. For all
307 searches, the N-terminal post-translation modifications were considered. Then, all the
308 *.puf files were additionally searched in “Absolute mass” mode using 1000 Da for
309 precursor search window. For identification of endogenous biomolecules, we validated
310 automatically all the peptidofoms/proteoforms with E value $<1E^{-8}$. Furthermore, we

311 validated all hits presenting a C score > 3 (LeDuc et al., 2014). The comparison of the
312 masses observed by ICM-MS and the average masses identified by Top-Down was
313 performed with a max mass tolerance of 0.05% for proteins.

314 In TD results list, the entry names and gene names were recovered from the UniProtKB
315 accession numbers from *Ovis aries* annotated proteins using the Retrieve/ID mapping of
316 Uniprot (<http://www.uniprot.org/uploadlists/>) and listed. Proteins identified in *Ovis*
317 were mapped to the corresponding *Bos taurus* taxon by identifying the reciprocal-best-
318 BLAST hits using blastp resource (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Only protein
319 sequences with 100% identity and 100% query cover were retained. The gene name and
320 accession number of identified proteins were recovered from the NCBI database.
321 Protein functions and cellular location were recovered from UniProtKB (Swiss-prot)
322 (<https://www.uniprot.org/uniprot/>, 01/27/2019) Last, the potential role of proteins
323 identified in membrane lipid rafts was assessed using the RaftProt Database V2
324 (Mammalian Lipid Raft Proteome Database, <http://raftprot.org/>, 01/27/2019) by
325 downloading the list of lipid-raft associated proteins detected in bovine experiments
326 (evidence based on Gene Name and UniprotID).

327 **3. Results**

328 **3.1. Binding to BOECs and subsequent P4-induced release decreased the** 329 **abundance of BSPs on sperm and increased sperm membrane fluidity**

330 Spermatozoa treated with P4 alone (P4 group) and spermatozoa unbound after
331 incubation with BOECs (BOEC group) did not display any change in BSP abundance
332 compared with controls (CTRL group). By contrast, a significant decrease in the
333 abundance of BSP-3 and BSP-5 was evidenced on spermatozoa bound to BOECs and
334 then released by P4 (BOEC-P4 group) compared with CTRL (fold changes of

335 normalized values of 2.89 and 2.76 for BSP-3 and BSP-5, $p < 0.05$ and $p < 0.01$,
336 respectively), while the abundance of BSP-1 tended to decrease (fold change of 2.16, p
337 > 0.05) (Fig. 1). In addition, FRAP analyses evidenced an increase in membrane fluidity
338 in BOEC-P4 sperm cells compared with the CTRL group ($p < 0.05$) (Fig. 2).

339 **3.2. Binding to BOECs and subsequent P4-induced release changed sperm** 340 **lipidomic profiles**

341 A total of 206 lipid molecular species with a mass range between 400 and 1000 Da were
342 detected among experimental groups. Of those, 123 masses were differential when
343 considering all possible paired comparisons between the four experimental groups (Fig.
344 3A). The action of P4 alone did not induce any significant change in sperm lipid
345 composition compared with the control group (P4 vs. CTRL). By contrast, spermatozoa
346 bound to BOECs and then released by P4 (BOEC-P4) showed 116 and 34 differential
347 masses compared with spermatozoa treated by P4 alone (P4) and those just manipulated
348 (CTRL), respectively. In addition, the incubation with BOECs without binding (BOEC
349 group) induced 33 differential masses as compared with CTRL, among which a high
350 proportion (29/33) was already identified in the BOEC-P4 vs. CTRL comparison (Fig.
351 3A). The hierarchical clustering of differentially abundant lipid masses evidenced a
352 closeness in lipid profiles between the BOEC-P4 and BOEC groups compared with the
353 CTRL and P4 groups (Fig. 4A).

354 **3.3. Identification of differentially abundant lipids**

355 A total of 84 lipids, listed in Supplementary Table 1, could be annotated. Most of those
356 were phosphatidylcholines (PC) (35/84), among which the PC(36:2) at m/z 786.6 and
357 PC(36:1) at m/z 788.6 exhibited the highest fold changes between conditions (see
358 Supplementary Table 1 for all paired-wise ratios between normalized values). A high

359 proportion of sphingomyelins (SM) (14/84) was also identified, followed by
360 lysophosphatidylcholines (LPC) (9/84). The remaining masses identified were
361 cholesteryl esters (CE), fatty acyl carnitines (CAR), ceramides (Cer), diacylglycerols
362 (DAG), lysophosphatidylethanolamines (LPE), phosphatidylethanolamines (PE) and
363 triacylglycerols (TAG). A detailed classification of the lipids identified in this analysis
364 and their corresponding percentages of abundance is showed in the Figure 5.

365 **3.4. Binding to BOECs and subsequent P4-induced release changed sperm** 366 **proteomic profiles**

367 In total, 200 protein molecular species with a mass range between 2 and 20 kDa were
368 detected in spermatozoa from the four experimental groups, among which 137 were
369 differential after paired-wise comparisons (Fig. 3B). As for lipidomics, the highest
370 numbers of differential masses were evidenced when comparing BOEC-P4 with
371 spermatozoa treated by P4 alone (P4 group) and CTRL (104 and 98 differential masses,
372 respectively). Comparing with the CTRL group, the P4 group and spermatozoa unbound
373 after incubation with BOECs (BOEC group) displayed 62 and 34 differential masses,
374 respectively. The BOEC-P4 group displayed specific changes in 11 and 14 masses when
375 compared with CTRL and P4, respectively (Fig. 3B). As for lipidomics, the clustering
376 of differential proteins evidenced common changes between the BOEC-P4 and BOEC
377 groups as compared with the CTRL and P4 groups, which also shared similar features
378 (Fig. 4B).

379 **3.5. Identification of differentially abundant proteins**

380 As presented in Supplementary Table 2, a total of 36 m/z were identified as fragments
381 of 32 proteins (AKAP4, CALM, ODF2 and SPESP1 were identified by two peptides).
382 In addition, 5 m/z were identified as fragments originated from 2 or 3 different proteins.

383 Among the proteins identified, five of them (ODF2, ODF3, Sp17, SPAM1 and SPESP1)
384 corresponded to fragments of sperm-specific proteins and nine were found to be altered
385 specifically in the comparison between CTRL and BOEC-P4 (Acrosin, NDUFA6,
386 PHB2, Sp17, ODF2, TPPP2, TUBA3C, OTOF and HDAC9). The known location of
387 the identified proteins included the sperm surface (SPAM1, SPA17), flagellum (ODFs,
388 AKAP4, several subunits of tubulin such as TUBB4B), mitochondria (COX6A1,
389 ACO2, MDH2, PHB2), the equatorial segment (SPES1), nucleus (CLP1, HDAC9,
390 DCAKD) and acrosome (PKM). In addition, according to the Lipid raft proteome
391 database for mammals, prohibitin-2 (PHB2) and tubulin beta 4B-chain (TUBB4B) were
392 found to play roles in lipid rafts.

393 **4. Discussion**

394 The exact mechanism by which spermatozoa detach from the sperm reservoir
395 and the consequences of cell release on sperm physiology are still poorly understood.
396 The main findings of the present work were that spermatozoa released from bovine
397 oviduct epithelial cells by the action of P4 displayed a decrease in the abundance of
398 BSP-3 and -5, an increased membrane fluidity and a number of changes in sperm
399 phospholipids and proteins with a range of biological functions.

400 The model of BOEC monolayers used in this study was previously shown to
401 display epithelial cell markers such as cytokeratin (Van Langendonck et al., 1995) and
402 improve early bovine embryo development rate and quality despite partial
403 dedifferentiation during culture (Cordova et al., 2014; Schmaltz-Panneau et al., 2014).
404 Although partially dedifferentiated, BOECs monolayers allowed a reliable and
405 reproducible counting of bound sperm cells in several previous studies (Gualtieri and
406 Talevi, 2003; Lamy et al., 2017; Osycka-Salut et al., 2017; Talevi and Gualtieri, 2001).

407 It is of note that the functional sperm reservoir is located in the distal part of the
408 oviduct, namely the isthmus (Sostaric et al., 2008), while we used BOECs from the
409 complete oviduct. Bovine spermatozoa were reported to interact with OECs from both
410 the ampulla and isthmus but with different dynamics (Ardon et al., 2016). A previous
411 study also demonstrated that sperm-binding capacity of porcine oviductal cells cultured
412 in an air-liquid interphase changed according to the hormonal environment (Chen et al.,
413 2013). Further studies using more physiological BOEC models are needed to evaluate if
414 the P4 action on bound sperm differ according to the oviductal region and mimicked
415 estrous cycle stage.

416 Although sperm capacitation may start immediately after ejaculation *in vivo*, a
417 majority of studies suggest that capacitation of spermatozoa occurs mainly in the
418 oviduct (Holt and Fazeli, 2010; Killian, 2004; Rodriguez-Martinez and Barth, 2007). It
419 is of note that the medium in which sperm incubations were undertaken contained
420 bicarbonate and serum albumin, both reported to be involved in the sperm capacitation
421 process (Aitken and Nixon, 2013). Furthermore, the spermatozoa used were frozen-
422 thawed, a process that could affect biochemical pathways involved in modulating sperm
423 function and in part related to capacitation (Cormier and Bailey, 2003). Therefore, the
424 control group in the present study cannot be considered *sensu stricto* as a ‘non-
425 capacitated’ group. Spermatozoa in the BOEC group were in vicinity with BOEC
426 without establishing a stable binding after 30 min of co-incubation, including free
427 spermatozoa in the medium, spermatozoa slightly attached to BOEC, or attached and
428 then spontaneously detached from BOEC during incubation. Finally, our study design
429 allowed to study the changes induced by co-incubation with BOEC without P4 action
430 (BOEC group), by P4 alone without stable BOEC binding (P4 group), and finally by the
431 sequential binding to BOEC then P4-induced release (BOEC-P4 group).

432 Binder of Sperm Proteins (BSP) -1, -3 and -5 are a family of proteins from the
433 seminal plasma playing major roles, among others, in the binding of spermatozoa to the
434 oviductal epithelium (Plante et al., 2016a). Immediately after ejaculation, spermatozoa
435 are coated with BSPs, which bind directly to the choline-containing phospholipids such
436 as phosphatidylcholines and sphingomyelins in the sperm membrane (Divyashree and
437 Roy, 2018; Plante et al., 2016b). In the present study, a sharp decrease in the abundance
438 of BSP-3 and -5 was evidenced on spermatozoa bound to BOECs and then released by
439 P4 (BOEC-P4 spz) compared with controls, while BSP-1 tended to decrease in the same
440 group. In accordance, BSPs were found to be lost on bovine spermatozoa after heparin-
441 induced capacitation by other authors (Chiu et al., 2013; Gwathmey et al., 2003). It is
442 likely that in our conditions, the loss of BSPs at the sperm surface was one of the
443 mechanisms by which spermatozoa detach from the BOECs. In parallel with the decline
444 of BSPs on BOEC-P4 spermatozoa, an increase in sperm membrane fluidity and
445 significant changes in the phospholipid profiling were evidenced, highlighting the
446 interaction of BSPs proteins with the phospholipids of the sperm membrane. Indeed,
447 BSP-1 and BSP-3 bind specifically to phospholipids containing the phosphorylcholine
448 group, while BSP-5 binds preferentially to phospholipids containing the
449 phosphorylcholine moiety and to phosphatidylethanolamine, phosphatidylserine,
450 phosphatidylinositol, phosphatidic acid and cardiolipin (Desnoyers and Manjunath,
451 1992). In this way, the loss of BSPs after the sperm release from the BOECs may cause
452 a destabilization of the membrane phospholipids. We performed some FRAP
453 experiments using the sensitive dye DiI C12(3), a lipophilic carbocyanine that
454 incorporates into membranes and diffuses laterally within them, resulting in the staining
455 of the entire cell. The advantages of using this staining is that it has an extremely high
456 extinction coefficient and short excited-state lifetime in lipid environments, allowing to

457 distinguish the live cells against the death ones (in contrast with other stainings like
458 Merocyanine 540). Taken together, these results strongly suggest that the detachment of
459 BSPs from the sperm membrane at the time of P4-induced release triggered somehow
460 the remodeling of sperm membrane lipids and finally lead to an increase in sperm
461 membrane fluidity, a process related to sperm capacitation.

462 To our knowledge, this is the first study exploring the lipidomic and proteomic
463 consequences of P4 alone and P4 action after attachment of sperm cells to BOECs. By
464 lipidomics, 34 masses were found differential when comparing BOEC-P4 with CTRL
465 spermatozoa while the treatment by P4 alone did not induce any change in sperm
466 lipidomics. Of interest, the highest number of differential masses (116 masses) was
467 evidenced by comparing the BOEC-P4 group with the P4 group. These results may
468 suggest that the action exerted by P4 on sperm phospholipids modifications is strongly
469 linked to their preliminary binding to BOECs. Probably, the binding to BOECs
470 modified the sperm ability to respond to P4 action or to activate P4-dependent pathways
471 that lead to the remodeling of sperm membranes. Among these differential masses, we
472 could identify 84 lipids. Most of them corresponded to phosphatidylcholines (PC) and
473 sphingomyelins (SM), reaching fold changes of up to 18.5 for PC(36:1) and 8.8 in the
474 case of SM(d40:1) when confronting the BOEC-P4 and CTRL sperm groups. These
475 findings highlight the high impact of P4-induced release on sperm phospholipid
476 composition. A positive association between the increased abundance of several
477 phospholipids and sperm motility was evidenced in equine spermatozoa after thawing
478 (Cabrera et al., 2018). Of interest, five of those phospholipids (PC(34:1), SM(38:1),
479 PC(38:4), PC(35:1) and PC(40:7)) were also identified in the present study as being
480 upregulated in the BOEC-P4 group of spermatozoa compared with P4 and CTRL
481 groups. As BOEC-P4 spermatozoa were previously shown to have an increased

482 fertilizing competence (Lamy et al., 2017), further studies could explore the potential of
483 the differential PC and SM evidenced in this study as biomarkers of the sperm
484 fertilizing ability in the bovine.

485 Sperm lipid rafts are dynamic membrane microdomains enriched with
486 phospholipids, glycosphingolipids and cholesterol, and known to be involved in cell
487 adhesion (Khalil et al., 2006). In addition, lipid rafts contain signaling proteins
488 responsible for various intracellular functions and pathway activation during the sperm
489 capacitation process (Kawano et al., 2011). By using a bovine mammalian lipid raft
490 proteome database, two proteins related to lipid rafts were evidenced among differential
491 proteins: prohibitin-2 (PHB2) and tubulin- β chain 4 (TUBB4B). PHB2 is a membrane
492 protein linked to the mitochondria and shown to act as a chaperone involved in protein
493 and lipid membrane scaffolds (Leonhard et al., 2000). Our results in the present work
494 show a five-fold decrease in PHB2 abundance in the P4 group (ratio of 0.2) but only a
495 two-fold decrease in the BOEC-P4 group (BOEC-P4:CTRL ratio of 0.54) compared
496 with controls. In our opinion these results are quite interesting, since PHB2 is known to
497 be involved in mitochondrial activity (Chai et al., 2017) but also in steroid hormone
498 signaling, functioning as an estrogen receptor (ER)-selective coregulatory factor and
499 able to potentiate the inhibitory activities of antiestrogens and repress the activity of
500 estrogens (He et al., 2008). Therefore, although a similar interaction between PHB2 and
501 PR-related pathway is currently not known, our results suggest a possible role in
502 steroid-related signal transduction of PHB2 during capacitation.

503 By proteomics, a total of 137 differential masses were found between the
504 different sperm groups, among which the highest proportion (98 masses) corresponded
505 to the BOEC-P4 vs. CTRL comparison. This high proportion of masses confirms the
506 strong effect resulting from the combination of sperm attachment to BOECs and

507 subsequent release induced by P4 on sperm physiology. Interestingly, 34 protein masses
508 and 33 lipid masses were found differentially abundant between the BOEC and CTRL
509 groups. Since a high proportion of these differential masses was shared when comparing
510 BOEC-P4 vs. CTRL groups, we consider that these findings may illustrate the existence
511 of a “BOECs effect”, by which short periods of sperm binding to and release from the
512 cells could induce molecular changes, although further experiments should be
513 conducted to decipher the exact changes and mechanisms-

514 Among the differential proteins, those with the highest fold-changes in the
515 BOEC-P4 vs. CTRL comparison were flagellar proteins: the A-Kinase anchoring
516 protein 4 (AKAP4; BOEC-P4:CTRL ratio of 4.2) and dynein heavy chain 7 (DNAH7;
517 ratio of 3.6), both more abundant in the BOEC-P4 and in the BOEC groups compared
518 with controls (BOEC:CTRL ratios of 2.7 and 2.8, respectively). AKAP4 and DNAH7
519 are major components of the sperm fibrous sheath (*i.e.* the extent of the principal piece
520 region of the sperm flagellum) that plays important roles in sperm motility (Baccetti et
521 al., 2005; Moretti et al., 2007). Furthermore, AKAP-4 is involved in the cAMP/PKA
522 and PKC/ERK1-2 signaling pathways leading to tyrosine phosphorylation, actin
523 polymerization and acquisition of sperm motility (Rahamim Ben-Navi et al., 2016).
524 AKAP-4 is the mature form obtained from pro-AKAP-4 after the removal of 188 amino
525 acids from the N-terminal domain, which allows AKAP-4 to bind AKAP-3 by the C-
526 terminal domain. In the present study, one of the AKAP-4 peptide (of 4960.6 Da) that
527 was found to be more abundant in the BOEC-P4 group compared with controls
528 corresponds to this C-terminal region of AKAP-4. These results are concordant with
529 those found by others authors (Romero-Aguirregomezcorta et al., 2019), showing an
530 increase in sperm hypermotility at the time of the sperm release from BOECs by the
531 action of P4.

532 DNAH7 is another functional component of sperm motility that was upregulated
533 in BOEC-P4 spermatozoa. Specifically, the corresponding m/z was identified as a
534 fragment from the carboxy-terminal region of the dynein heavy chain (DNAH7), which
535 possesses all the elements necessary to produce movement with the energy from the
536 ATP hydrolysis (Roberts et al., 2013). Dyneins are cytoskeletal proteins anchored to the
537 sperm outer microtubules held responsible for generating the force required to produce
538 the beating pattern of the flagellum (Castaneda et al., 2017; Gibbons and Rowe, 1965).
539 Alterations in the gene encoding dynein heavy chain 1 (*DNAH1*) have been shown to
540 cause flagellar abnormalities leading to infertility in some human populations (Sha et
541 al., 2017). Also potentially involved in sperm mobility, fumarate hydratase (FH), an
542 enzyme involved in the Krebs citric acid cycle (Coughlin et al., 1998) and related to
543 ATP production, was found upregulated in BOEC-P4 (ratio of 2.2) spermatozoa
544 compared with controls. This is in agreement with a previous study showing a
545 correlation between down-regulation of major proteins related to ATP production,
546 including FH, and deficiencies of the flagellum in spermatozoa from asthenozoospermic
547 patients (Martinez-Heredia et al., 2008). In summary, BOEC-P4 spermatozoa showed
548 decreased levels of ODF-2 and -3, increased levels of AKAP-4, DNAH7 and FH,
549 proteins related with energy production and sperm motility, and of Sp17, a protein
550 involved in oocyte recognition but also probably in PKA signaling. Taken together, our
551 results are concordant with previous results from our group that reported a higher
552 fertilizing ability of BOEC-P4 spermatozoa (Lamy et al., 2017). However, the ability of
553 those proteins to become biomarkers of male fertility in the bovine remains to be
554 evaluated.

555 **5. Conclusions**

556 In conclusion, P4 at physiological concentrations triggered in vitro the release of
557 a sperm subpopulation characterized by a significant loss of surface BSPs and an
558 increase in membrane fluidity, both events potentially related to capacitation. Moreover,
559 those sperm cells bound to oviduct epithelial cells and then released by P4 presented
560 important changes in phospholipid and protein composition when compared with
561 controls. Our current hypothesis is that P4 acts through progesterone receptors on the
562 sperm membrane to activate specific transduction pathways leading to the selection of a
563 responsive sperm subpopulation with increased fertilizing ability. A number of potential
564 biomarkers of sperm fertility was identified and remain to be validated for application in
565 the field.

566 **Author contributions**

567 MSD and PM conceived the work; all authors curated the data; MSD, MRS, NB
568 and PM performed the formal analysis; NB and PM acquired the funding; MRS
569 performed cell culture experiments; MRS and MCB performed and analyzed FRAP
570 experiments; MRS, VL and XD performed and analyzed mass spectrometry
571 experiments; MRS, GT and XD performed and analyzed Western Blot experiments;
572 MRS created and edited all manuscript figures; NB, PM and MSD administered the
573 project and provided the resources; NB, MSD and PM supervised the work; all authors
574 validated the data; MRS and MSD wrote the original draft with the involvement of all
575 the co-authors; NB and PM critically corrected the draft; all authors reviewed and edited
576 the manuscript; all authors approved the final version of the present manuscript.

577 **Conflict of interest**

578 The authors declare that there is no conflict of interest regarding the publication
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806 **Figure legends**

807 **Figure 1. Abundance of BSP-1,-3,-5 on sperm cells.** Means \pm SEM of
808 normalized values for BSP1, BSP3 and BSP5 (n=3 replicates) quantified by western
809 blot. *P<0.05; **P<0.01, significance compared with the CTRL group.

810 **Figure 2. Evaluation of sperm membrane fluidity by Fluorescence Recovery**
811 **After Photobleaching (FRAP) analysis.** Histogram showing sperm fluidity medians
812 and percentiles 25 and 75% for spermatozoa released by P4 after attachment to BOECs
813 (BOEC-P4) and just manipulated sperm cells (CTRL) Each point represents a sperm
814 cell (n= 6 replicates) *P<0.05.

815 **Figure 3. Distribution of differential lipids and proteins in spermatozoa.** A)
816 Venn diagram of all differential masses (m/z, 123 masses in total) from lipidomic
817 analysis among the three comparisons in which differential masses were identified (the
818 CTRL vs. P4 comparison retrieved no difference): CTRL vs. BOEC (33); CTRL vs.
819 BOEC-P4 (34); BOEC-P4 vs. P4 (116); B) Venn diagram of all differential masses
820 (m/z, 151 masses in total) from proteomic analysis among four comparisons between
821 experimental groups: CTRL vs. BOEC (34); CTRL vs. BOEC-P4 (98); CTRL vs. P4
822 (62), BOEC-P4 vs. P4 (104).

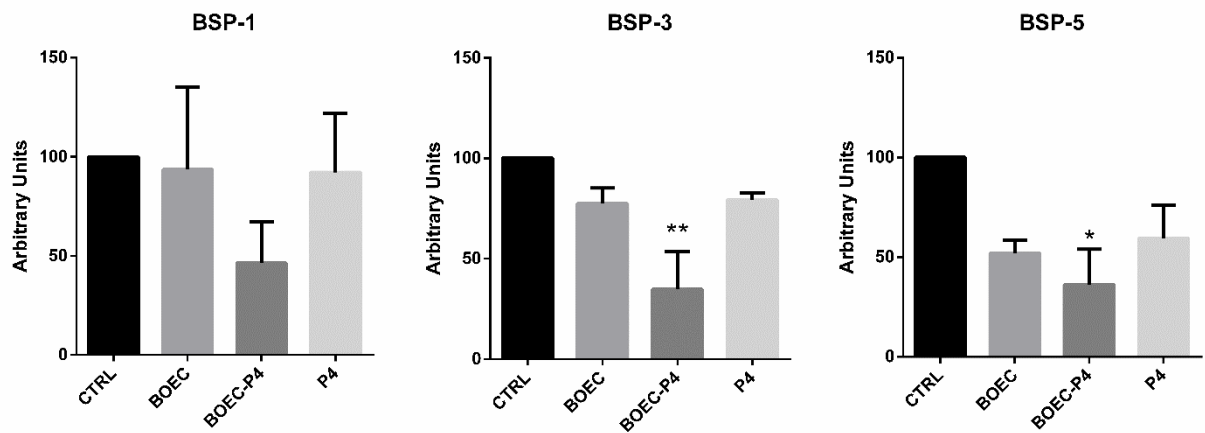
823 **Figure 4. Hierarchical clustering of differential lipids and proteins in**
824 **spermatozoa.** A) Heat map representation of differentially abundant lipid masses
825 among experimental groups (CTRL, BOEC, BOEC-P4 and P4); B) Heat map
826 representation of differentially abundant differential protein masses among experimental
827 groups (CTRL, BOEC, BOEC-P4 and P4).

828 **Figure 5. Lipids classification.** Diagram showing the lipids classification
829 including the percentage of each lipid class found after the analysis of lipidomic
830 experiments.

831 **Supplementary Data**

832 **Supplementary Table 1. Identification of lipids detected as differential by**
833 **ICM-MS in bovine spermatozoa.** The MALDI masses (m/z) correspond to the
834 observed masses by ICM-MS, confronted to the theoretical mass (Da) and the % of
835 delta mass (<0.1). Fold changes (FC) are ratios of mean normalized intensity values
836 between experimental groups: spermatozoa unbound or released from BOECs after a
837 short period of binding (BOEC), spermatozoa released from BOECs by P4 action
838 (BOEC-P4), treated with P4 without BOECs (P4) or just manipulated (CTRL).

839 **Supplementary Table 2. Identification of proteins detected as differential by**
840 **ICM-MS in bovine spermatozoa.** The MALDI masses (m/z) correspond to the
841 observed masses by ICM-MS, confronted to the theoretical mass (Da) and the % of
842 delta mass (<0.05%). Fold changes (FC) are ratios of mean normalized intensity values
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845 (BOEC-P4), treated with P4 without BOECs (P4) or just manipulated (CTRL).



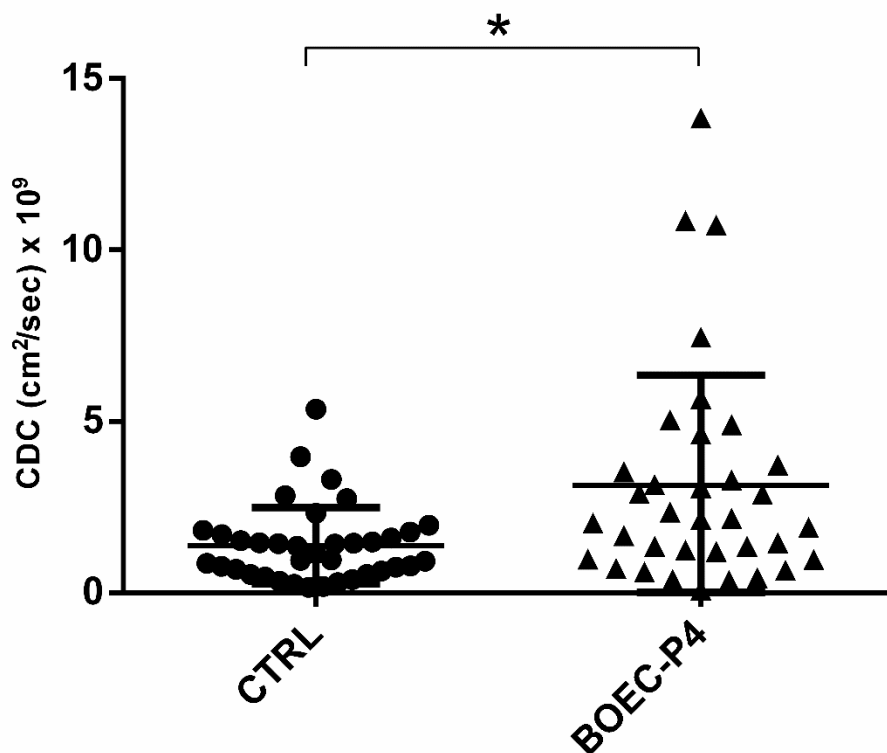
846

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849 **P<0.01, significance compared with the CTRL group.

850

851 2-columns fitting image

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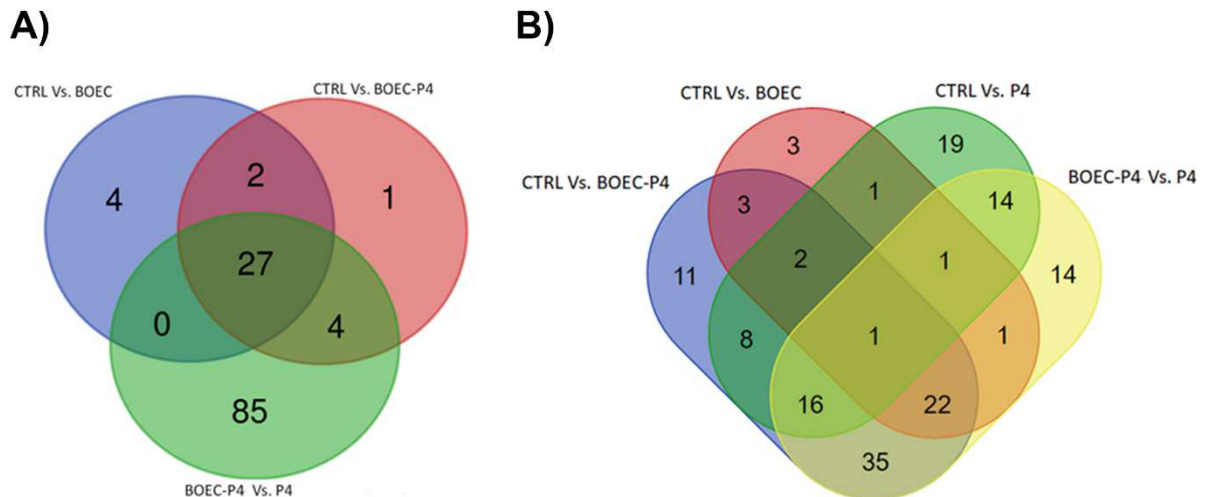


853

854 **Figure 2. Evaluation of sperm membrane fluidity by Fluorescence Recovery After**
 855 **Photobleaching (FRAP) analysis.** Histogram showing sperm fluidity medians and
 856 percentiles 25 and 75% for spermatozoa released by P4 after attachment to BOECs
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 858 cell (n= 6 replicates) *P<0.05.

859

860 1-column fitting image

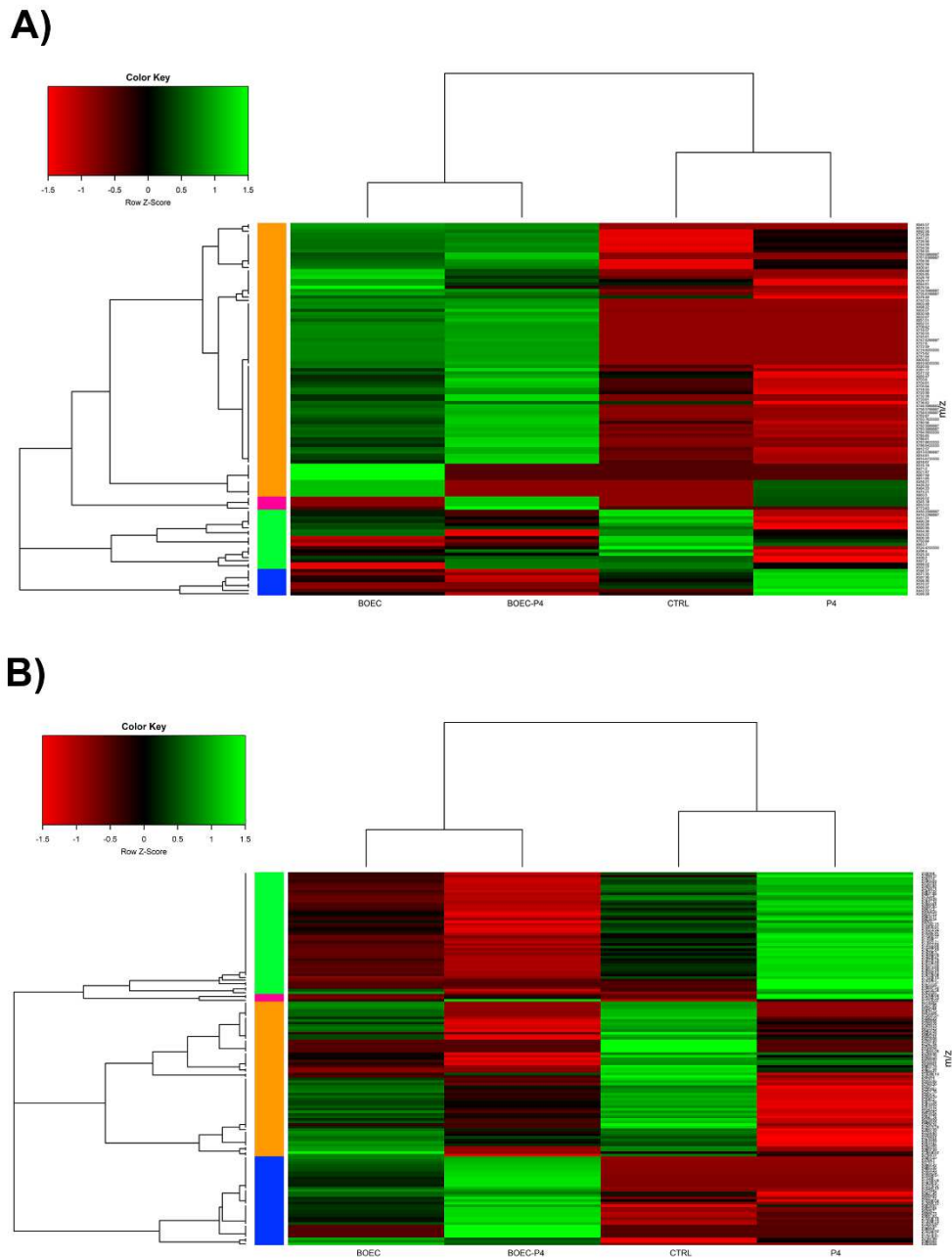


861

862 **Figure 3. Distribution of differential lipids and proteins in spermatozoa.** A) Venn
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 864 among the three comparisons in which differential masses were identified (the CTRL
 865 vs. P4 comparison retrieved no difference): CTRL vs. BOEC (33); CTRL vs. BOEC-P4
 866 (34); BOEC-P4 vs. P4 (116); B) Venn diagram of all differential masses (m/z, 151
 867 masses in total) from proteomic analysis among four comparisons between
 868 experimental groups: CTRL vs. BOEC (34); CTRL vs. BOEC-P4 (98); CTRL vs. P4
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870

871 2-columns fitting image



872

873 **Figure 4. Hierarchical clustering of differential lipids and proteins in spermatozoa.**

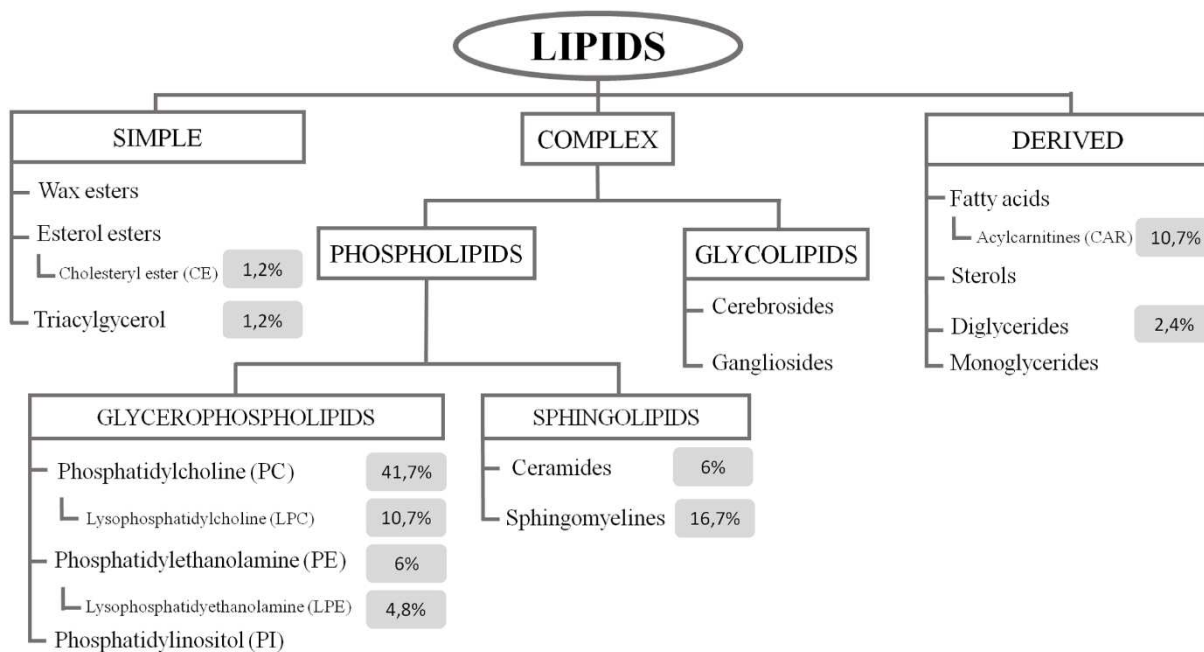
874 A) Heat map representation of differentially abundant lipid masses among experimental

875 groups (CTRL, BOEC, BOEC-P4 and P4); B) Heat map representation of differentially

876 abundant differential protein masses among experimental groups (CTRL, BOEC,

877 BOEC-P4 and P4).

878 2-columns fitting image



879

880 **Figure 5. Lipids classification.** Diagram showing the lipids classification including the
 881 percentage of each lipid class found after the analysis of lipidomic experiments.

882

883 2-columns fitting image