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1 **Avian uterine fluid proteome: exosomes and biological processes potentially involved in**  
2 **sperm survival**

3

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15

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18 **Abstract**

19 Uterine fluid is an aqueous milieu to which sperm are exposed during their storage and ascent.  
20 In this study a bottom up proteomic strategy and bioinformatic analysis of hen uterine fluid was  
21 performed to improve understanding of this fluid and its potential role in sperm survival  
22 mechanisms. The proteomic data were submitted to ProteomeXchange. Among the 913 proteins  
23 identified, 160 are known to be secreted and 640 are referenced in exosomes databases. We  
24 isolated exosomes from avian uterine fluid, analyzed them using electron microscopy and  
25 targeted several exosomes markers (ANXA1/2/4/5, VCP, HSP90A, HSPA8, PARK7, and  
26 MDH1) using immunoblotting. Electron microscopy and immunohistochemistry were also  
27 used to analyze uterovaginal junctions for the exosomal proteins ANXA4, VCP and PARK7.  
28 Exosomes were observed both at the surface epithelium and inside sperm storage tubules. Our  
29 data were compared to two previously published studies on proteomic of hen uterine fluid, and  
30 with one study describing the proteomic content of rooster seminal plasma and sperm. In  
31 conclusion, we demonstrated for the first time that avian uterine fluid contains exosomes. These  
32 may play a key role in preserving sperm functions within the female genital tract. Their presence  
33 in the sperm storage tubules may represent an important mechanism regarding interaction  
34 between the female genital tract and sperm.

35

## 36 Introduction

37 Avian species have the capacity to store sperm for several weeks in the female genital tract  
38 (Bakst et al., 1994; Birkhead & Moller, 1993). Sperm reservoirs, mainly located in the mucosa  
39 of the utero-vaginal junction (UVJ), are called sperm storage tubules (SST) (Bakst, 2011).

40 The female avian reproductive tract (oviduct) consists of the infundibulum, magnum, white  
41 isthmus, uterus and vagina, which are associated with the egg components. The infundibulum  
42 ensures the deposition of the vitelline membrane outer layer. The developing egg then enters  
43 the magnum, where the egg white is produced (0.5 to 3.5 hours post ovulation). In the isthmus,  
44 eggshell membranes are synthesized (3.5 to 5 hours post ovulation). Then eggshell calcification  
45 takes place in the uterus within an 18 hours period. At that time, the egg mass in the uterus  
46 compresses the UVJ folds that are positioned towards the uterus, resulting in its mucosa being  
47 contiguous with those of the uterine tissue (Bakst & Akuffo, 2009), as shown schematically in  
48 Figure 1. Finally, 24 hours after ovulation, the egg transits through the vagina and is laid. After  
49 mating, at the same time as the daily process of egg formation, sperm pass through the vagina  
50 and are stored in the uterovaginal SST (UVJ-SST) for several days. Then, the sperm transit  
51 from the UVJ-SST to the infundibulum, which is the site of fertilization (Sasanami et al., 2013).

52 Uterine fluid (UF) is the only reproductive fluid described yet in the avian reproductive tract. It  
53 represents the aqueous environment in which sperm is exposed during its ascent from the vagina  
54 to the infundibulum. Uterine fluid has been widely investigated over the past few years in  
55 relation to the egg formation (Gautron et al., 2019). It is a selective transudate of serum which  
56 also contains locally produced factors, mainly related to the metabolic activity of uterine  
57 epithelial cells. It contains several key proteins involved in the eggshell mineralization process  
58 that occurs from 5 hours after ovulation (Marie et al., 2015; Sun et al., 2013). It is also composed  
59 of bactericidal factors, providing an aseptic package for embryo development (Gautron et al.,  
60 2019; Gautron et al., 2007; Silphaduang et al., 2006; Wellman-Labadie et al., 2008).

61 Additionally to the fact that the secretory activity of the reproductive tract depends to the egg  
62 formation, UF has been shown to promote the maintenance of sperm motility and viability  
63 (Ahammad et al., 2013; Brillard et al., 1987). Actually, Ahammad et al. (2013) demonstrated  
64 that the stage of the secretory activity of the female reproductive tract has an incidence on the  
65 sperm storage efficiency. They showed that the filling of UVJ-SST is more effective when  
66 insemination is performed during initialization of eggshell mineralization (i.e. 5-6 hours after  
67 ovulation) than the calcifying phase (Ahammad et al., 2013). Thus, sperm may be more  
68 efficiently stored when inseminated at the beginning of the eggshell formation, when the  
69 secretory activity of the uterus increases and the UF is highly secreted. Under these conditions  
70 hens exhibit a longer period of fertile egg production (Brillard et al., 1987). Consequently, it  
71 has been postulated that UF constituents are essential for sperm storage efficiency in birds.  
72 Furthermore, we can hypothesize that some key proteins localized at the surface epithelium or  
73 in the lumen of the SST may originate from other regions of the genital tract such as by UF.  
74 Then these may adhere to the epithelium in order to enhance sperm retention within SST,  
75 preserve sperm function and/or regulate their release. Nevertheless, the mechanisms involved  
76 are still poorly understood.

77 Many biological fluids have been shown to vehicle molecules not only as soluble/secreted  
78 forms, but also engulfed in cell-derived membranous extracellular vesicles (EV). These are  
79 involved in the transfer of molecules from one cell to another, and thus play an important role  
80 in cell-to-cell signaling (Valadi et al., 2007). To date, in mammals, only a few studies have  
81 described the presence of EV in the fluid from of the female genital tract. In birds, Baskt and  
82 Bauchan (2015) demonstrated the presence of 30-130 nm EV in the lumen of turkey UVJ-SST  
83 (Bakst & Bauchan, 2015), and more recently Huang et al (2017) showed that SST cells produce  
84 exosomes *in vitro* in the presence of sperm (Huang et al., 2017).

85 In this study, we hypothesized that UF contained molecules that may preserve sperm during  
86 their storage and ascent in the female genital tract. We aimed to determine the protein  
87 composition of UF using proteomic analysis, to examine its EV content, and thus understand  
88 better the potential underlying mechanisms involved in the sustained sperm survival. We  
89 demonstrated that avian UF contained EV, particularly exosomes, which may play a role in the  
90 preservation of sperm functions during storage and ascent. Their presence in the SST may  
91 represent a mechanism of genital tract-sperm interaction involved in sperm survival.

92

## 93 **Material and Methods**

### 94 ***Birds***

95 Mature female domestic hens from a light broiler-type strain (Beaumont et al., 1992) were used  
96 in this study. Breeding procedures and handling protocols were carried out in accordance with  
97 the European Union Council Directives regarding practices of animal care and use, practices of  
98 the French Ministry of Agriculture on animal experimentation, under the supervision of an  
99 authorized scientist (Authorization # 37035). The facilities at the Institut National de la  
100 Recherche Agronomique, UE-PEAT 1295, are officially authorized to rear and euthanize birds  
101 (B27-175-1 dated 28/08/2012). At 50 weeks of age, hens were placed in individual cages  
102 equipped with automatic devices to record the time of oviposition. They were kept under a 16L:  
103 8D photoperiod, and fed a layer mash ad libitum. The protocol of bird management and  
104 collection was approved by the local ethics committee (Comité d'éthique de Val de Loire n°19)  
105 and the French Ministry of Research under agreement number # 443.

### 106 ***Uterine fluid collection***

107 UF collection was scheduled 10h after oviposition and after confirming the presence of an egg  
108 in utero. Egg expulsion was induced by intravenous injection of prostaglandin F2A at 50µg/hen.  
109 During egg expulsion UF was collected in a plastic tube placed at the entrance of the everted  
110 vagina, from 10 virgin hens as described previously (Gautron et al., 1997). An aliquot of each  
111 UF was immediately diluted with 5x Laemmli buffer (5v:1v) (312.5 mM Tris-HCl pH 6.8, 10  
112 % SDS, 12.5 % β-mercaptoethanol, 50 % glycerol, bromophenol blue), and then boiled for 5  
113 min before storage at -20°C. Two other aliquots of fluid per hen were diluted with PBS (1v:1v)  
114 to limit calcium carbonate and proteins precipitation, in order to perform in solution digestion  
115 MS analysis, and to measure protein concentration.

### 116 ***Tissue Collection***

117 Avian UVJ were collected from 6 hens for immunohistochemistry and transmission electron  
118 microscopy. Hens were euthanized by cervical dislocation. The genital tract section from uterus  
119 to vagina was excised as one segment. Connective tissue was removed to expose the  
120 uterovaginal junction. Samples of UVJ mucosa containing the UVJ-SST were collected from  
121 each hen and processed.

### 122 ***SDS-PAGE with fractionation***

123 The protein concentrations of samples were determined using a Pierce™ BCA protein assay  
124 kit, Thermofisher™. For exhaustive identification, the aliquots of UF collected from 10 females  
125 and diluted in Laemmli buffer (see above) were pooled by mixing the same amount of proteins  
126 from individual samples. Fifty µg of proteins from the pool of UF were fractionated by SDS-  
127 PAGE (10%) and stained with Coomassie Blue staining (PageBlue™ Protein Staining Solution,  
128 Fermentas®). Each lane was sectioned into 30 slices, which was cut into approximately 1 mm<sup>3</sup>  
129 pieces.

### 130 ***In-gel and liquid digestion***

131 For liquid digestion 5 µg of UF sample was pooled by mixing the same amount of proteins from  
132 individual samples collected from 10 females. For in-gel digestion each gel slice was washed  
133 in water/ACN (1:1) for 5 min and in ACN for 10 min. For both digestions, cystein reduction  
134 and alkylation were performed by successive incubations in 10 mM dithiothreitol/50 mM  
135 NH<sub>4</sub>HCO<sub>3</sub> for 30 min at 56°C and 55 mM iodoacetamide/50 mM NH<sub>4</sub>HCO<sub>3</sub> for 20 min at room  
136 temperature in the dark. For in-gel digestion, gel slices were washed by incubation in 50 mM  
137 NH<sub>4</sub>HCO<sub>3</sub>/ACN (1:1) for 10 min and by incubation in ACN for 15 min. Proteins were digested  
138 overnight in 25 mM NH<sub>4</sub>HCO<sub>3</sub> with 12.5 ng/µl trypsin (Sequencing Grade, Roche, Paris). For  
139 liquid digestion, the resulting peptides solution was dried, reconstituted with 13µL of 0.1% FA,  
140 2% ACN, and sonicated for 10 min before MS analysis. For in-gel digestion, the resulting  
141 peptides were extracted from gel by successive incubations in 0.1% FA/ACN (1:1) for 10 min  
142 and in ACN for 5 min. The two extracts were pooled, dried, reconstituted with 13µL of 0.1%  
143 FA, 2% ACN, and sonicated for 10 min before MS analysis.

#### 144 *NanoLC-MS/MS*

145 Peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass  
146 spectrometry (nanoLC-MS/MS). All experiments were performed on a LTQ Orbitrap Velos  
147 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate®  
148 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, The  
149 Netherlands) controlled by Chromeleon Software (version 6.8 SR11 ; Dionex, Amsterdam, The  
150 Netherlands). Five microliters of each sample were loaded on an LCPackings trap column  
151 (Acclaim PepMap 100 C<sub>18</sub>, 100 µm inner diameter x 2cm long, 3µm particles, 100Å pores).  
152 Mobile phases consisted of (A) 0.1% FA, 97.9% water, 2% ACN (v/v/v) and (B) 0.1% FA, 15.9  
153 % water, 84% ACN (v/v/v). Peptides were desalted and preconcentrated for 10 min at 5µL/min  
154 with 4% solvent B. The peptide separation was conducted using a LCPackings nano-column  
155 (Acclaim PepMap C<sub>18</sub>, 75µm inner diameter x 50 cm long, 3µm particles, 100Å pores). The  
156 gradient consisted of 4-55% B for 90 min, 55 to 99% B for 1 min, constant 99% B 20 min and  
157 return to 4% B in 1 min. The column was re-equilibrated for 15 min at 4% B between runs. The  
158 nanoflow rate was set at 300 nl/ min. Data were acquired using Xcalibur software (version 2.1;  
159 Thermo Fisher Scientific, San Jose, CA). The instrument was operated in positive mode in data-  
160 dependent mode. Resolution in the Orbitrap was set at R = 60,000. In the scan range of m/z  
161 300-1800, the 20 most intense peptide ions with charge states  $\geq 2$  were sequentially isolated  
162 (isolation width, 2 m/z; 1 microscan) and fragmented using Collision Induced Dissociation  
163 (CID). The ion selection threshold was 500 counts for MS/MS, and the maximum ion  
164 accumulation times allowed were 500 ms for full scans and 25 ms for CID-MS/MS. Target ion  
165 quantity for FT full MS was 1e6 and for MS<sup>2</sup> it was 1e4. The resulting fragment ions were  
166 scanned at the “normal scan rate” with  $q = 0.25$  activation and activation time of 10 ms.  
167 Dynamic exclusion was activated for 30 s with a repeat count of 1. The lock mass was enabled  
168 for accurate mass measurements.

#### 169 *Protein identification and data validation*

170 Raw data files were converted to MSF with Proteome Discoverer software (version 1.4; Thermo  
171 Fischer Scientific, San Jose, USA). A precursor mass range of 350–5000 Da and signal to noise

172 ratio of 1.5 were the criteria used for generation of peak lists. The MS proteomics data were  
173 converted using a PRIDE Converter (version 2.5.3) (Deutsch et al., 2017; Perez-Riverol et al.,  
174 2019) and deposited with the ProteomeXchange Consortium  
175 (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the  
176 dataset identifier [*submitted*] and project [*submitted*]. The peptide and fragment masses  
177 obtained were matched automatically against the Chordata section of a locally maintained copy  
178 of nrNCBI (1601319 sequences). MS/MS ion searches were performed using MASCOT  
179 Daemon and search engine (version 2.3; Matrix Science, London, UK). The parameters used  
180 for database searches included trypsin as a protease with two missed cleavages allowed, and  
181 carbamidomethylcysteine, oxidation of methionine and N-terminal protein acetylation as  
182 variable modifications. The tolerance of the ions was set at 5 ppm for parent and 0.8 Da for  
183 fragment ion matches. Mascot results were incorporated in Scaffold 4 software (version 4.3,  
184 Proteome Software, Portland, USA). Peptide identifications were accepted if they could be  
185 established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.  
186 Peptides were considered distinct if they differed in sequence. Protein identifications were  
187 accepted if they could be established at greater than 95.0% probability as specified by the  
188 Protein Prophet algorithm and contained at least two identified peptides. A false discovery rate  
189 was calculated as < 1% at the peptide or protein level. The abundance of identified proteins was  
190 estimated by calculating the emPAI using Scaffold software. A phylogenetic tree was constructed  
191 with Clustal Omega and similar *Gallus gallus* proteins were searched in nr databases using blast  
192 in order to clarify the scaffold groups.

### 193 ***Statistical, data mining and bioinformatics analysis***

194 Proteomic data were extracted from Scaffold software and analyzed using R language  
195 (<http://cran.r-project.org>) after elimination of keratin and trypsin, as they were contaminants or  
196 resulted from the digestion process, respectively. For each protein the identifier, symbol and  
197 description of the annotated gene were extracted from nr NCBI database. Overlapping and  
198 continuous peptides from the same sequence were concatenated to create a longer peptide  
199 (reconstituted peptide), in order to remove the peptidic redundancy and to generate a higher  
200 strength for the next step of analysis. Longer peptide sequences were blasted against nr NCBI  
201 database limited to *Gallus gallus* taxon, using the blastp program (BLAST+ suite) (Camacho  
202 et al., 2009). The alignment result of each peptide for each protein was expressed as a global  
203 score of similarity (SIsc) and identity (IDsc). Only a perfect match (IDsc =100%) with a *Gallus*  
204 *gallus* protein allowed removal of redundancy inside protein groups. Proteins which were not  
205 strictly identified in the *Gallus gallus* database were: a) considered as orthologous to *Gallus*  
206 *gallus* proteins when the blastp result indicated the same protein description and name as the  
207 Chordata Scaffold result, and b) considered as unknown *Gallus gallus* proteins when the blastp  
208 result did not share any similar name or protein description. The resulting file constituted the  
209 non-redundant proteome presented in the present study. Only proteins that display SIsc higher  
210 than 91% (in terms of their blast results, including the size of peptides and the profile of the  
211 match, i.e. number of mismatches and gaps) were considered orthologous to the previously  
212 identified proteins. The SecretomeP 2.0 (Bendtsen et al., 2004)  
213 (<http://www.cbs.dtu.dk/services/SecretomeP/>), as well as the Signal P 4.1 (Petersen et al., 2011)

214 (<http://www.cbs.dtu.dk/services/SignalP>) servers were used as a prediction method of the  
215 secretion pathway (signal peptide-dependent or independent or via exosomes) for each proteins  
216 obtained in the present study. Our data were then compared to an exosome database which we  
217 constituted by merging Exocarta (human, rat, mouse), UniprotKB (chicken) and KEGG  
218 (chicken) databases (personal data). Extraction of conserved and functional domains from  
219 protein sequences using BioMart allow to classify proteins by molecular function  
220 (<http://www.ensembl.org/biomart/martview>). According to their molecular function, to the GO-  
221 term annotation from Uniprot and Genecards (chicken and mammals), and to the literature,  
222 proteins were categorized in three main putative functions of interest as mineralization,  
223 immunity and sperm survival. The molecular function of total UF and exosomal proteins were  
224 compared using Fisher's exact test. Our identified peptides were also compared to published  
225 quantitative proteome lists from avian UF and eggshell (Marie et al., 2015; Sun et al., 2013)  
226 using BLAST+ suite, and from rooster's seminal plasma and sperm using protein ID.

### 227 ***EV preparation***

228 EV were obtained by serial centrifugations as previously described (Thery et al., 2006). Briefly,  
229 UF were pooled (n= 2 to 5 animals/replicate) and the volume was completed to 5 ml with PBS  
230 before being centrifuged at 100 g for 15 min, followed by 12,000g for 15min to remove cells  
231 and cell debris. Two successive ultracentrifugations at 100,000g for 90min (Beckman L8-M  
232 with SW41T1 rotor) provided an exosome pellet. The pellet was resuspended in 50µl of PBS  
233 and stored at -20°C until analysis. An aliquot of each initial pool and ultracentrifugation  
234 supernatant were also kept at -20°C.

### 235 ***Transmission electron microscopy analysis of EV and uterovaginal junction***

236 EV were analyzed as whole-mounted vesicles deposited on EM copper/carbon grids for 5min,  
237 and contrasted for 10s in 1% uranyl acetate. Grids were examined with a Hitachi HT7700  
238 electron microscope operated at 80kV (Elexience – France), and images were acquired with a  
239 charge-coupled device camera (AMT). Tissue samples from uterovaginal junctions were fixed  
240 with 4% glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.2, for 4 h at room temperature.  
241 Following fixation, samples were transferred to cacodylate 0.1M buffer containing osmic acid  
242 2% for 2 h at 20°C. Samples were then dehydrated in graded baths of ethanol (70 to 100%) and  
243 embedded in epon resin. Polymerisation was performed at 60°C for 48 h. Ultrathin sections of  
244 70nm were prepared with an Ultracut Leica ultramicrotome. Sections were counterstained using  
245 uranyl acetate and lead citrate and observed using a CM10 Philips TEM at 80Kv. Images were  
246 acquired using a Megaview III camera coupled with AnaySIS soft imaging system software.

### 247 ***Western blotting of EV proteins***

248 The protein concentration was determined in each sample of UF and EV using the Pierce®  
249 BiCinchoninic Acid protein assay kit (Life Technologies SAS, Saint Aubin, France) using  
250 bovine serum albumin as the protein standard and according to the manufacturer's instructions.  
251 Aliquots of 10µg of proteins from the UF samples, ultra-centrifuged UF (EV-depleted) samples,  
252 and isolated-EV samples that were added with 5x Laemmli buffer (5v:1v) and boiled at 95°C  
253 for 5 minutes were loaded and separated on a 10% SDS-PAGE, before being transferred to

254 nitrocellulose filters. The membranes were washed with TBS (10mM Tris, 150mM NaCl, pH  
255 7.4) containing 0.1% (v/v) Tween-20 (TBS-T), incubated for 1 hour in the blocking solution  
256 (5% (w/v) non-fat dry milk in TBS-T), and then overnight in the blocking solution containing  
257 anti-VCP (1:1000; mouse monoclonal; ab11433, Abcam, Paris, France), anti-HSP90A (1:1000;  
258 rat monoclonal; ADI-SPA-840-D, Enzo life sciences, Villeurbanne, France), anti-HSPA8  
259 (1:500; rabbit polyclonal; bs-5117R, Bioss, Interchim, Montluçon, France), anti-MDH1  
260 (1:1000; rabbit polyclonal; CSB-PA013621ESR1HU, Cusabio, Clinisciences, Nanterre,  
261 France), anti-PARK7 (1:1000; rabbit polyclonal; NB300-270 Novus biologicals, Bio-Techne,  
262 Lille, France), anti-ANXA2 (1:1000; rabbit polyclonal; CSB-PA001840HA01HU, Cusabio,  
263 Clinisciences, Nanterre, France), anti-ANXA5 (1:1000; rabbit polyclonal; CSB-  
264 PA06384A0Rb, Cusabio, Clinisciences, Nanterre, France), anti-ANXA1 (1:1000; rabbit  
265 polyclonal; sc-11387, Santa cruz biotechnology, Heidelberg, Germany), anti-ANXA4 (1:1000;  
266 rabbit polyclonal; CSB-PA001845ESR2HU, Cusabio, Clinisciences, Nanterre, France), anti-  
267 BPIFB3/OCX36 (1/5000), or anti-OC17 (1/1000) both rabbit polyclonal kindly provided by J.  
268 Gautron. The membranes were then sequentially washed with TBS-T, incubated for 1 hour in  
269 the blocking solution, then for 1 hour with peroxidase-conjugated secondary antibody, i.e. goat  
270 anti-rabbit IgG (A6154, Sigma-Aldrich, Saint Quentin Fallavier, France) or goat anti-mouse  
271 IgG (A4416 Sigma-Aldrich) or goat anti-rat IgG (112-036-003 Jackson ImmunoResearch,  
272 Interchim), diluted 1/5000 in the blocking solution and finally washed with TBS-T. The  
273 peroxidase activity was detected with the ECL select™ Western Blotting Detection Reagent  
274 (GE Healthcare, Velizy-Villacoublay, France) and the signal was captured using the  
275 ImageMaster VDS-CL bio imaging system (Amersham Biosciences/GE Healthcare).

### 276 *Immunohistochemistry on uterovaginal tissue*

277 Following fixation for 24 hours in 4% saline buffered (PBS) formalin, tissue samples (UVJ  
278 mucosa) were transferred to two successive baths of 70% ethanol (2x30 min), and then moved  
279 to an automated tissue processor system (Leica TP1020 Semi-enclosed Benchtop Tissue  
280 Processor). The samples were then embedded in paraffin (Leica EG1150 Modular Tissue  
281 Embedding Center, Leica Microsystems Richmond, Inc., Richmond, IL). Four to six 7-µm thick  
282 sections were collected in sequence onto slides treated with 0.01% poly-L-Lysine in water, air-  
283 dried overnight, stored at room temperature for 12 hours and then incubated overnight at 60°C.  
284 The staining procedures consisted in deparaffinization for 5 min in toluene, followed by  
285 progressive rehydration (2 min each of 100%, 95%, 80% and 70% ethanol bathes,) and removal  
286 of excess ethanol by water baths. After rehydration, the slides were treated with 1% citrate  
287 based unmasking solution H3300 (Vector Laboratories, Burlingame, CA) diluted as  
288 recommended by the manufacturer. The slides were rinsed once in TBS for 5 min and then  
289 placed in a bath with normal horse serum blocking solution (Vector) for 20 min. The slides  
290 were incubated overnight at 4°C with the primary antibodies, anti-VCP (1:500; mouse  
291 monoclonal; ab11433, Abcam, Paris, France), anti-PARK7 (1:500; rabbit polyclonal; NB300-  
292 270 Novus biologicals, Bio-Techne, Lille, France) or anti-ANXA4 (1:500; rabbit polyclonal;  
293 CSB-PA001845ESR2HU, Cusabio, Clinisciences, Nanterre, France) diluted in TBS with 5%  
294 non-fat dry milk. The slides were then washed three times in TBS (5 min), and incubated for  
295 30 min with the secondary antibody ImmPRESS™ HRP Anti-Rabbit/Mouse IgG (Vector).

296 They were rinsed in TBS (5 min), incubated with peroxidase ImmPACT NovaRED (Vector)  
297 and finally rinsed in distilled water (5 min). The sections were counterstained with  
298 Papanicolaou stain (Sigma). Coverslips were applied with aqueous mounting medium after  
299 dehydration through graded alcohol baths (70%, 80% 95%, 100%; 20s each) and the sections  
300 were incubated in toluene for 1 min. Sections were examined using an Axioplan Carl Zeiss  
301 microscope. Images were acquired using a digital monochrome camera (Spot-Flex, Diagnostic  
302 Instruments) coupled with the SPOT 5.2 imaging software. Negative controls for  
303 immunohistochemistry were performed by the substitution of the primary antibody by an  
304 isotypic-specific immunoglobulin at the same concentration. Figures are representative of  
305 several observations performed on three hens per conditions.

306

## 307 Results

308 In the present study, a global proteomic inventory of soluble proteins present in the UF of light-  
309 broiler-type hens was performed using GeLC-MS/MS followed by SDS-PAGE fractionation  
310 and then shotgun proteomic. After elimination of redundancies, a total of 913 UF proteins were  
311 identified (supplementary data 1). Their abundance within the sample was determined using the  
312 emPAI calculation. The two main components were Lysozyme and Albumin that displayed an  
313 emPAI value of 1000 and 999.99, respectively. Of the 913 proteins, 837 are already listed in  
314 the *Gallus gallus* database (supplementary data 1 part 1), 37 presented an orthologous protein  
315 (supplementary data 1 part 2), 39 corresponded to proteins not listed in the *Gallus gallus*  
316 database (supplementary data 1 part 3). A total of 875 proteins have already been identified in  
317 Aves species, compared to 38 proteins in Mammalia (n=26), Reptilia (n=7), Actinopterygii  
318 (n=3), Amphibian (n=1) or Cephalochordata (n=1) (Figure 2A). Of the 39 proteins not  
319 identified either in *Gallus gallus* or orthologous to *Gallus gallus* proteins, 18 have already been  
320 identified in Aves, whereas others were identified in Mammalia (n=15), Reptilia (n=3),  
321 Actinopterygii (n=2), or Amphibian (n=1) species. Among the 18 proteins identified in Aves  
322 species, some are related to the same family as *Gallus gallus* such as *Meleagris gallopavo* (n=7  
323 proteins), or to a neighboring order (Passeriformes, n=9; Psittaciformes, n=1; Falconiformes,  
324 n=1). One of these 18 proteins displayed 90.7% similarity with ALDOA that was annotated in  
325 *Falco peregrinus*, whereas all the others displayed between 40.6% and 90% similarity with  
326 already sequenced avian proteins.

### 327 *Uterine fluid proteins which overlap with those from laying-type hens*

328 A few years ago, Sun et al., 2013 and Marie et al., 2015 revealed the UF protein composition  
329 for laying hens (White Leghorn and ISA-Hendrix, respectively) using a bottom-up proteomic  
330 approach (Marie et al., 2015; Sun et al., 2013). The proteome described in the current study and  
331 in these two previous studies were combined resulting in the identification of 1,230 avian UF  
332 proteins. Of to the 913 UF proteins described in the current study, we identified 153 (16.8%)  
333 proteins common to the three studies (i.e. with a protein sequence similarity coefficient higher  
334 than 91%) (Figure 2B). Their emPAI values ranged from 1,000 (rank 1) to 0.037 (rank 900).  
335 Moreover, 174 of our identified proteins were shared with one of the two previous studies. Out  
336 of these 327 shared proteins, 312 corresponded to a protein listed in the *Gallus gallus* database,  
337 10 possessed a *Gallus gallus* orthologous protein and five corresponded to proteins that are not  
338 listed in the *Gallus gallus* database yet (supplementary data 1). None of the peptides from the  
339 remaining 586 proteins (64.1%) revealed in our study had previously been identified in avian  
340 UF; they displayed emPAI values from 21.667 (rank 16) to 0.015 (rank 908).

### 341 *Analysis of uterine fluid proteins in relation to cellular localization*

342 Intracellular and extracellular localization of the 913 UF proteins identified in the present study  
343 are presented in Figure 3. The use of SecretomeP and SignalP bioinformatic tools demonstrated  
344 a total of 476/913 proteins (52.1%) that have already been categorized as secreted proteins.  
345 These either contained a signal peptide (155) or not (321). To date, the remaining 437 proteins  
346 (47.9%) have not been referenced as potentially secreted proteins in either database  
347 (supplementary data 1). Of these 437 proteins, 324 (74.1%) have previously been observed in

348 biological fluids within EV named exosomes (UniprotKB, Exocarta and KEGG databases).  
349 Among the 913 UF proteins identified in the present study, 18 of the top 25 of the EV/exosome  
350 markers (Exocarta DB) have been found. Their emPAI values varied from 999.62 (rank 2) to  
351 0.36 (rank 458). Moreover, we identified some proteins involved in multivesicular bodies  
352 (MVB) biogenesis such as Alix (PDCD6IP; emPAI=1.27; rank=162), TSG101 (emPAI=0.24;  
353 rank=578), and several proteins from the HSP70 family such as HSPA8 (emPAI=22.06;  
354 rank=17) and HSPA2 (emPAI=12.56; rank=24). Finally, to date only 113/913 proteins (12.4%)  
355 have not been categorized as secreted (Figure 3; supplementary data 1). These are mostly  
356 localized in cellular components such as cytosol, lysosomes, cytoskeleton and centrosome,  
357 when considering the GO-term enrichment in FunRich (human) or Genomatix (chicken)  
358 databases (data not shown). Their emPAI values ranged between 170.55 (rank 3) and 0.01 (rank  
359 907). Out of these 113 proteins as yet not identified as secreted, 12 have already been identified  
360 in at least one of the two avian UF proteomics studies (Marie et al., 2015; Sun et al., 2013). Out  
361 of these, 108/113 proteins are listed in the *Gallus gallus* database, 2/113 have been identified  
362 in the Aves class and possess a *Gallus gallus* orthologous protein (ADPRHL1 and RPS6KA3)  
363 and 3/113 correspond to proteins that are not listed in the *Gallus gallus* database  
364 (Immunoglobulin A heavy chain variable region), but are already listed in the Aves class  
365 (Loc102114577 and MAPRE3).

#### 366 ***Analysis of EV/exosomes from UF and in SST***

367 The EV fraction purified from UF was analyzed using transmission electron microscopy (Figure  
368 4A). It contained several vesicles of various sizes (20.695 - 244.628 nm). The majority ranged  
369 from 30 to 100 nm and was morphologically consistent with exosomes (Figure 4B).  
370 Immunodetection (Figure 4C) of exosomal and non-exosomal proteins demonstrated that VCP,  
371 HSP90A, HSPA8, ANXA2, MDH1, ANXA5, ANXA1, ANXA4 and PARK7 were present in  
372 the avian EV fraction, whereas BPIFB3 and OC-17 were not. Moreover, HSPA8, MDH1 and  
373 PARK7 signals were observed in EV-depleted UF.

374 TEM analysis of UVJ revealed the presence of exosomes (30-100 nm) at the surface of the  
375 epithelium (Figure 5A). Within SST, we observed intracellular MVB containing exosomes  
376 (<100 nm) (Figure 5B). We also detected apocrine blebs containing microvesicles (100-1000  
377 nm) (Figure 5C) in the lumen of SST.

#### 378 ***Immunohisto-localization of VCP, PARK7 and ANXA4 in SST***

379 SST are tubular glands localized in the UVJ whose role is to store sperm. Negative controls for  
380 immunohistochemistry demonstrated the absence of non-specific signals for all antibodies  
381 (Figure 6A). VCP, PARK7 and ANXA4, which are all known as exosome markers, were  
382 visualized within epithelial cells and lumen of SST (Figure 6B-D). A high ANXA4 signal was  
383 mainly observed on the apical part of the epithelium (Figure 6D).

#### 384 ***Analysis of uterine fluid proteins in relation to molecular functions***

385 The molecular functions of the 913 avian UF proteins identified in the present study were  
386 determined using InterPro, to extract the functional domains of each protein, followed by

387 Uniprot description to attribute the molecular function. This showed a wide range of activities  
388 (Figure 7A). The major molecular function encompassed enzymes involved in various  
389 metabolic processes, such as glucose synthesis (MDH1, GPI, PGK, PKM, ALDOA, ENO1,  
390 etc.), lipid homeostasis (FASN, LYPLA1 and 2, PLA2G4A, PLCD1, etc.) and glutathione  
391 metabolism (GSTA3, GSTM2 and 3, GPX1 and 3, etc.). The UF also contained structural  
392 proteins mostly participating in cytoskeletal assembly (12 actin isoforms, 6 tubulin isoforms,  
393 GSN, etc.). Moreover, we observed the presence of a large number of chaperon proteins (HSP70  
394 family HSPA2/ 4/ 4L and 8, DNAJA4/ B6/ B1/ B13 and C10, HYOU1, SGT1, STIP1,  
395 HSP90AA1, HSP90B1, subunits 2/ 3/ 4/ 5/ 6A/ 7 and 8 of T-complex protein 1, TCP1, PDIA3/  
396 4 and 6, etc.). It is noteworthy that 29 and 27 proteins were associated with multivesicular body  
397 (MVB) biogenesis and membrane trafficking, respectively.

### 398 *Analysis of uterine fluid proteins in relation to biological processes*

399 Potential functions of the 913 proteins were examined with particular emphasis on eggshell  
400 mineralization, molecular defense against pathogens and sperm survival via exosomes.

401 The first functional group concerned mineralization (Table 1). It was composed of 59 proteins,  
402 of which 28 may be involved in the regulation of protein activity since they corresponded to  
403 proteases, phosphatases or chaperons related to mineralization. The other 31 proteins from this  
404 group possessed either calcium binding or proteoglycan/proteoglycans binding domains, or  
405 were included in collagen fiber (ECM components). They were major UF proteins that  
406 displayed emPAI between 1,000 (LYZ) and 0.03 (ROS1).

407 The second group concerned molecular defense (Table 2). It was made up of 48 proteins of  
408 which five contained microbial degrading components, five were involved in decreasing  
409 bioavailability of iron and vitamins, 27 displayed microbial protease inhibiting activity, 10  
410 belonged to the immunoglobulin superfamily and one is known to mediate the immune  
411 response.

412 The third group contained proteins that potentially mediate sperm survival via the exosomes  
413 (Figure 7B). This exosome cluster was significantly ( $p < 0.001$ ) enriched with proteasomal  
414 proteins and comprises proteins involved in the ubiquitin pathway. It also contained chaperones  
415 or chaperone binding proteins including subunits from the TCP1-ring complex as well as  
416 HSPA8, HSPA2, HSP90AA1 and VCP. Moreover, it included antioxidant proteins comprising  
417 ALB, PRDX1, PRDX6, GSTA3, GSTO1, and GSTO1, and annexins. These latter include  
418 ANXA2, ANXA8, ANXA5 and ANXA1, which all possess phospholipase A2 inhibitor  
419 activity, and/or calcium binding properties.

### 420 *Uterine fluid proteins which overlap with avian semen proteins*

421 It is noteworthy that 396/913 proteins that we described in avian UF have been identified in  
422 rooster seminal plasma (143; 16%) or sperm (99; 15%), or both (154; 24%, Labas et al., 2015).  
423 Furthermore, 330/396 proteins belong to the exosome cluster, and to seminal plasma (125/143;  
424 20%) or sperm (64/99; 10%) or both (141/154; 22%) (Figure 8).

425 The 125 proteins referenced in both exosomes and the seminal plasma were composed of 34  
426 proteins involved in vesicle-mediated transport, 25 proteins involved in homeostasis, 24  
427 proteins from metabolic pathways, five proteins possessing a serpin domain, eight proteins from  
428 complement and coagulation cascades and four proteins presenting antioxidant properties. The  
429 most abundant proteins from this group were LYZ, ACTB, PRDX6, GDI2, GC, GSTO1,  
430 ANXA8, ANXA5, AKR7A2 and SERPINF1.

431 The 64 proteins referenced in the exosomes and the sperm contained seven chaperones or  
432 chaperon binding proteins, nine proteins from proteasome or ubiquitin pathways, 19 proteins  
433 involved in metabolic process comprising six proteins from TCA cycle, and five proteins  
434 involved in fatty acid degradation. The most abundant proteins of this group included HSPA8  
435 and TUBB4B.

436 The 141 proteins referenced in exosomes and in both the seminal plasma and the sperm were  
437 composed of 11 proteasomal proteins, 12 chaperons or chaperon binding proteins including  
438 eight subunits from the TCP1-ring complex, nine proteins exhibiting antioxidant properties, 31  
439 proteins involved in metabolic pathways comprising 11 proteins taking part in glycolysis. The  
440 most abundant proteins were ALB, ACTG1, CKB, PRDX1, HBAA, ALDOC, ENO1, ANXA2,  
441 HSPA2, YWHAE, GAPDH and HSP90AA1.

442 Moreover, 66 non-exosomal proteins were identified in UF and either seminal plasma, sperm  
443 or both (Figure 8). Among them, the six main proteins were HBG2, UBC, Ig gamma chain  
444 (clone 36), PIT54, SPINK2 and immunoglobulin alpha heavy chain.

445

## 446 Discussion

447 In the present study, we performed an in-depth inventory of proteins present in avian UF using  
448 classical proteomic approaches. We demonstrated for the first time that avian UF contains  
449 extracellular vesicles, mainly exosomes. These EV represent a mechanism of oviduct-sperm  
450 interaction that may play an essential role in the preservation of sperm functions and sperm  
451 survival within the SST.

452 We compared our dataset, obtained with domestic hens from a light broiler-type strain (n=913  
453 identified proteins) to those of Sun et al. (2013) and Marie et al. (2015) both relating to laying  
454 hens (n=550 and 308 protein sequences, respectively), revealing a list of 1,230 proteins of avian  
455 UF proteome. Our study allowed the number of proteins recently listed to be doubled (Marie et  
456 al., 2015).

### 457 *Proteins linked to shell formation that are potentially involved in sperm function*

458 Our study demonstrated that major proteins from the UF are involved in shell formation, as  
459 previously demonstrated (Marie et al., 2015). These proteins comprise eggshell matrix proteins  
460 unique to the process of shell formation such as ovocleidins (MEPE, also known as OC-116  
461 and OC-17), ovocalyxins (LOC771972, RARRES1, and OCX36) and hydrolases, dehydratases,  
462 proteases, protease inhibitors, chaperones, proteins possessing calcium binding domains, and  
463 proteins involved in the composition of the extracellular matrix (proteoglycans, proteoglycans  
464 binding proteins, collagen modulating proteins; Table 1). All these are known to play a key role  
465 in shell formation (Gautron et al., 2007; Hincke et al., 2012; Nys et al., 2004). The UF collected  
466 during the initiation of mineralization (6-10 hours post oviposition) was shown to improve  
467 sperm motility *in vitro* (Ahammad et al., 2013; Brillard et al., 1987). Moreover, this stage was  
468 shown to be an optimal window for artificial insemination regarding the fertility of hens  
469 (Brillard et al., 1987). Therefore, it is reasonable to hypothesize that UF components involved  
470 in the constitution of shell organic matrix may be important for sperm survival and ascent  
471 through the uterus.

472 In mammals, ECM components like collagen and proteoglycan binding protein (FN1) may have  
473 an influence on the ability of sperm to stick to the extracellular matrix (Koehler et al., 1980).  
474 Our study revealed the presence of proteoglycans (MEPE, TSKU, GPC4, HSPG2) and proteins  
475 with abilities to bind proteoglycans (FN1, SDCBP, SERPINF1, HAPLN3, VTN, SERPIND1,  
476 SDCBP2), that may display antimicrobial functions. We also identified several proteins  
477 involved in the modulation of collagen fibers (PLOD1, PLOD2, MMP2, TNXB). Interestingly,  
478 knowing that MEPE is a protein core of dermatan sulfate proteoglycan, and that sulfated  
479 glycosaminoglycans are known to modulate sperm-oviduct binding and to trigger sperm release  
480 in mammals (Talevi & Gualtieri, 2001), this protein may have a similar role in avian species.  
481 Moreover, it has been demonstrated recently that some proteoglycan binding proteins (FN1)  
482 interact with exosomes when in the presence of heparan sulfate glycosaminoglycan (Dismuke  
483 et al., 2016). These proteins, observed here in avian UF, could thus support the EV cargo and  
484 improve communication between the female genital tract and sperm.

485 MEPE is assumed to be a calcium chelator (Hincke et al., 1999) and it thus could regulate  
486 calcium availability for sperm. This is in accordance with Froman's model which suggests that  
487 sperm motility is strongly linked to extracellular calcium (Froman, 2003). Moreover, in the  
488 present study we identified numerous calcium binding proteins (ALB, OVOT, ANXA2, OVAL,  
489 EDIL3, ANXA5, HPX, CALM, MFGE8 and GSN). A previous study (Fujihara & Koga, 1984)  
490 demonstrated that calcium, used as an additive to sperm diluent, protects fowl sperm from  
491 deformation, lipid peroxidation and maintains fertility and motility *in vitro*. Albumin (ALB)  
492 exhibits antioxidant activity (Gum et al., 2004) and impacts fowl sperm motility *in vitro*  
493 (Blesbois & Caffin, 1992), whereas MFGE8, localized on sperm plasma membrane, is  
494 considered as a key modulator of sperm-egg binding *in vitro* (Raymond et al., 2009). Our work  
495 also demonstrated the presence of CALR protein that has been described as a regulator of  
496 intracellular calcium of sperm during hyperactivation and acrosome reaction in mammals (Dun  
497 et al., 2012; Nakamura et al., 1993).

498 Other proteins involved in the remodeling of membrane events like PDIA3, HSP90B/B1 (Dun  
499 et al., 2012), or kazal domain proteins like SPINK5, SPINK7 and SPINK2 (Jalkanen et al.,  
500 2006; Lee et al., 2011; Slowinska et al., 2015) have been identified in avian UF. There, these  
501 proteins may participate, at least in part, to membrane changes leading to the functional  
502 modulation of sperm during storage and ascent before fertilization.

503 Carbonic anhydrase 2 (CA2) that has been described in the present study, has been previously  
504 hypothesized to be related to the modulation of pH in the lumen of SST in turkey, hens and  
505 quail (Holm & Ridderstrale, 1998; Holm et al., 1996). It thus may be of major interest for sperm  
506 survival during its storage in the female genital tract by lowering of the pH in SST leading to  
507 the quiescence during storage.

508 Finally, UF proteins involved in eggshell mineralization could impact sperm features (plasma  
509 membrane, calcium levels), luminal features (pH, EV), and interactions (cell-cell binding) and  
510 consequently regulate sperm survival and/or motility in the female genital tract.

### 511 ***Proteins exhibiting antimicrobial activities potentially involved in sperm function***

512 We identified several antimicrobial proteins in the UF collected 10h post oviposition. They are  
513 involved in the degradation of microbial components (LYZ, OC-17, BPIFCB, BPIFB3 and  
514 LOC102118097), in the decrease in iron and vitamin bioavailability (OVOT, GC, VTG2, AVD  
515 and VTG1), or in the inhibition of proteases (RARRES1, SPINKs, CST3, AMBP, OVALY,  
516 OVAX, SERPINs, OVSTL, C3, C5, A2M, LOC418892) (Réhault-Godbert et al., 2011). These  
517 antimicrobial proteins most probably act to maintain a bacteria-free environment for sperm  
518 during its storage and transit in the female genital tract, and participate to the sperm acceptance.

519 Among these proteins, several are known to regulate the shell mineralization process, such as  
520 calcium binding proteins (OVOT), ECM components (i.e. proteoglycan binding proteins) or  
521 others (LYZ, SPINKs, RARRES1, CST3, OC-17, SERPINs, OVSTL). SPINKs have been  
522 demonstrated to protect sperm from the proteolytic action of proteases from dead or damaged  
523 sperm in the epididymis, the testis, and the seminal plasma (Jalkanen et al., 2006; Lee et al.,  
524 2011; Slowinska et al., 2015). Therefore, the presence of SPINKs in the avian UF may

525 contribute to the local proteases/proteases inhibitors homeostasis, regulate the proteolytic  
526 processing of the sperm surface proteins, and/or participate to sperm membrane changes during  
527 storage and ascent before fertilization.

528 Moreover, our study revealed several fragments of immunoglobulins. The immunoglobulin Ig  
529 gamma chain has recently been linked to sperm survival and has been proposed as a seminal  
530 marker of fertility in roosters (Labas et al., 2015). We also identified avidin in hen's UF, a  
531 protein which supports long term sperm storage in uterovaginal SST in turkey (Foye-Jackson  
532 et al., 2011; Long et al., 2003). Thus, both proteins may participate to the sustained sperm  
533 survival during storage in SST.

#### 534 ***Exosomal proteins potentially involved in sperm survival***

535 To our knowledge, our study is the first to demonstrate the presence of exosomes in avian UF  
536 collected 10 hours post-oviposition. Exosomes are extracellular vesicles released from cells that  
537 participate in several cellular processes and exhibit pleiotropic biological functions (Colombo  
538 et al., 2014). To date, they have been described in almost all body fluids, such as blood, urine,  
539 saliva and milk. In the female genital tract, exosomes have already been described and isolated  
540 from human and ovine uterine luminal fluid as well as from bovine oviductal fluid (Alminana  
541 et al., 2017; Burns et al., 2014; Ng et al., 2013). In avian species, they were observed in the  
542 lumen of UVJ-SST (Bakst & Bauchan, 2015), and were demonstrated to be secreted in vitro by  
543 UVJ and vaginal cells (Huang et al., 2017). Therefore, one can hypothesize that exosomes from  
544 avian UF could provide an alternative mode of communication between epithelial cells of the  
545 genital tract and sperm cells during their ascent and storage in the female genital tract since  
546 exosomes are known to deliver ubiquitous and specific proteins, lipids, RNA and ions to  
547 recipient cells.

548 We isolated exosomes from avian UF and demonstrated that they contained HSPA8, HSP90A,  
549 VCP, annexins, PARK7 and MDH1. Moreover, in the UF we identified the antioxidant proteins  
550 PRDX1, PRDX6, GSTA3 and SOD1, the chaperones HSPA8, HSPA2 and HSP90AA1, and  
551 proteins involved in metabolic pathways like CKB, ALDOC, LDHB, GSTA3, GSTO1, GSTM3  
552 and MDH1. All are known to be exosomal proteins as referenced in the *Exocarta* database.

553 Most of these components have already been shown to regulate sperm function, as reviewed in  
554 mammals (Dun et al., 2012; Yu & Huang, 2015) and avian species (Breque et al., 2003; Hiyama  
555 et al., 2014).

556 Exosomes have been observed within SST, fusing with sperm plasma membrane, suggesting  
557 that they may sustain sperm storage in the SST (Bakst & Bauchan, 2015). Moreover, we  
558 demonstrated that ANXA4, VCP and PARK7 were relevant markers of avian genital tract  
559 exosomes. ANXA4 was localized at the apical part of SST epithelial cells and VCP and PARK7  
560 were localized intracellularly. Considering that in mammals annexins have been proposed as  
561 sperm receptors (Ignotz et al., 2007; Talevi & Gualtieri, 2010), we can hypothesize that  
562 exosomes are key components for sperm survival during long term storage in the hen's genital  
563 tract.

564 In the mammalian male, seminal plasma exosomes have been demonstrated to regulate fluidity  
565 of the sperm membrane (Piehl et al., 2013), and to ensure the transfer of key molecules involved  
566 in calcium signaling to sperm (Park et al., 2011). As a result of their fusogenic properties,  
567 exosomes from seminal plasma are capable of transferring molecules to sperm, thereby altering  
568 the sperm's cholesterol: phospholipid ratio, sperm function relative to their maturation, motility  
569 properties, and antioxidant capabilities (Sullivan et al., 2005). It has been proposed that  
570 exosomes may constitute a pool of enzymes/proteins used to replenish the enzymes/proteins  
571 which are insufficiently represented or have been lost from sperm (Ronquist et al., 2013).  
572 Similarly, we could hypothesize that exosome components may be transferred from UF  
573 exosomes to stored sperm to guarantee sperm membrane properties, motility, antioxidant  
574 capabilities, and fecundity before fertilization. Nevertheless, the impact of UF exosome  
575 components on sperm functions requires further investigation.

### 576 ***Overlapping between avian UF and semen proteins***

577 We compared our data to proteomic analysis of rooster's seminal plasma and sperm (Labas et  
578 al., 2015) in order to identify key proteins potentially involved in sperm survival. Several  
579 antioxidant proteins (ALB, PRDX1, SOD1, GPX3 and TXNL1) were common between UF,  
580 seminal plasma and sperm (Labas et al., 2015). SOD and GPX have been suggested to prevent  
581 peroxidation of sperm lipids in the hen's oviduct (Breque et al., 2003). Metabolic pathway  
582 proteins (CKB, ALDOC, ENO1, GAPDH, LDHB, GSTA3, MDH1, PGK1, TPI1, PKM,  
583 PGAM1, GSS, GPI, PFKP, GOT1, GSTM2, LDHA, MDH2 and ALDH9A1) were also  
584 common to UF, seminal plasma and sperm. In human seminal plasma, glycolytic enzymes may  
585 enable sperm to produce ATP (Ronquist et al., 2013).

586 In conclusion, the present work improves our knowledge of the protein content of avian UF and  
587 using proteomics demonstrates for the first time that avian UF contains EV, mainly exosomes.  
588 The latter were purified and immunodetection associated with transmission microscopy  
589 approaches supported this result. Exosomes may play a key role in the preservation of sperm  
590 functions, by participating in long-term sperm storage in chicken. Moreover, the proteins we  
591 identified in the UF which are linked to shell formation may constitute a support for sperm  
592 survival. The presence of antimicrobial proteins may ensure a bacteria-free environment for  
593 sperm as well as sperm acceptance.

### 594 **Authors' contribution**

595 CR participated to the conception of the study, coordinated and participated to the experimental  
596 procedures and collection of the biological samples, performed experiments, analyzed data and  
597 wrote the manuscript. AB actively participated to the analysis of the proteomic data. LC actively  
598 participated to the collection of the biological samples, performed experiments, and corrected  
599 the manuscript. GH, AG and VL performed the nano LC MS/MS analysis and protein  
600 identification. JG participated to the conception of the study and collection of the biological  
601 samples. NG conceived the study and participated in its design, coordination and in the  
602 collection of biological samples, found the financial support and corrected the manuscript.

### 603 **Declaration of interest**

604 The authors declare no conflict of interest.

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821 **Legends**

822 **Table 1.** UF proteins potentially linked to the process of shell mineralization clustered in 3  
823 groups according to their functions.

824 **Table 2.** UF proteins linked to antimicrobial activities clustered in 5 groups according to their  
825 functions.

826 **Figure 1.** Schematic representation of the hen's genital tract at ovulation (A) and fluid  
827 collection, 10 hours post oviposition (B).

828 **Figure 2.** Proteomic analysis of avian UF. (A) Distribution of the 913 proteins from the UF in  
829 relation to taxonomy. (B) Venn diagram of proteins identified in the present study and studies  
830 from Marie et al. (2015) and Sun et al. (2013).

831 **Figure 3.** Venn diagram of identified proteins in the UF categorized either as not secreted  
832 (orange, 437 proteins), or secreted containing a signal peptide (PS, pink, 155 proteins) or not  
833 (NCP, yellow, 321 proteins), and EV/exosomal proteins (4643 proteins, UniprotKB, Exocarta  
834 and KEGG databases).

835 **Figure 4.** Analysis of EV from the UF. (A) Purification of the EV containing exosomes  
836 (arrowheads) observed by transmission electron microscopy. (B) Distribution of the size of EV.  
837 (C) Immunodetection of exosomal (VCP, HSP90A, HSPA8, ANXA2, MDH1, ANXA5,  
838 ANXA1, ANXA4, PARK7) and non-exosomal (BPIFB3, OC-17) proteins from UF. Selected  
839 proteins observed in UF by mass spectrometry were analyzed using western blot of the UF (lane  
840 1), EV-depleted UF (lane 2), and EV (lane 3) using primary antibodies directed against proteins  
841 either previously observed in exosomes, or not. Numbers on the left of each blot represent  
842 molecular weight (kDa) from a simultaneous protein standard run. All lanes contained 10 µg  
843 of proteins.

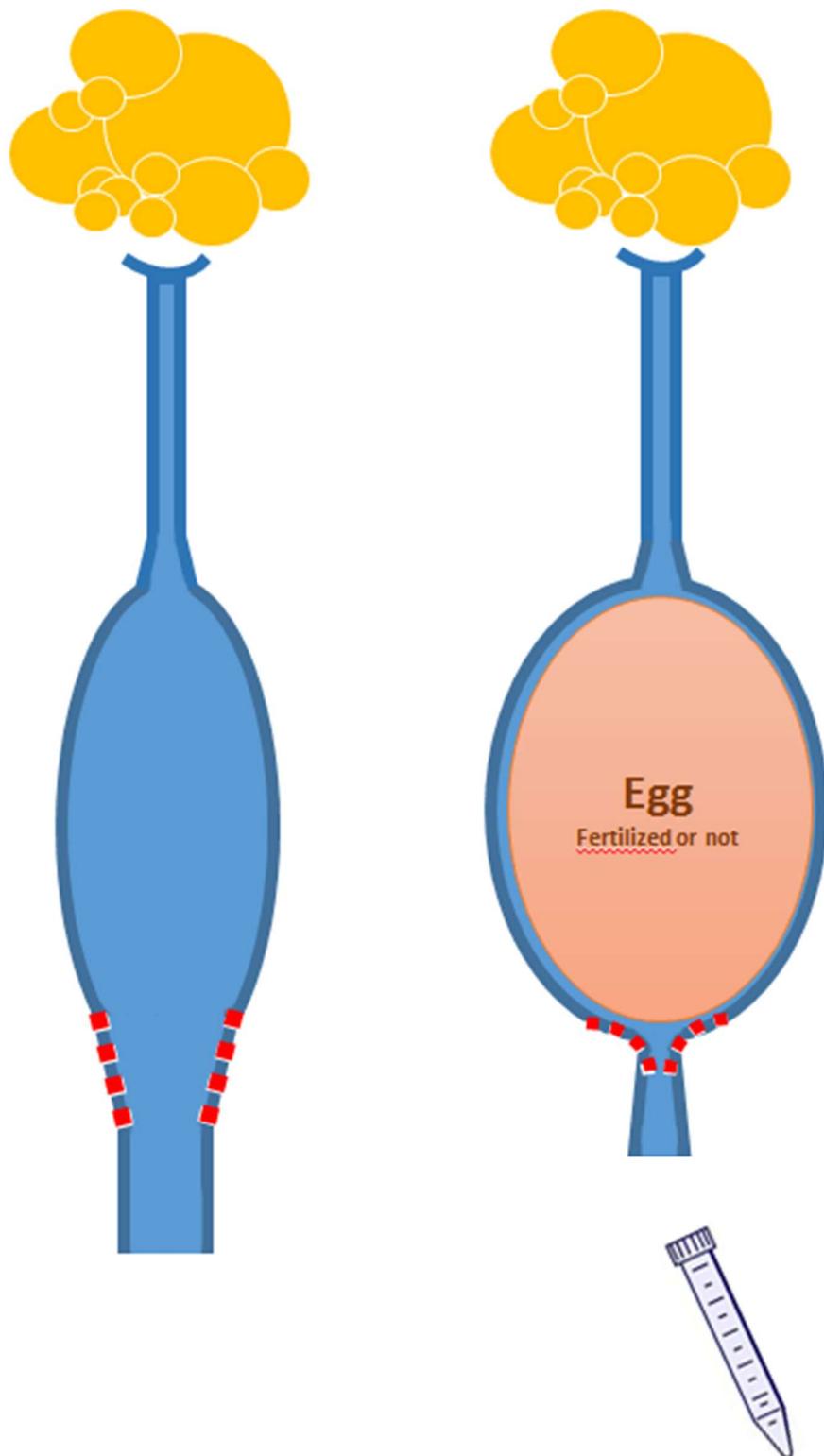
844 **Figure 5.** Transmission electron microscopy of the uterovaginal junction. (A) Exosomes  
845 (arrowheads) (< 100 nm) were observed at surface epithelium of uterovaginal junction. (B)  
846 Intracellular MVB containing exosomes (< 100 nm) were observed. (C) Blebs containing  
847 microvesicles (100-1000 nm) were present in the lumen of the SST. The figure is representative  
848 of numerous observations. Bl: Bleb; Lu: Lumen; MVB: Multivesicular Bodies.

849 **Figure 6.** Immunohistochemistry on UVJ and SST. (A) Negative controls for  
850 immunohistochemistry performed with mouse (left) and rabbit (right) immunoglobulins instead  
851 of the primary antibody. (B) VCP positive signal was observed in UVJ and SST epithelium.  
852 (C) PARK7 positive signal was present in UVJ and SST epithelium. (D) ANXA4 positive signal  
853 was observed in the apical SST epithelium and the UVJ cilia. The figure is representative of  
854 numerous observations. sst: sperm storage tubules; lu: lumen; UVJ ep: pseudostratified  
855 epithelium from uterovaginal junction.

856 **Figure 7.** Pie chart representing molecular functions of the 913 proteins from the avian UF(A)  
857 and the 640 proteins referenced in exosome databases (B). MVB: Multivesicular Bodies.

858 **Figure 8.** Venn diagram of the 913 proteins identified in UF categorized either as non-exosomal  
859 (blue, 589 proteins), or exosomal (green, 640 proteins), compared to proteins identified in  
860 seminal plasma (SP, grey, 607 proteins) and spermatozoa (SPZ, yellow, 822 proteins) from  
861 roosters. Data provided by Labas et al., (2015).

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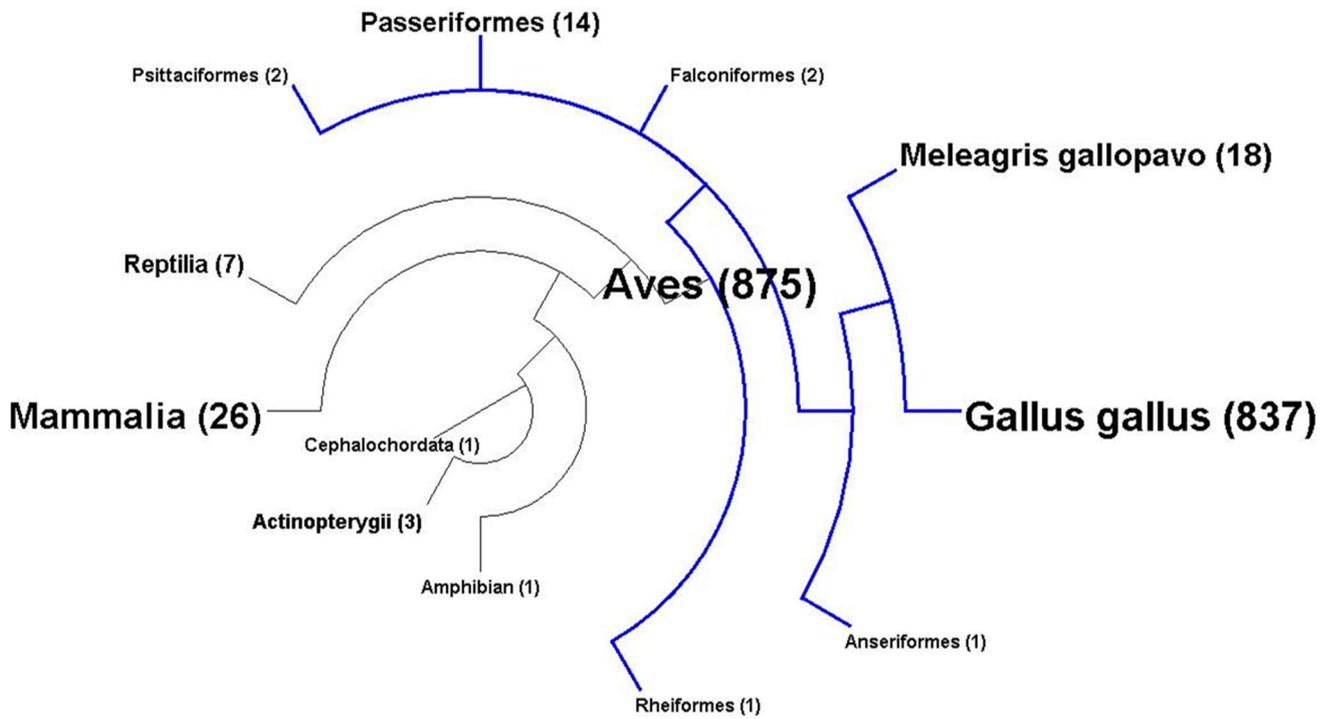


863

864 Figure 1

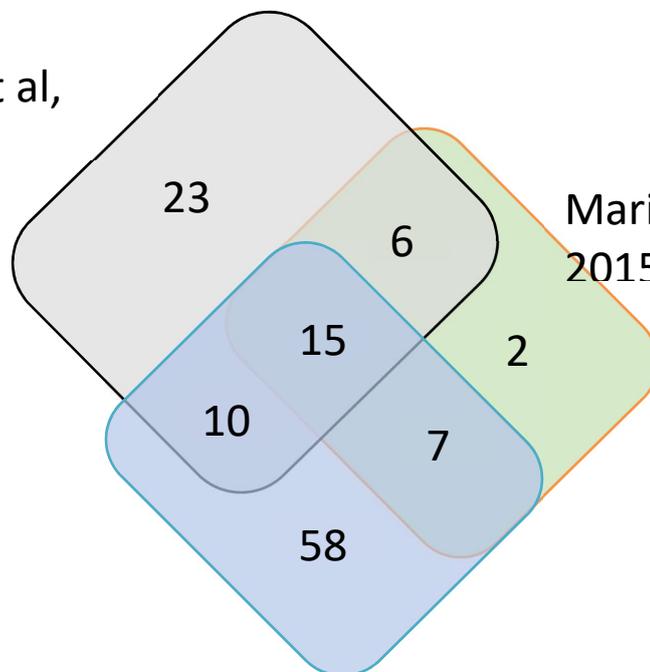
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**A**



**B**

Sun et al,  
2013



Current  
study

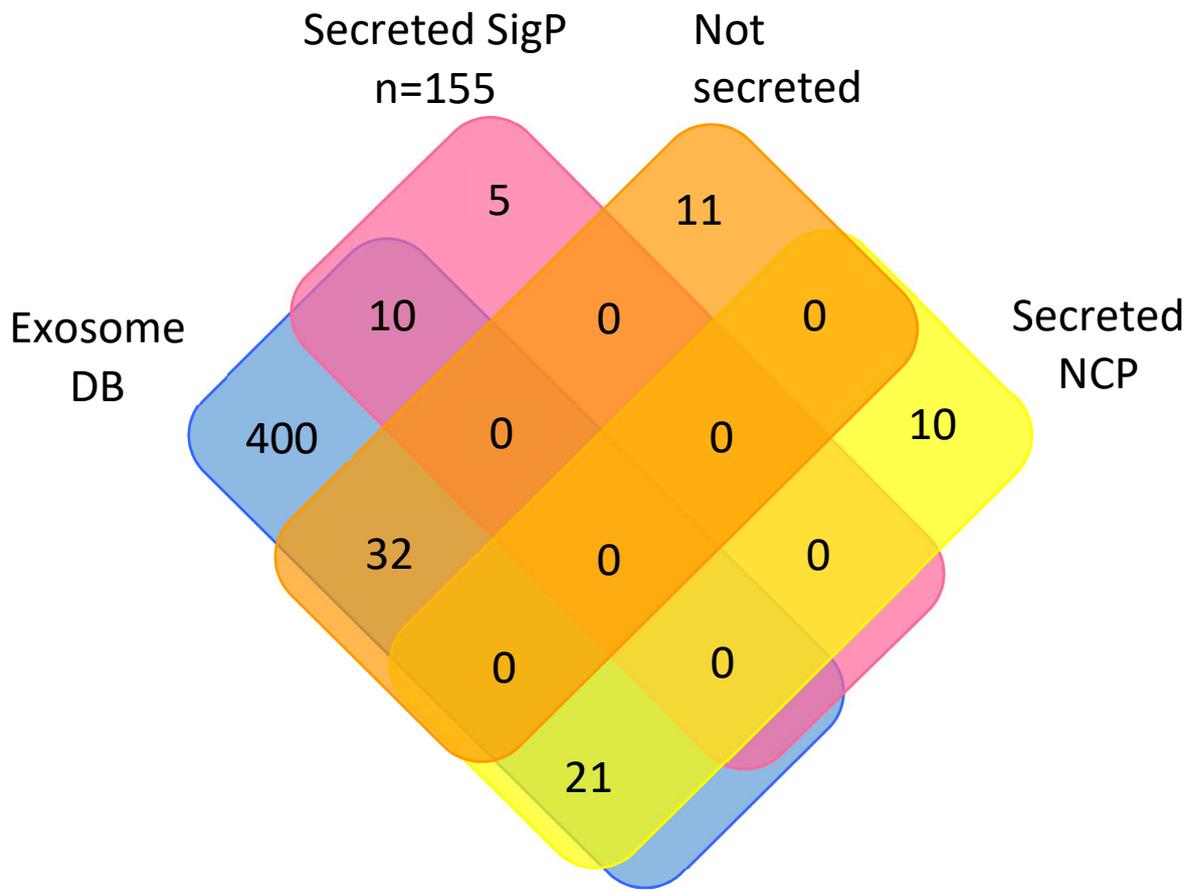
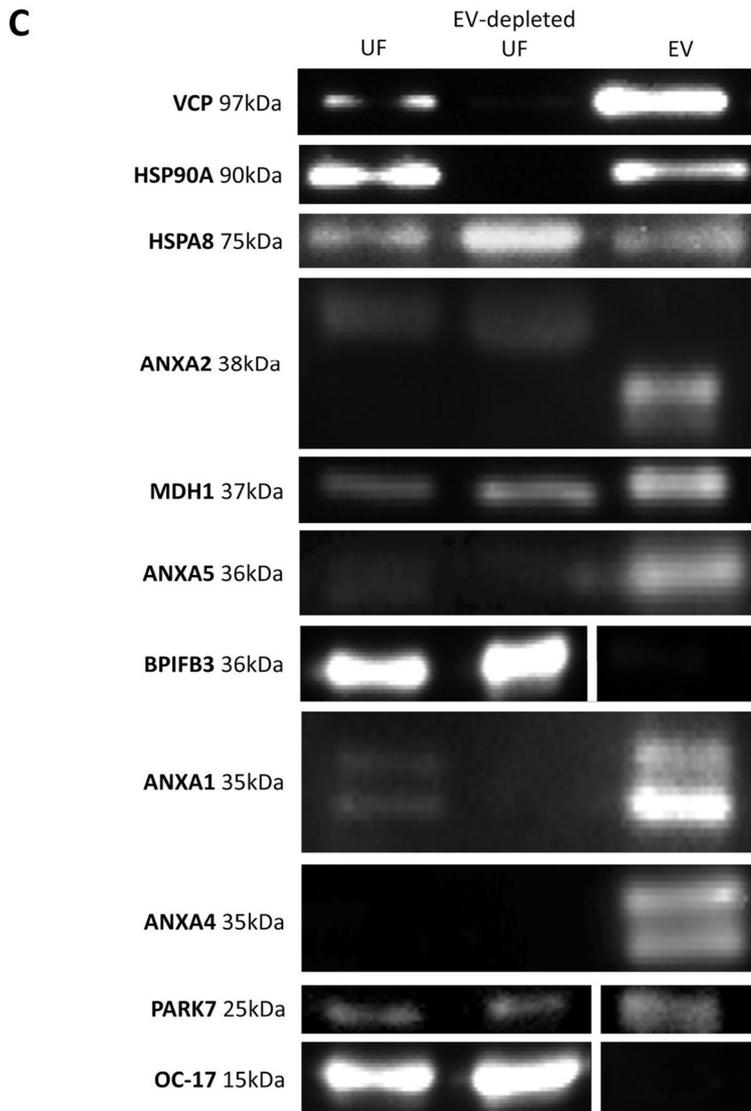
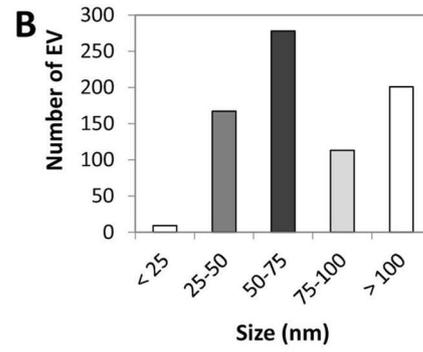
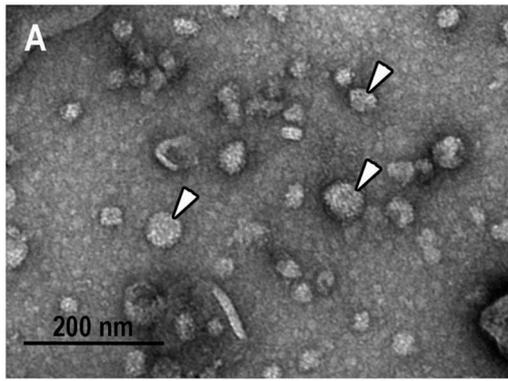


Figure 3



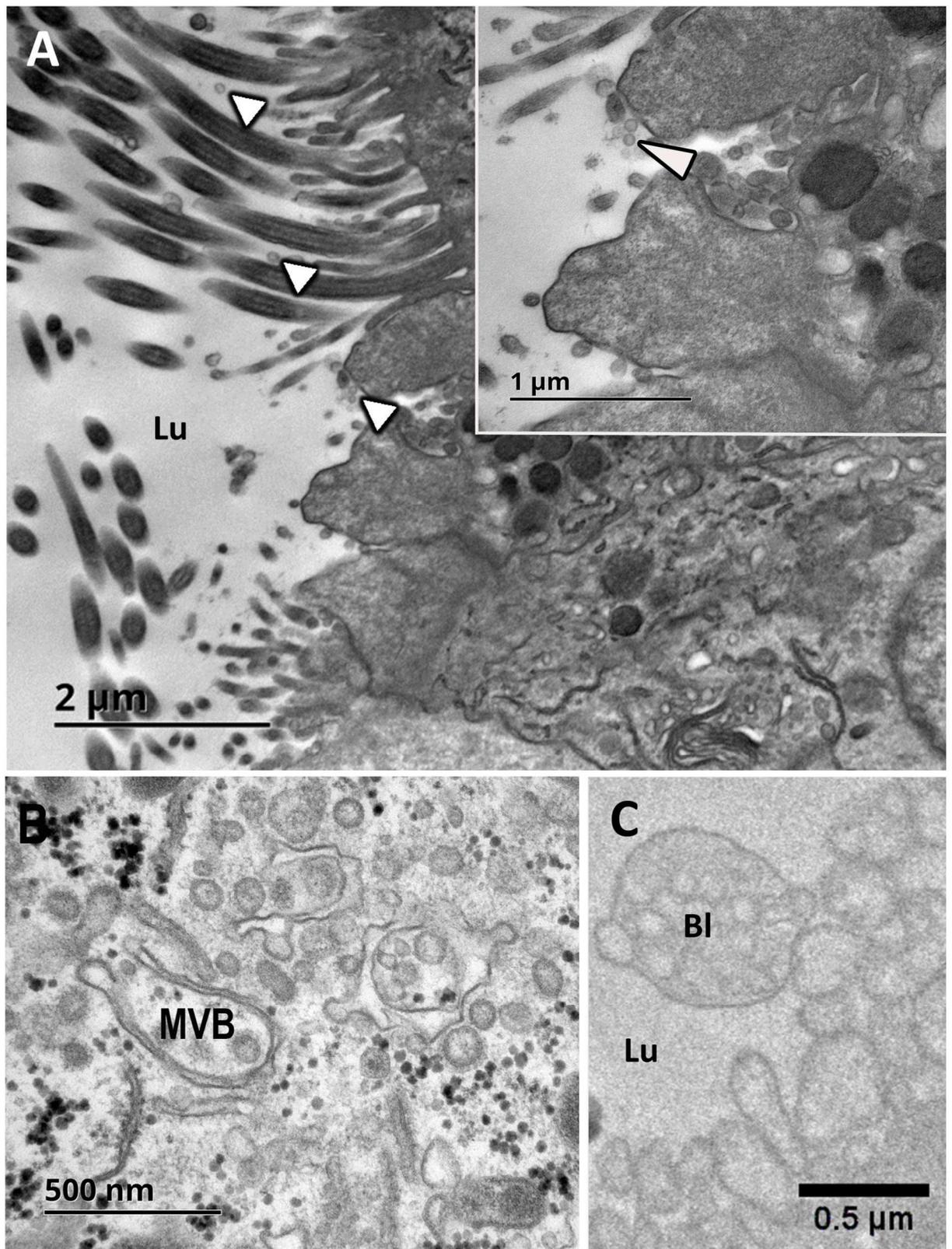
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Figure 4

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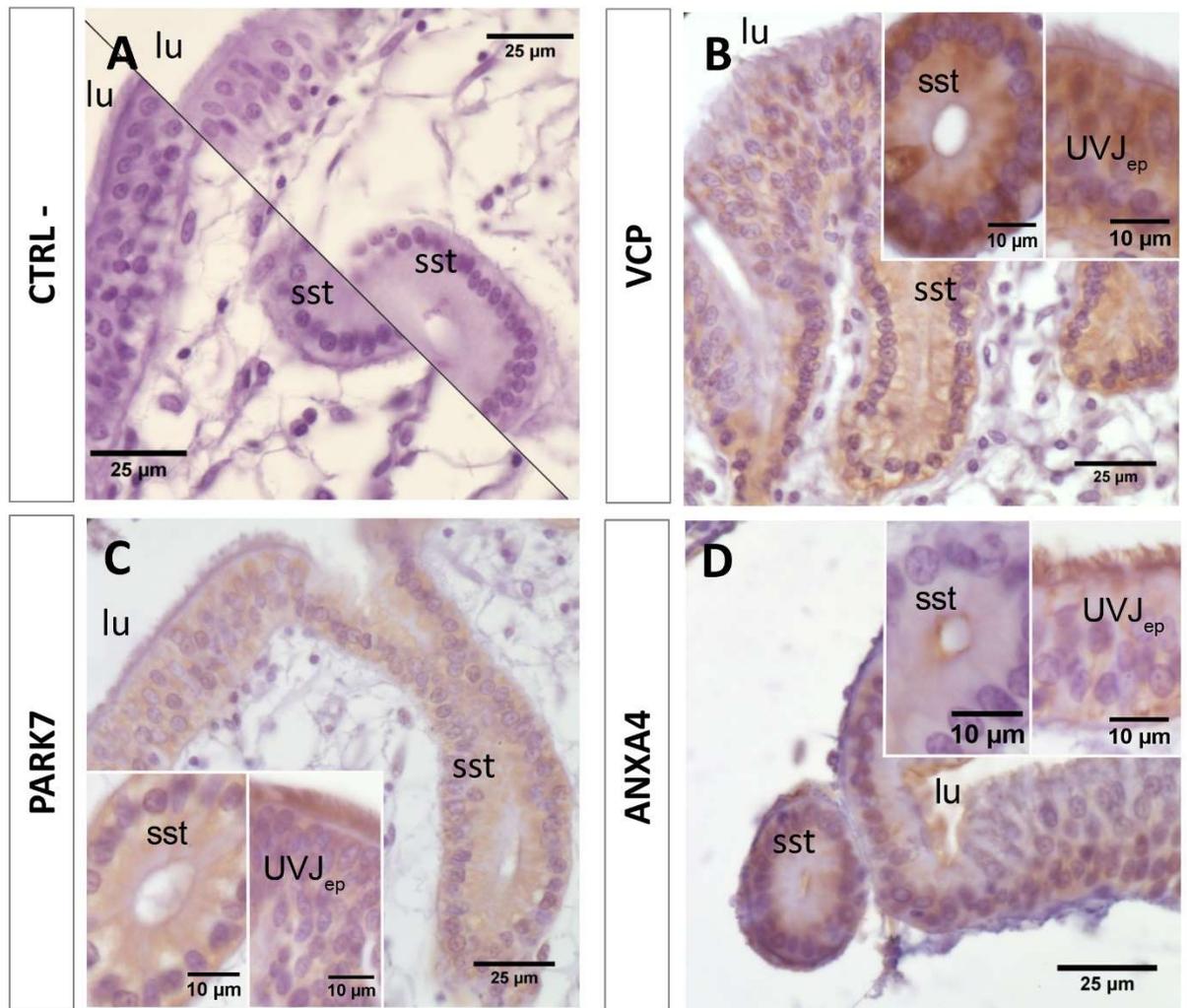


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Figure5

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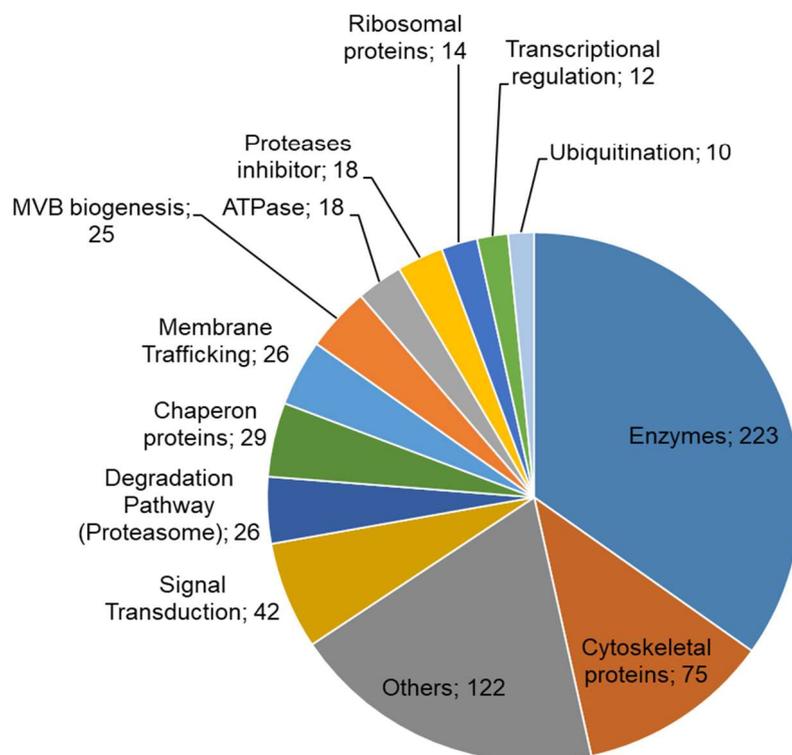
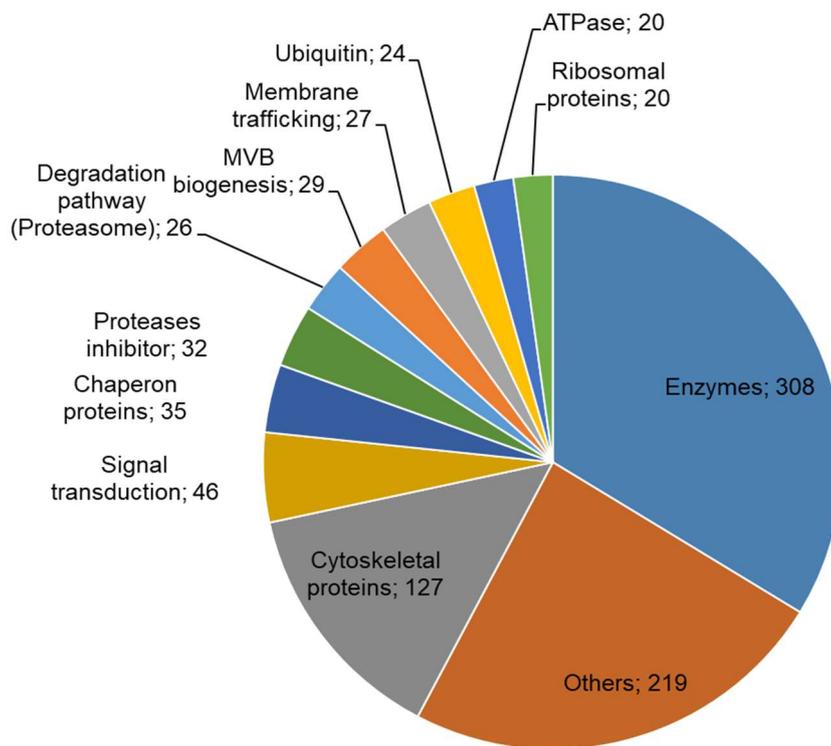


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Figure 6

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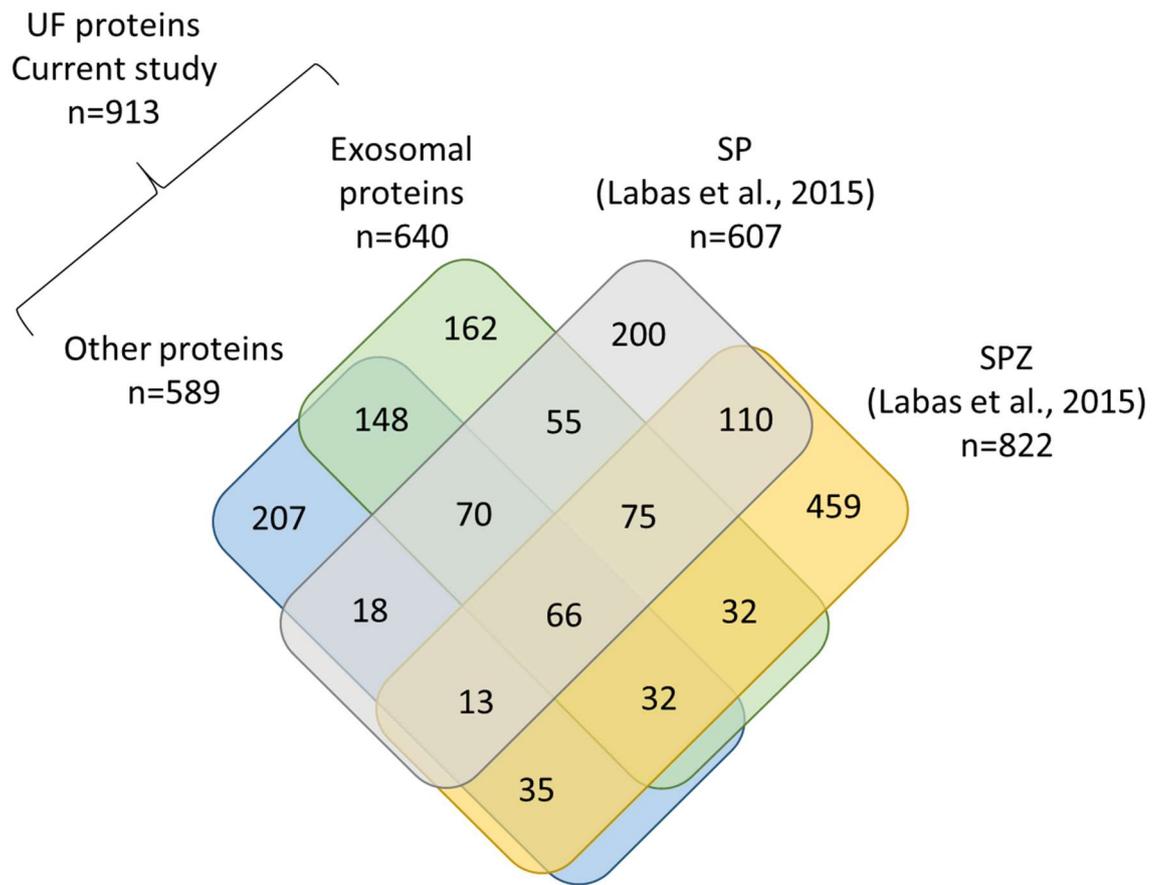
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Figure 7

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Figure 8

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