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

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Abstract

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

Introduction

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

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

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

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

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

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

Material and Methods

Birds

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

Uterine fluid collection

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

Tissue Collection

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

SDS-PAGE with fractionation

The protein concentrations of samples were determined using a Pierce™ BCA protein assay kit, Thermofisher™. For exhaustive identification, the aliquots of UF collected from 10 females and diluted in Laemmli buffer (see above) were pooled by mixing the same amount of proteins from individual samples. Fifty µg of proteins from the pool of UF were fractionated by SDS-PAGE (10%) and stained with Coomassie Blue staining (PageBlue™ Protein Staining Solution, Fermentas®). Each lane was sectioned into 30 slices, which was cut into approximately 1 mm³ pieces.

In-gel and liquid digestion

For liquid digestion 5 µg of UF sample was pooled by mixing the same amount of proteins from individual samples collected from 10 females. For in-gel digestion each gel slice was washed in water/ACN (1:1) for 5 min and in ACN for 10 min. For both digestions, cysteine reduction and alkylation were performed by successive incubations in 10 mM dithiothreitol/50 mM NH₄HCO₃ for 30 min at 56°C and 55 mM iodoacetamide/50 mM NH₄HCO₃ for 20 min at room temperature in the dark. For in-gel digestion, gel slices were washed by incubation in 50 mM NH₄HCO₃/ACN (1:1) for 10 min and by incubation in ACN for 15 min. Proteins were digested overnight in 25 mM NH₄HCO₃ with 12.5 ng/µl trypsin (Sequencing Grade, Roche, Paris). For liquid digestion, the resulting peptides solution was dried, reconstituted with 13 µL of 0.1% FA, 2% ACN, and sonicated for 10 min before MS analysis. For in-gel digestion, the resulting peptides were extracted from gel by successive incubations in 0.1% FA/ACN (1:1) for 10 min and in ACN for 5 min. The two extracts were pooled, dried, reconstituted with 13 µL of 0.1% FA, 2% ACN, and sonicated for 10 min before MS analysis.

NanoLC-MS/MS

Peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS). All experiments were performed on a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, The Netherlands) controlled by Chromeleon Software (version 6.8 SR11 ; Dionex, Amsterdam, The Netherlands). Five microliters of each sample were loaded on an LCPackings trap column (Acclaim PepMap 100 C₁₈, 100 µm inner diameter x 2cm long, 3µm particles, 100Å pores). Mobile phases consisted of (A) 0.1% FA, 97.9% water, 2% ACN (v/v/v) and (B) 0.1% FA, 15.9 % water, 84% ACN (v/v/v). Peptides were desalted and preconcentrated for 10 min at 5µL/min with 4% solvent B. The peptide separation was conducted using a LCPackings nano-column (Acclaim PepMap C₁₈, 75µm inner diameter x 50 cm long, 3µm particles, 100Å pores). The gradient consisted of 4-55% B for 90 min, 55 to 99% B for 1 min, constant 99% B 20 min and return to 4% B in 1 min. The column was re-equilibrated for 15 min at 4% B between runs. The nanoflow rate was set at 300 nl/ min. Data were acquired using Xcalibur software (version 2.1; Thermo Fisher Scientific, San Jose, CA). The instrument was operated in positive mode in data-dependent mode. Resolution in the Orbitrap was set at R = 60,000. In the scan range of m/z 300-1800, the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated (isolation width, 2 m/z; 1 microscan) and fragmented using Collision Induced Dissociation (CID). The ion selection threshold was 500 counts for MS/MS, and the maximum ion accumulation times allowed were 500 ms for full scans and 25 ms for CID-MS/MS. Target ion quantity for FT full MS was 1e6 and for MS² it was 1e4. The resulting fragment ions were scanned at the “normal scan rate” with $q = 0.25$ activation and activation time of 10 ms. Dynamic exclusion was activated for 30 s with a repeat count of 1. The lock mass was enabled for accurate mass measurements.

Protein identification and data validation

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

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

Statistical, data mining and bioinformatics analysis

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

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

EV preparation

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

Transmission electron microscopy analysis of EV and uterovaginal junction

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

Western blotting of EV proteins

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

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

Immunohistochemistry on uterovaginal tissue

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

Results

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

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

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

Analysis of uterine fluid proteins in relation to cellular localization

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

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

Analysis of EV/exosomes from UF and in SST

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

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

Immunohisto-localization of VCP, PARK7 and ANXA4 in SST

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

Analysis of uterine fluid proteins in relation to molecular functions

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

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

Analysis of uterine fluid proteins in relation to biological processes

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

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

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

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

Uterine fluid proteins which overlap with avian semen proteins

It is noteworthy that 396/913 proteins that we described in avian UF have been identified in rooster seminal plasma (143; 16%) or sperm (99; 15%), or both (154; 24%, Labas et al., 2015). Furthermore, 330/396 proteins belong to the exosome cluster, and to seminal plasma (125/143; 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, YWHAЕ, 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

Discussion

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

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

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

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

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

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

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

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

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

Proteins exhibiting antimicrobial activities potentially involved in sperm function

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

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

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

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

Exosomal proteins potentially involved in sperm survival

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

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

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

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

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

Overlapping between avian UF and semen proteins

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

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

Authors' contribution

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

Declaration of interest

604 The authors declare no conflict of interest.

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624 **References**

- 625 Ahammad, M. U., Miyazato, T., Nishino, C., Tatemoto, H., Okura, N., Okamoto, S., . . .
626 Nakada, T. (2013). Effects of Fluid Secreted from the Uterus on Duration of Fertile
627 Egg Production in Hens, and Survivability and Penetrability of Fowl Sperm in vitro.
628 *Journal of Poultry Science*, 50(1), 74-82. doi:10.2141/jpsa.0120045
- 629 Alminana, C., Corbin, E., Tsikis, G., Alcantara-Neto, A. S., Labas, V., Reynaud, K., . . .
630 Mermillod, P. (2017). Oviduct extracellular vesicles protein content and their role
631 during oviduct-embryo cross-talk. *Reproduction*, 154(3), 153-168. doi:10.1530/REP-
632 17-0054
- 633 Bakst, M. R. (2011). Physiology and endocrinology symposium: role of the oviduct in
634 maintaining sustained fertility in hens. *J Anim Sci*, 89(5), 1323-1329.
635 doi:10.2527/jas.2010-3663
- 636 Bakst, M. R., & Akuffo, V. (2009). Morphology of the turkey vagina with and without an egg
637 mass in the uterus. *Poult Sci*, 88(3), 631-635. doi:10.3382/ps.2007-00527
- 638 Bakst, M. R., & Bauchan, G. (2015). Apical blebs on sperm storage tubule epithelial cell
639 microvilli: their release and interaction with resident sperm in the turkey hen oviduct.
640 *Theriogenology*, 83(9), 1438-1444. doi:10.1016/j.theriogenology.2015.01.016
- 641 Bakst, M. R., Wishart, G. J., & Brillard, J. P. (1994). Oviductal sperm selection, transport,
642 and storage in poultry. *Poultry Science Reviews*, 5, 117-143.
- 643 Beaumont, C., Brillard, J. P., Millet, N., & De Reviers, M. (1992). Comparison of various
644 characteristics of duration of fertility in hens. *Br Poult Sci*, 33(3), 649-661.
645 doi:10.1080/00071669208417503

- 646 Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G., & Brunak, S. (2004). Feature-based
647 prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel*,
648 17(4), 349-356. doi:10.1093/protein/gzh037
- 649 Birkhead, T. R., & Moller, A. P. (1993). Sexual Selection and the Temporal Separation of
650 Reproductive Events - Sperm Storage Data from Reptiles, Birds and Mammals.
651 *Biological Journal of the Linnean Society*, 50(4), 295-311. doi:DOI 10.1111/j.1095-
652 8312.1993.tb00933.x
- 653 Blesbois, E., & Caffin, J. P. (1992). 'Serum like' albumin of fowl seminal plasma and effects
654 of albumin on fowl spermatozoa stored at 4 degrees C. *Br Poult Sci*, 33(3), 663-670.
655 doi:10.1080/00071669208417504
- 656 Breque, C., Surai, P., & Brillard, J. P. (2003). Roles of antioxidants on prolonged storage of
657 avian spermatozoa in vivo and in vitro. *Mol Reprod Dev*, 66(3), 314-323.
658 doi:10.1002/mrd.10347
- 659 Brillard, J. P., Galut, O., & Nys, Y. (1987). Possible causes of subfertility in hens following
660 insemination near the time of oviposition. *Br Poult Sci*, 28(2), 307-318.
661 doi:10.1080/00071668708416963
- 662 Burns, G., Brooks, K., Wildung, M., Navakanitworakul, R., Christenson, L. K., & Spencer, T.
663 E. (2014). Extracellular vesicles in luminal fluid of the ovine uterus. *PLoS One*, 9(3),
664 e90913. doi:10.1371/journal.pone.0090913
- 665 Camacho, C., Coulouris, G., Avagyan, V., MA, N., Papadopoulos, J., Bealer, K., & Madden,
666 T. L. (2009). BLAST+: architecture and applications. *BMC Bioinformatics*, 10(421),
667 1-9. doi:10.1186/1471-2105-10-421
- 668 Colombo, M., Raposo, G., & Thery, C. (2014). Biogenesis, secretion, and intercellular
669 interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*,
670 30(1530-8995 (Electronic)), 255-289. doi:10.1146/annurev-cellbio-101512-122326
- 671 Deutsch, E. W., Csordas, A., Sun, Z., Jarnuczak, A., Perez-Riverol, Y., Ternent, T., . . .
672 Vizcaino, J. A. (2017). The ProteomeXchange consortium in 2017: supporting the
673 cultural change in proteomics public data deposition. *Nucleic Acids Res*, 45(D1),
674 D1100-D1106. doi:10.1093/nar/gkw936
- 675 Dismuke, W. M., Klingeborn, M., & Stamer, W. D. (2016). Mechanism of Fibronectin
676 Binding to Human Trabecular Meshwork Exosomes and Its Modulation by
677 Dexamethasone. *PLoS One*, 11(10), e0165326. doi:10.1371/journal.pone.0165326
- 678 Dun, M. D., Aitken, R. J., & Nixon, B. (2012). The role of molecular chaperones in
679 spermatogenesis and the post-testicular maturation of mammalian spermatozoa. *Hum*
680 *Reprod Update*, 18(4), 420-435. doi:10.1093/humupd/dms009
- 681 Foye-Jackson, O. T., Long, J. A., Bakst, M. R., Blomberg, L. A., Akuffo, V. G., Silva, M. V.,
682 . . . McMurtry, J. P. (2011). Oviductal expression of avidin, avidin-related protein-2,
683 and progesterone receptor in turkey hens in relation to sperm storage: effects of
684 oviduct tissue type, sperm presence, and turkey line. *Poult Sci*, 90(7), 1539-1547.
685 doi:10.3382/ps.2010-01159
- 686 Froman, D. (2003). Deduction of a model for sperm storage in the oviduct of the domestic
687 fowl (*Gallus domesticus*). *Biol Reprod*, 69(1), 248-253.
688 doi:10.1095/biolreprod.102.013482
- 689 Fujihara, N., & Koga, O. (1984). Prevention of the Production of Lipid Peroxide in Rooster
690 Spermatozoa. *Animal Reproduction Science*, 7(4), 385-390. doi:Doi 10.1016/0378-
691 4320(84)90023-X
- 692 Gautron, J., Guyot, N., Brionne, A., & Réhault-Godbert, S. (2019). CHAPTER 14 Bioactive
693 Minor Egg Components. In *Eggs as Functional Foods and Nutraceuticals for Human*
694 *Health* (pp. 259-284): The Royal Society of Chemistry.

- 695 Gautron, J., Hincke, M. T., & Nys, Y. (1997). Precursor matrix proteins in the uterine fluid
696 change with stages of eggshell formation in hens. *Connect Tissue Res*, 36(3), 195-210.
697 doi:10.3109/03008209709160220
- 698 Gautron, J., Murayama, E., Vignal, A., Morisson, M., McKee, M. D., Rehault, S., . . . Hincke,
699 M. T. (2007). Cloning of ovocalyxin-36, a novel chicken eggshell protein related to
700 lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and
701 plunc family proteins. *J Biol Chem*, 282(8), 5273-5286. doi:10.1074/jbc.M610294200
- 702 Gum, E. T., Swanson, R. A., Alano, C., Liu, J., Hong, S., Weinstein, P. R., & Panter, S. S.
703 (2004). Human serum albumin and its N-terminal tetrapeptide (DAHK) block oxidant-
704 induced neuronal death. *Stroke*, 35(2), 590-595.
705 doi:10.1161/01.STR.0000110790.05859.DA
- 706 Hincke, M. T., Gautron, J., Tsang, C. P., McKee, M. D., & Nys, Y. (1999). Molecular cloning
707 and ultrastructural localization of the core protein of an eggshell matrix proteoglycan,
708 ovocleidin-116. *J Biol Chem*, 274(46), 32915-32923. Retrieved from
709 <http://www.ncbi.nlm.nih.gov/pubmed/10551857>
- 710 Hincke, M. T., Nys, Y., Gautron, J., Mann, K., Rodriguez-Navarro, A. B., & McKee, M. D.
711 (2012). The eggshell: structure, composition and mineralization. *Front Biosci*
712 *(Landmark Ed)*, 17, 1266-1280. Retrieved from
713 <http://www.ncbi.nlm.nih.gov/pubmed/22201802>
- 714 Hiyama, G., Matsuzaki, M., Mizushima, S., Dohra, H., Ikegami, K., Yoshimura, T., . . .
715 Sasanami, T. (2014). Sperm activation by heat shock protein 70 supports the migration
716 of sperm released from sperm storage tubules in Japanese quail (*Coturnix japonica*).
717 *Reproduction*, 147(2), 167-178. doi:10.1530/REP-13-0439
- 718 Holm, L., & Ridderstrale, Y. (1998). Localization of carbonic anhydrase in the sperm-storing
719 regions of the turkey and quail oviduct. *Histochem J*, 30(7), 481-488. Retrieved from
720 <http://www.ncbi.nlm.nih.gov/pubmed/10192531>
- 721 Holm, L., Ridderstrale, Y., & Knutsson, P. G. (1996). Localisation of carbonic anhydrase in
722 the sperm storing regions of the domestic hen oviduct. *Acta Anat (Basel)*, 156(4), 253-
723 260. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9078396>
- 724 Huang, A., Isobe, N., & Yoshimura, Y. (2017). Changes in localization and density of CD63-
725 positive exosome-like substances in the hen oviduct with artificial insemination and
726 their effect on sperm viability. *Theriogenology*, 101, 135-143.
727 doi:10.1016/j.theriogenology.2017.06.028
- 728 Ignatz, G. G., Cho, M. Y., & Suarez, S. S. (2007). Annexins are candidate oviductal receptors
729 for bovine sperm surface proteins and thus may serve to hold bovine sperm in the
730 oviductal reservoir. *Biol Reprod*, 77(6), 906-913. doi:10.1095/biolreprod.107.062505
- 731 Jalkanen, J., Kotimäki, M., Huhtaniemi, I., & Poutanen, M. (2006). Novel epididymal
732 protease inhibitors with Kazal or WAP family domain. *Biochem Biophys Res*
733 *Commun*, 349(1), 245-254. doi:10.1016/j.bbrc.2006.08.023
- 734 Koehler, J. K., Nudelman, E. D., & Hakomori, S. (1980). A collagen-binding protein on the
735 surface of ejaculated rabbit spermatozoa. *J Cell Biol*, 86(2), 529-536. Retrieved from
736 <http://www.ncbi.nlm.nih.gov/pubmed/6995466>
- 737 Labas, V., Grasseau, I., Cahier, K., Gargaros, A., Harichaux, G., Teixeira-Gomes, A. P., . . .
738 Blesbois, E. (2015). Qualitative and quantitative peptidomic and proteomic
739 approaches to phenotyping chicken semen. *J Proteomics*, 112, 313-335.
740 doi:10.1016/j.jprot.2014.07.024
- 741 Lee, B., Park, I., Jin, S., Choi, H., Kwon, J. T., Kim, J., . . . Cho, C. (2011). Impaired
742 spermatogenesis and fertility in mice carrying a mutation in the Spink2 gene expressed
743 predominantly in testes. *J Biol Chem*, 286(33), 29108-29117.
744 doi:10.1074/jbc.M111.244905

- Long, E. L., Sonstegard, T. S., Long, J. A., Van Tassell, C. P., & Zuelke, K. A. (2003). Serial analysis of gene expression in turkey sperm storage tubules in the presence and absence of resident sperm. *Biol Reprod*, 69(2), 469-474. doi:10.1095/biolreprod.102.015172
- Marie, P., Labas, V., Brionne, A., Harichaux, G., Hennequet-Antier, C., Nys, Y., & Gautron, J. (2015). Quantitative proteomics and bioinformatics analysis provide new insight into protein function during avian eggshell biomineralization. *J Proteomics*, 113, 178-193.
- Nakamura, M., Moriya, M., Baba, T., Michikawa, Y., Yamanobe, T., Arai, K., . . . Kobayashi, T. (1993). An endoplasmic reticulum protein, calreticulin, is transported into the acrosome of rat sperm. *Exp Cell Res*, 205(1), 101-110. doi:10.1006/excr.1993.1063
- Ng, Y. H., Rome, S., Jalabert, A., Forterre, A., Singh, H., Hincks, C. L., & Salamonsen, L. A. (2013). Endometrial Exosomes/Microvesicles in the Uterine Microenvironment: A New Paradigm for Embryo-Endometrial Cross Talk at Implantation. *PLoS One*, 8(3). doi:ARTN e58502
- 10.1371/journal.pone.0058502
- Nys, Y., Gautron, J., Garcia-Ruiz, J. M., & Hincke, M. T. (2004). Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *Comptes Rendus Palevol*, 3(6-7), 549-562. doi:10.1016/j.crpv.2004.08.002
- Park, K. H., Kim, B. J., Kang, J., Nam, T. S., Lim, J. M., Kim, H. T., . . . Kim, U. H. (2011). Ca²⁺ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. *Sci Signal*, 4(173), ra31. doi:10.1126/scisignal.2001595
- Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., . . . Vizcaino, J. A. (2019). The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res*, 47(D1), D442-D450. doi:10.1093/nar/gky1106
- Petersen, T. N., Brunak, S., von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods*, 8(10), 785-786. doi:10.1038/nmeth.1701
- Piehl, L. L., Fischman, M. L., Hellman, U., Cisale, H., & Miranda, P. V. (2013). Boar seminal plasma exosomes: effect on sperm function and protein identification by sequencing. *Theriogenology*, 79(7), 1071-1082. doi:10.1016/j.theriogenology.2013.01.028
- Raymond, A., Ensslin, M. A., & Shur, B. D. (2009). SED1/MFG-E8: a bi-motif protein that orchestrates diverse cellular interactions. *J Cell Biochem*, 106(6), 957-966. doi:10.1002/jcb.22076
- Réhault-Godbert, S., Hervé-Grépinet, V., Gautron, J., Cabau, C., Nys, Y., & Hincke, M. (2011). 9 - Molecules involved in chemical defence of the chicken egg. In *Improving the Safety and Quality of Eggs and Egg Products* (pp. 183-208): Woodhead Publishing.
- Ronquist, K. G., Ek, B., Stavreus-Evers, A., Larsson, A., & Ronquist, G. (2013). Human prostasomes express glycolytic enzymes with capacity for ATP production. *Am J Physiol Endocrinol Metab*, 304(6), E576-582. doi:10.1152/ajpendo.00511.2012
- Sasanami, T., Matsuzaki, M., Mizushima, S., & Hiyama, G. (2013). Sperm storage in the female reproductive tract in birds. *J Reprod Dev*, 59(4), 334-338. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/23965601>
- Silphaduang, U., Hincke, M. T., Nys, Y., & Mine, Y. (2006). Antimicrobial proteins in chicken reproductive system. *Biochem Biophys Res Commun*, 340(2), 648-655. doi:10.1016/j.bbrc.2005.12.054
- Slowinska, M., Bukowska, J., Hejmej, A., Bilinska, B., Kozłowski, K., Jankowski, J., & Ciereszko, A. (2015). Hepatocyte growth factor activator is a potential target

- 795 proteinase for Kazal-type inhibitor in turkey (*Meleagris gallopavo*) seminal plasma.
 796 *Theriogenology*, 84(3), 425-436 e423. doi:10.1016/j.theriogenology.2015.03.026
- 797 Sullivan, R., Saez, F., Girouard, J., & Frenette, G. (2005). Role of exosomes in sperm
 798 maturation during the transit along the male reproductive tract. *Blood Cells Mol Dis*,
 799 35(1), 1-10. doi:10.1016/j.bcmd.2005.03.005
- 800 Sun, C., Xu, G., & Yang, N. (2013). Differential label-free quantitative proteomic analysis of
 801 avian eggshell matrix and uterine fluid proteins associated with eggshell mechanical
 802 property. *Proteomics*, 13(23-24), 3523-3536. doi:10.1002/pmic.201300286
- 803 Talevi, R., & Gualtieri, R. (2001). Sulfated glycoconjugates are powerful modulators of
 804 bovine sperm adhesion and release from the oviductal epithelium in vitro. *Biol*
 805 *Reprod*, 64(2), 491-498. Retrieved from
 806 <http://www.ncbi.nlm.nih.gov/pubmed/11159351>
- 807 Talevi, R., & Gualtieri, R. (2010). Molecules involved in sperm-oviduct adhesion and release.
 808 *Theriogenology*, 73(6), 796-801. doi:10.1016/j.theriogenology.2009.07.005
- 809 Thery, C., Amigorena, S., Raposo, G., & Clayton, A. (2006). Isolation and characterization of
 810 exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*,
 811 *Chapter 3*(1934-2616 (Electronic)), Unit 3 22. doi:10.1002/0471143030.cb0322s30
- 812 Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., & Lotvall, J. O. (2007).
 813 Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of
 814 genetic exchange between cells. *Nat Cell Biol*, 9(6), 654-659. doi:10.1038/ncb1596
- 815 Wellman-Labadie, O., Lakshminarayanan, R., & Hincke, M. T. (2008). Antimicrobial
 816 properties of avian eggshell-specific C-type lectin-like proteins. *FEBS Lett*, 582(5),
 817 699-704. doi:10.1016/j.febslet.2008.01.043
- 818 Yu, B., & Huang, Z. (2015). Variations in Antioxidant Genes and Male Infertility. *Biomed*
 819 *Res Int*, 2015, 513196. doi:10.1155/2015/513196

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Legends

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

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

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

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

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

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

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

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

Figure 7. Pie chart representing molecular functions of the 913 proteins from the avian UF(A) 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).

862

863

864 Figure 1

865

A

B

Sun et al,
2013

Marie et al.,
2015

Current
study

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Figure 4

Figure5

Figure 6

Figure 7

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Figure 8