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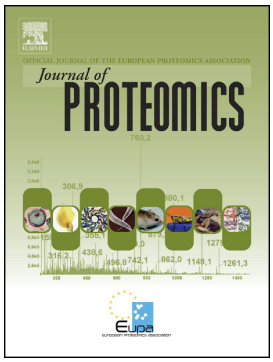

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Multifaceted roles of the egg perivitelline layer in avian reproduction: functional insights from the proteomes of chicken egg inner and outer sublayers

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ABSTRACT

The avian egg perivitelline layer (PL) is a proteinaceous structure that encloses the egg yolk. It consists of the inner and the outer perivitelline layers (IPL and OPL, respectively) that are assumed to play distinct roles in bird reproduction. To gain insight into their respective function, we analyzed the proteome of IPL and OPL in chicken unfertilized eggs after mechanical separation, using a GeLC-MS/MS strategy. Of the 412 proteins identified, 173 proteins were uniquely recovered in IPL and 98 proteins in OPL, while 141 proteins were identified in both sublayers. Genes coding the most abundant proteins were shown to be expressed either in the liver/ovary (IPL formation) or in the oviduct (OPL formation), but rarely in both. The presence of oviduct-specific proteins (including LYZ, VMO1, AvBD11,

PTN, OVAL and LOC10175704) in IPL strongly suggests that they participate in the physical association of IPL to OPL, whose tight attachment was further evidenced by analyses of IPL/OPL interfaces (by scanning electron microscopy). Functional annotation of identified proteins revealed functions associated with fertilization and early development for IPL, while OPL would rather participate in egg defense and embryogenesis. Collectively, our data highlight the complementary functions of IPL and OPL that are major determinants of bird reproductive success.

SIGNIFICANCE

The present study unveils for the first time the individual proteomes of the two sublayers composing the chicken egg perivitelline layer (PL), which allowed to assign their respective putative biological roles in avian reproduction. The combination of proteomics with gene expression and ultrastructural analyses provides insightful data on the structure and biochemistry of the avian PL. The functional annotation of PL proteins highlights the multifaceted biological functions of this structure in reproduction including fertilization, embryonic development, and antimicrobial protection. This work will stimulate further research to validate predicted functions and to compare the physiology and the functional specificities of PL in egg-laying species.

Keywords: Egg, *Gallus gallus*, chicken, inner perivitelline layer, outer perivitelline layer, proteomics, functional annotation, gene expression, ultrastructure, avian reproduction.

ABBREVIATIONS

CM	continuous membrane
ECM	extracellular matrix

GO	gene ontology
IPL	inner perivitelline layer
NSAF	Normalized Spectral Abundance Factor
OPL	outer perivitelline layer
PL	perivitelline layer
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SEM	scanning electron microscopy
TEM	transmission electron microscopy

INTRODUCTION

Vertebrate and invertebrate eggs are surrounded by extracellular coats that play pivotal roles in reproduction, such as fertilization, immune and physical protections, nutrition and development [1-3]. These egg coats consist of multiple layers with high diversity among species in terms of structure, molecular content and functions [1, 3]. In vertebrates, these structures have been classified as preovulatory and postovulatory coats depending on the time and site of synthesis [1]. The former are usually produced by the oocyte and the follicular cells during oogenesis, while the latter are deposited after fertilization by the oviductal tract and conceptus. The preovulatory coat (oocyte coat), called zona pellucida (ZP) in mammals and vitelline envelope in non-mammals, consists of a protein matrix of cross-linked glycoprotein filaments composed of ZP proteins and mediates the sperm-egg interaction prior to fertilization [4].

In birds, the ovum is surrounded by different specific egg coats, namely the perivitelline layer (also known in literature as (peri)vitelline membrane or yolk membrane), the chalaziferous layer, the albumen, the eggshell membranes and the calcified eggshell. Although the perivitelline layer (PL) should only refer to a preovulatory ZP-related coat, the avian PL is unique in that two avian-specific postovulatory coats, the continuous membrane and the outer

perivitelline layer (OPL), are closely associated with the preovulatory ZP-related coat, thereby called the inner perivitelline layer (IPL), during egg formation in the oviduct. In chicken, IPL and OPL are two fibrous proteinaceous layers separated by a very thin granular continuous membrane (Fig. 1) [5]. Facing the oolemma (oocyte cell membrane) and yolk content, the IPL has a thickness of 1.0-3.5 µm and consists of a three-dimensional network of thick fibers. Oriented towards the chalaziferous layer and the albumen, the OPL is 3.0-8.5 µm thick and comprises several superimposed sublayers, each composed of a lattice-work of thin fibrils. These structural differences are supposed to result from difference in protein content [6-8]. The chicken IPL mainly includes four glycoproteins, initially named GP-I, GP-II, GP-III [9-11] and GP-IV [8]. GP-I and GP-II likely correspond to the ZP (Zona Pellucida) glycoproteins ZP3/ZPC/gp42 and ZP1/ZPB1/gp97, respectively [12, 13]. These two major IPL constituents aggregate into helical fibrils, which further assemble into bundles to form thick fibers [14]. Other ZP glycoproteins including ZPD [15] and ZP2 [16] were also reported in the IPL. The IPL is formed in the ovary, between the granulosa cells and the oolemma during folliculogenesis [17]. Proteins composing the IPL are of various origins including the liver (ZP1, [12]), the oocyte (ZP2, [18]), the granulosa (ZP2, ZP3, ZP4 and ZPD, [16]) and possibly theca cells [16]. The IPL participates in the fertilization process via ZP proteins. The chicken OPL is mainly composed of ovomucin (MUC5B, MUC6), lysozyme C (LYZ), and the vitelline membrane outer layer proteins VMO1 and VMO2 (now referred as AvBD11) [9, 19]. Ovomucin is assumed to constitute the backbone of the OPL [6] and its disulfide bonds are essential to PL structural integrity [8]. LYZ is thought to contribute to the OPL structure via electrostatic interactions with ovomucin [6, 20] and it has been suggested in quail that AvBD11 mediates the binding of the OPL with the IPL through interactions with ZP1 and ZP3 proteins [21]. The OPL together with the continuous membrane are deposited onto the IPL of ovulated yolk by secretory cells in the upper oviduct (infundibulum) [22].

Given the antimicrobial properties of LYZ, AvBD11 and ovomucin (MUC5B, MUC6), the OPL is supposed to have an important role in the antimicrobial protection of the embryo and the yolk content [23-25]. In addition, the OPL acts as a physical barrier to prevent pathological polyspermy and subsequent embryonic death [26, 27].

Besides these major proteins, the chicken PL contains a myriad of minor components. Indeed, 137 different proteins were identified in the proteome of the whole PL [28]. However, the distribution of most of these proteins between the two sublayers remains to be elucidated. Indeed, only four proteins have been unambiguously associated with IPL (ZP1, ZP2, ZP3, ZPD) and four other proteins to OPL (Ovomucin, LYZ, VMO1, AvBD11). In addition, with the development in instrumentation, software, and methodology applied to proteomic studies during the last decade, and as mass spectrometry data intimately depend on databank updates and on the release of new genome assemblies, we suspect that the list of these 137 proteins published in 2008 may be underestimated [28]. We believe that the biological functions of the PL are probably more complex than initially described.

Thus, the objective of the study was to provide an updated and more exhaustive list of the protein composition of the whole PL, and more importantly to obtain a clear overview of the proteins that characterize IPL and OPL, respectively. We used in-depth mass spectrometry analyses combined to the functional annotation of identified proteins, we explored the expression of genes related to some proteins identified OPL and IPL in several reproductive tissues to assess their tissue specificity, and we performed histological analyses of IPL and OPL by electron microscopy. From all these data, we present an integrative view of the structural and molecular specificities of IPL and OPL. Combined results from the various approaches also highlight the tight association between the two sublayers and allowed the identification of proteins that might elicit this OPL/IPL tangle. These data provide new results that will stimulate research hypotheses to decipher the respective functions of IPL and OPL in

fertilization, in egg defense and in assisting the early stages of embryonic development in birds.

MATERIAL AND METHODS

A diagram describing the experimental design is presented in Fig. 2.

IPL and OPL sampling

OPL and IPL samples from three freshly laid unfertilized eggs of 60-week laying hens (Isa-Hendrix, St Brieuc, France) were used to analyze the PL proteins. Eggs were manually broken, albumen were delicately separated from the yolk, and OPL/IPL separation was achieved as described elsewhere [29]. The resulting IPL and OPL samples obtained from three independent eggs were individually stored in microtubes at -80°C, until use.

Solubilization and electrophoretic separation of proteins

IPL and OPL samples were freeze-dried, and one mg of each sample was mixed with 400 µL of 50 mM Tris pH 7, 500 mM NaCl, and grinded twice for 5 min at 30 Hz (Mixer Mill MM400, Retsch, Hann, Germany). Samples were treated with a 5X SDS-PAGE sample buffer (0.25 M Tris-HCl, 0.05% bromophenol blue, 50% glycerol, 5% SDS, 5% beta-mercaptoethanol, pH 6.8) in a final volume of 500 µL (2 µg/µL), and boiled for 5 min [29]. Proteins (20 µg/lane) were fractionated on an SDS polyacrylamide gel (4-20% gradient gel) and stained with Coomassie Brilliant Blue to verify the homogeneity of the OPL and IPL biological replicates (Fig. 3), prior to mass spectrometry analyses.

Mass Spectrometry Analysis

Proteins (50 µg) were briefly separated by SDS-PAGE for about 2 cm on a 10% polyacrylamide gel (Mini-Protean II, BioRad) and stained with Coomassie Brilliant Blue G250. Five protein bands (per sample) were cut out and treated by in-gel digestion with trypsin. Each slice was rinsed separately in water and then acetonitrile. Proteins were then reduced with dithiothreitol, alkylated with iodoacetamide, and incubated overnight at 37°C in 25 mM NH_4HCO_3 with 12.5 ng/µl trypsin (Sequencing grade, Roche, Paris, France), as described previously [30]. Peptides were pooled and dried using a SPD1010 speedvac system. Peptide mixtures associated to each band and in-solution samples were analyzed by nanoLC-MS/MS.

For nano-LC fragmentation, protein or peptide samples were first desalted and concentrated onto a µC18 Omix (Agilent) before analysis. The chromatography step was performed on a NanoElute (Bruker Daltonics) ultra high pressure nano flow chromatography system. Peptides were concentrated onto a C18 pepmap 100 (5mm x 300µm i.d.) precolumn (Thermo Scientific) and separated at 50°C onto an Aurora reversed phase Reprosil column (25cm x 75µm i.d.) packed with 1.6 µm C18 coated porous silica beads (Ionopticks). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1% formic acid in 99.9% acetonitrile (v/v) (B). The nanoflow rate was set at 400 nl/min, and the gradient profile was as follows: from 2 to 15% B within 60 min, followed by an increase to 25% B within 30 min and further to 37% within 10 min, followed by a washing step at 95% B and reequilibration. MS experiments were carried out on a TIMS-TOF pro mass spectrometer (Bruker Daltonics) with a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). The system was calibrated each week and mass precision was better than 1 ppm. A 1400 spray voltage with a capillary temperature of 180°C was typically employed for ionizing. MS spectra were acquired in the positive mode in the mass range from 100 to 1700 m/z. In the experiments described here, the mass spectrometer was operated in PASEF mode with exclusion of single

charged peptides. A number of 10 PASEF MS/MS scans was performed during 1.25 s from charge range 2-5.

Database searching was performed using the Mascot 2.6.1 program (Matrix Science, London, UK) with a *Gallus gallus* Uniprot database (including 19121 entries, *Gallus gallus* genome assembly GRCg6a). The sequence of the beta-microseminoprotein-like (LOC101750704 or MSMB3, gene ID: 101750704) was added manually for the search, since this egg protein is absent from *Gallus gallus* genome assembly GRCg6a (protein sequence: MKFLLVFCLILFSRTLCDARCYFRTSSKYGCISNRNLYVFGAVWKTEDCYQCKCKMN AMVCCSLVSIPKNYDRVNCVGLFHKKSCSIRVVKKTDRDISCKVYNGVG), although it was unambiguously identified in chicken egg, as demonstrated in a previous study [31] [32]. The variable modifications allowed were as follows: C-Carbamidomethyl, acetylation (K), methionine oxidation and deamidated (NQ). "Trypsin" was selected and two miscleavages were allowed. Mass accuracy was set to 10 ppm and 0.05 Da for MS and MS/MS mode, respectively.

Mascot results obtained from the target and decoy databases searches were subjected to Scaffold software (v 4.8.9, Proteome Software, Portland, USA) using the protein cluster analysis option (assemblage of proteins into clusters based on shared peptide evidence). Peptide and protein identifications were accepted if they could be established at greater than 95.0 % probability as specified by the Peptide Prophet algorithm [33] and by the Protein Prophet algorithm [34], respectively. Protein identifications were accepted if they contained at least two identified peptides. The abundance of identified proteins was estimated by calculating the NSAF (Normalized Spectral Abundance Factor) [35] using Scaffold Q+ software (version 4.8.9, Proteome Software, Portland, USA).

Quantitative values of Normalized Weighed Spectra (NWS) were then exported to PerSPECtives software for differential analysis using t-tests. Proteins were considered as

differential when p-value<0.05 and Log2(FoldChange)>1 or <-1 (fold change > 2 or <0.5, respectively) with at least 5 spectra for quantification with "Weighted Spectrum Count". Keratins were not taken into consideration in the analysis (Table S1).

Functional Annotation

The biological functions and the location associated with each identified protein were obtained using data provided by the protein database Uniprot (http://www.uniprot.org), the GO consortium (http://www.geneontology.org/), National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) and published articles. The reason for such an approach instead of using classical gene ontology tools is that chicken-specific proteins are usually poorly annotated and not referenced. Consequently, their role is highly underestimated, although they play a major physiological role. This strategy was applied recently to decipher the liver transcriptome of laying hens [36]. To achieve the functional annotation, the 412 identified proteins were first annotated according to their summarized location either “cell” (intracellular or membrane-associated proteins) or “secreted”. Each protein was then manually annotated with a generic term corresponding to different functions. The term “cell metabolism” includes proteins associated with “cell” location and involved in many functions such as synthesis, degradation or transport of biomolecules, vesicular trafficking, signal transduction, membrane-associated receptors, transporters or channels, etc. The term “cell structure” includes structural components with “cell” location involved in the maintenance of the cell shape and in cell division and motility, such as proteins of the cytoskeleton. “Regulation of development” refers to the development of the embryo and extraembryonic sacs, and includes “secreted” proteins involved in anatomical development, in angiogenesis or proteins of the ECM involved in cell adhesion and migration. “Ovarian function” includes “cell” or “secreted” proteins with known activity in follicular tissue

remodeling, vitellogenesis or hormone regulation. “Fertilization” contains extracellular actors involved in the recognition and interaction of spermatozoa to the oocyte. “Immunity” includes “secreted” components having a putative function in the antimicrobial defense of the egg and the embryo. “Stress response” encompasses “secreted” proteins involved in a stress response such as proteins with chaperone activity. “Nutrient supply” includes “secreted” proteins providing amino-acids or vitamins for the developing embryo. “Blood homeostasis” refers to “cell” or “secreted” proteins associated with blood cells or plasma, respectively. “Proteolysis and regulation” comprises proteases and antiproteases, whose function remains unclear in the context of the avian egg physiology. The remaining proteins with unknown functions are annotated with the term « unknown ».

Ethical statement, animal handling and housing

Experiments were performed in compliance with European Communities Council Directives concerning the practice for the care and use of animals for scientific purposes and the French Ministry of Agriculture on animal experimentation, and under the supervision of authorized scientists (authorization no. 7323, delivered by the “Direction Départementale de la Protection des Populations d'Indre et Loire France”). Poultry Experimental Facility, PEAT, INRAE (doi : 10.15454/1.5572326220867292E12), where the birds were housed, has an agreement to rear birds and to euthanize animals (decree no. C31-175-1 of August 28, 2012, delivered by the “Préfecture d'Indre et Loire”). The experimental protocol was accepted by the Val de Loire Ethical Committee (French National Ethics Committee for Animal Experimentation no. 19) and the French Ministry under no. 16099-015902.

Tissue samplings

Tissues were collected from 60-week-old laying hens (ISA-Hendrix). It is noteworthy that eggs used for « IPL and OPL sampling » were collected from the same animals. Hens were housed in individual furnished cages equipped with automatic devices for recording laying times. Animals were fed ad libitum (layer mash) using a commercial feed for layers. Laying hens were subjected to a cycle of 14 h of light/10 h of darkness. Animals were euthanized with Dolethal® (Vetoquinol, Magny-vernois, France) at 9-10 hours post-ovulation. Several tissues were collected: liver, granulosa cells and theca from follicles F1, F2 and F3, infundibulum, junction between infundibulum and magnum (including the upper part of the magnum), and magnum (middle part of the magnum). Tissues were immersed in liquid nitrogen immediately after sampling and were kept at −80 °C until further use.

Tissue specificity of OPL and IPL-associated genes: RNA extraction and RT-qPCR

Total RNA was extracted from frozen chicken tissues. RNAs from liver and oviduct were extracted using NucleoSpin RNA® commercial kit (Macherey-Nagel, Düren, Germany), whereas RNAs from other tissues (theca and granulosa cells) were extracted using RNA now®(Ozyme, Montigny-le-Bretonneux, France) following manufacturer's instructions. Total RNAs from each sample were treated with TURBO DNA-free kit (Invitrogen, Carlsbad, CA, USA). As two different extraction methods were used to extract the RNAs depending on the tissue, all samples were concentrated to the same final concentration to avoid bias. RNA concentration was measured at 260 nm and RNA quality was assessed using a bioanalyzer RNA 6000 Nano (Agilent Technologies, Santa Clara, CA, USA). Using equal amounts of the RNA preparation (1 µg), the first-strand cDNA was synthesized with RNase H-MMLV reverse transcriptase (Superscript II, Invitrogen) and Oligo (dT)™ primers (Invitrogen). The cDNA was stored at –20°C. Primers to detect the expression of 20 candidate genes, and six housekeeping genes (*B2M, EIF3I, GAPDH, GUSB, TBP, SDHA*) were designed using Primer-

BLAST (NCBI, https://www.ncbi.nlm.nih.gov/) and synthesized (Eurogentec, Seraing, Belgium). The sequences of each pair of primers are given in Table 1. Their efficiencies and gene expression were obtained by RT-qPCR using LightCycler® 480 SYBR Green I Master and LightCycler® 480 instrument II (Roche, Basel, Switzerland). Eight biological replicates and three technical replicates were performed for each sample (except for granulosa cells and theca cells from follicle F3, which were run on 4 biological replicates). Demineralized water was used in all reactions as a negative control. GenNorm software was used for validation of housekeeping gene stabilities. The normalized quantities of candidate genes were calculated using the following formula: gene efficiency $^{(ctcalibrator - ctsample)}$ / geometric average quantity of housekeeping genes.

Analysis of OPL and IPL by scanning and transmission electron microscopy

OPL and IPL from four freshly-laid eggs of 32-week-old hens were obtained as described in « IPL and OPL sampling » paragraph. For scanning electron microscopy (SEM), the analysis was performed on the inner surface of OPL (in contact with IPL) and on the outer surface of IPL (in contact with OPL). For transmission electron microscopy (TEM), the analysis was performed on the OPL and IPL regardless of their orientation. Each sample was placed between two filter paper eyelets and the set was attached to a cork with two pins, looking at the orientation (SEM) or not (TEM). All these assemblies were placed in a 24-well plates and fixed in 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and then washed in PBS and post-fixed by incubation with 2% osmium tetroxide for 1 h. For SEM, membranes were then fully dehydrated in a graded series of ethanol solutions and dried in hexamethyldisilazane (HMDS, Sigma-Aldrich, Saint-Quentin-Fallavier, France). Finally, samples were coated with four nm carbon, using a GATAN PECS 682 apparatus (Pleasanton, CA, USA), prior to analyses under a Zeiss Ultra plus FEG-SEM scanning-electron

microscope (Oberkochen, Germany). For TEM, samples were then fully dehydrated in a graded series of ethanol solutions and embedded in Epon resin, which was allowed to polymerize from 37°C to 60°C. Ultra-thin sections were stained with 5% uranyl acetate and 5% lead citrate, and were analyzed using a transmission electron microscope (JEOL 1011, Tokyo, Japan).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020274.

RESULTS

Proteomic analyses of IPL and OPL

After sampling PL of three different unfertilized eggs, OPL and IPL were manually separated according to the procedure described elsewhere [29] and that has been optimized to obtain structurally intact OPL and IPL, while limiting material and protein loss. As shown in Fig. 3 on three biological replicates, IPL and OPL have very distinct SDS-PAGE protein profiles, suggesting that the protein composition of the two sublayers is likely to be different.

A proteomic analysis, based on a GeLC-MS / MS strategy was conducted on the three OPL and IPL biological replicates. The resulting data set (Table S2, Table S3 and Table S4) was carefully reviewed and curated to exclude keratins (Table S1) and redundant proteins. A total of 314 and 239 proteins were identified in IPL and OPL proteomes, respectively (Fig. 4A, Table S2, Table S3) corresponding to 412 non-redundant proteins (Table S4). Among the 412 identified proteins, 98 (23.8%) and 173 (42%) are specifically found in the OPL and in the IPL respectively, while 141 (34.2%) are shared by both sublayers (Fig. 4A). A label-free

relative quantification was performed on common proteins to further appreciate potential difference in abundance in IPL and OPL proteomes. As shown in Fig. 4A, 40 out of the 141 shared proteins are differentially distributed between the two sublayers with a significant P-value ($P<0.05$). Of note, two ZP proteins, namely ZP1 and ZP3 are significantly more abundant in IPL, while a number of proteins such as mucins (MUC6, LOC107053416/MUC5AC, MUC2, LOC395381/MUC5B), beta-defensins (AvBD11, OVODB1, OvoDA1), RARRES2, PTN, OVALX, VMO1, LOC101750704/MSMB3 are more abundant in OPL (Fig. 4A, Table S5). The scatter plots in Fig. 4B shows the log10 of the Normalized Spectral Abundance Factor (NSAF) [37] of individual proteins, as a function of the log10 of the percentage of protein sequence coverage for OPL and IPL. The ten most abundant proteins in OPL and IPL (framed in red in Fig. 4B) with their quantitative values (determined as % of total NSAF within the considered proteome) are compiled in Table 2. These sets of proteins (Table 2) represent about 82% and 87% of the total protein content identified in isolated OPL and IPL proteomes, respectively. It is noteworthy that six proteins (LYZ, VMO1, AvBD11, LOC101750704/MSMB3, PTN, OVAL) are common to top ten IPL and OPL proteins, with LYZ being the most abundant protein. Nevertheless, some noticeable differences can also be observed. In particular, RARRES1/OCX32, TIMP3, OLFML3 and OVALX are only found in the top ten OPL list while ZP1, ZP3, UMOD/ZPD and SLURP1/HEP21 are only identified in the top ten IPL proteins.

Investigation of the origin of proteins identified in IPL and OPL using RT-qPCR on reproductive tissues and analysis of IPL and OPL micrographs

To determine whether the presence these abundant proteins in IPL and OPL reflects a multisite synthesis or is rather due to remaining small pieces of IPL on the OPL or vice versa, we explored the expression of the corresponding genes in various reproductive tissues.

Fourteen candidates extracted from Table 2 (UMOD/ZPD, ZP3, ZP1, TIMP3, RARRES1/OCX32, OVAL, LYZ, PTN, SLURP1/HEP21, VMO1, OLFML3, OVALX, AvBD11, LOC101750704/MSMB3) were analyzed. In addition to this list, we also included LINC00954 (abundant ZP protein detected in IPL) and several other abundant molecules assumed to have structural roles (mucins LOC107053416/MUC5AC, LOC395381/MUC5B, MUC6) and protective functions (ovodefensins OvoDA1, OVODB1) in the PL. The liver and ovary-derived tissues, namely the theca and the granulosa cell layers of the three most mature follicles (F3 to F1), were examined for their expected participation in IPL synthesis. To investigate gene expression associated with OPL formation, we analyzed the expression of the candidate genes in infundibulum (OPL synthesis) and magnum (egg white secretion), as well as in the junction between these two segments (infundibulum-magnum junction). Comparisons of quantified gene expression are shown as a heatmap diagram in Fig. 5.

The highest Z-scores in tissues responsible for IPL synthesis (liver and ovary) were observed for zona pellucida protein-coding genes (LINC00954, ZP1, UMOD/ZPD, ZP3) and for TIMP3. LINC00954 and ZP1 genes are significantly over-expressed in the liver compared to other tissues. Interestingly, ZP1 gene is also over-expressed in the theca, but with a lower magnitude than in liver. ZP3, UMOD and TIMP3 genes are significantly overexpressed in granulosa cells, with a level gradually increasing from F3 to F1 stages for ZP3 and UMOD. No or very weak expression of ZP1, ZP3, UMOD/ZPD and LINC00954 genes was detected in the oviductal segments responsible for OPL and egg white synthesis, while TIMP3 gene is significantly expressed in infundibulum. The highest Z-scores in oviductal segments were observed for LOC107053416/MUC5AC, RARRES1/OCX32, OVAL, LYZ, LOC395381/MUC5B, PTN, VMO1, SLURP1/HEP21, OvoDA1, OLFML3, OVALX, AvBD11, LOC101750704, OVODB1 and MUC6. All these genes have no or very weak expression in liver, theca and granulosa, except OLFML3 that is significantly expressed in the

theca cells. LOC107053416/MUC5AC and RARRES1/OCX32 genes are significantly over-expressed in the infundibulum. The infundibulum-magnum junction is the segment with the highest expression level for PTN, SLURP1, VMO1, OLFML3 and OvoDA1 genes. OVAL, LYZ and LOC395381 genes are significantly over-expressed both in junction and in the magnum, while OVALX, AvBD11, LOC101750704, OVODB1 and MUC6 are significantly over-expressed in the magnum.

To better understand why some proteins produced only by the oviduct that is responsible for OPL synthesis, are also found in the IPL proteome (although less abundant), we analyzed the histological structure of IPL and OPL after separation by electron microscopy. Indeed, we hypothesized that fragments of OPL may remain tightly attached to IPL during the process of sublayers separation. TEM and SEM micrographs of the structure of the inner surface of OPL and the outer surface of IPL are shown in Fig. 6. Our results demonstrate that the continuous membrane (CM) remains associated with OPL or IPL after OPL/IPL separation (Fig. 6B and 6C). Interestingly, some protein fibers of OPL can be observed on the IPL-bound CM (Fig. 6C). The presence of the CM on one or the other of the two sublayers reflects the difficulty of separating the two structures, and gives new evidence of their tight association.

Functional annotation of the OPL and IPL proteins

The molecular functions and summarized location associated with identified PL proteins were retrieved using Gene Ontology (GO) terms (available in Uniprot database) and/or literature. All identified proteins were first classified according to their location, i.e “cell” or “secreted”. As shown in Fig. 7, the combined IPL+OPL proteome, representative of the whole PL, is composed of 271 cellular and 141 secreted proteins. This initial sorting was necessary considering that the PL is essentially composed of proteinaceous fibers and that the presence of cellular protein may reflect the passive incorporation of remaining ovarian cells during the

process of ovulation or of oviductal cells during OPL formation. Thus, the biological significance of these proteins could be controversial.

Among cellular proteins, 148 and 54 are IPL and OPL-specific, respectively, while 69 are shared by IPL and OPL (Fig. 7A). Interestingly, the proportion of cellular proteins is much higher in IPL-specific proteome (>85%) than in OPL-specific (55.1%) and shared OPL/IPL (48.9%) proteomes (Table S6). The 271 cellular proteins are represented by four functional categories: cell metabolism, cell structure, ovarian function and blood homeostasis. Based on the number of proteins per term, the most representative functions associated with cellular proteins are related to cell metabolism (202 out of 271, i.e. 74.5%) and cell structure (58 out of 271, i.e. 21.4%) (Fig. 7A, Table S6). The proteins associated with these functions are mainly found in IPL-specific proteome (Fig. 7A, Table S6). Among cellular proteins, seven IPL-specific proteins are assumed to be involved in ovarian function including vitellogenesis (VLDLR/LR8, STRA6, SLC44A1, SLC5A7, LOC430303). Moreover, two cellular proteins involved in blood homeostasis are found in IPL-specific proteome (HBA1) and in both IPL and OPL layers (HBBA). These proteins are likely passive contaminants resulting from the process of yolk formation from blood precursors or from the inclusion of non-visible blood spots at the surface of yolky follicles during vitellogenesis.

Among secreted proteins, 25 and 44 are IPL and OPL-specific, respectively, while 72 are common to both sublayers (Fig. 7B, Table S6). The 141 secreted proteins have various functions related to regulation of development, ovarian function, fertilization, proteolysis and regulation, immunity, stress response, nutrient supply and blood homeostasis. “Regulation of development” is the most represented function within the secreted proteins (59 out of 141, i.e. 41.8%). Among these proteins, 15.6% (22 out of 141 proteins) refer to anatomical development (PTN, OLFML3, RARRES2…), 19.9% (28 out of 141 proteins) are extracellular matrix (ECM) proteins (FN1, COL18A1, FBN2…) and 6.4% (9 out of 141 proteins) are

involved in angiogenesis (EDIL3, VEGFA, SCUBE1, ENPP2, THSD7A, SCUBE2, ECM1, HSPG2, ANGPTL3) (Fig. 7B, Table S6). Proteins associated with anatomical development or angiogenesis are either OPL-specific or found in both IPL/OPL sublayers while the number of ECM proteins seem to be equally distributed within the two layers (Fig. 7B, Table S6). "Immunity" represents 15.6% of the number of secreted proteins (22 proteins including LYZ, AvBD11, LOC101750704/MSMB3, OVALX…), which are mostly present either in both OPL and IPL, or specifically in OPL (Fig. 7B, Table S6). The "ovarian function" accounts for 9.9% of secreted proteins (14 out of 141 proteins), including potential roles in tissue remodeling (PLAT, PRSS23, SERPINF2, SERPINE2), vitellogenesis (CTSD, APOA1, APOV1, APOB, APOD, VTG1, VTG2, VTG3) and hormone regulation (CRHBP, FST). The proteins putatively involved in tissue remodeling are mainly found in OPL while the proteins associated with vitellogenesis are found in both sublayers or equally distributed between the two sublayers (Fig. 7B). The proteins involved in "proteolysis and regulation" and "blood homeostasis" represent 7.1% and 7.8% of the secreted proteins, respectively, and are found in OPL and IPL. As shown in Fig. 7B, the proteins involved in "fertilization" are mostly present in IPL (LINC00954, ZP2, ZP3L1, ZP3L2, ZP4) or within both layers (ZP1, ZP3, UMOD/ZPD, MFGE8, OVCH1) and represent 7.1% of the number of secreted proteins (10 out of 141 proteins). Among those shared by both layers, 3 out of 5 proteins (ZP1, ZP3, UMOD/ZPD) belong to the top ten most abundant proteins of IPL (Table 2). "Stress response" and "nutrient supply" represent 1.4% (GPX3, CLU) and 2.1% (OVAL, RBP, AVD) of the secreted proteins, respectively, and are either specific to OPL or found in both layers but none is specific to IPL. It is worth mentioning that the most abundant proteins identified in isolated OPL and IPL (Table 2) are mainly associated with "immunity" (LYZ, AvBD11, LOC101750704/MSMB3, OVALX), "fertilization" (ZP1, ZP3, UMOD/ZPD), "regulation of

development" (PTN, OLFML3) or have yet "unknown" functions (VMO1, SLURP1/HEP21, RARRES1/OCX32) (Table S6).

Of note, twelve proteins have "unknown" functions (Fig. 7B), including two cellular proteins (LRRN4, FAM234A) and 10 secreted proteins (VMO1, OVALY, LOC100859777/DMBT1L3, SLURP1/HEP21, RARRES1/OCX32, MEPE/OC-116, LUZP2, INHBE, PTGDS, VH1).

DISCUSSION

The chicken egg PL, also named the vitelline membrane in literature, is known to play pivotal roles in fertilization (sperm-egg binding), in early embryonic development [38] and in the physical and antimicrobial protection of the embryo. Using proteomics, 137 proteins were previously identified in PL [28]. In the chicken and in most birds studied, PL mainly consists of two sublayers sequentially deposited during egg formation, namely the inner perivitelline layer (IPL) (preovulatory zona pellucida-related layer) and the outer perivitelline layer (OPL) (postovulatory avian-specific layer). The objective of this study was to characterize the proteins composing each sublayer using in-depth quantitative proteomics, to gain insight into their respective physiological role in avian reproduction.

The proteome of PL contains 412 non-redundant proteins

A total of 412 different proteins was identified in PL when combining IPL and OPL proteomic data. Although the number of identified proteins is much higher than previously published [28], 6 proteins identified by Mann K. (2008) were not identified in the current study: ASAH1, ENTPD8, FAM3D, HPX, NTM and WFDC2 (Table S7). Nevertheless, using less stringent conditions (731 proteins identified using a threshold of one peptide for protein

identification instead of two peptides), four of these proteins (ASAH1, ENTPD8, HPX and NTM) could be detected in one or two sample replicates, but at very low abundance.

Comparative analyses of IPL and OPL proteomes reveals protein specificities associated with each sublayer

Two-hundred and thirty-nine proteins were identified in OPL *vs.* 314 in IPL, including 141 proteins that are common to both IPL and OPL. However, of these 141 proteins, some were significantly more abundant in one or the other sublayer.

Our results reveal that LYZ, VMO1 and AvBD11 are among the three most abundant proteins in OPL, representing 35%, 14%, 10%, of total NSAF values, respectively (Table 2). These percentages are in accordance with previous studies [6, 8, 20]. In our study, beta-ovomucin (MUC6) is within the top 20 most abundant proteins (out of 239) of OPL (Table S3), accounting for 0.59% of total NSAF values, while alpha-ovomucin (LOC395381/MUC5B) is present at a very low abundance. Nevertheless, the relative amounts of these two high molecular weight glycoproteins are probably underestimated due to their high degree of glycosylation and poor solubility.

The three known major IPL proteins ZP1, ZP3 and UMOD/ZPD identified in previous studies [12, 13, 15, 39] were indeed identified in our study in the ten most abundant proteins of IPL (Table 2).

Some proteins are suspected to participate in the physical association of OPL/IPL sublayers

Strikingly, a high number of proteins (141 out of 412) including the previously cited abundant proteins (LYZ, VMO1, AvBD11, ZP1, ZP3) were identified in both layers, and six proteins including LYZ, VMO1, AvBD11, PTN, LOC101750704 and OVAL are found within the top

ten proteins of both IPL and OPL (Table 2). The presence of overlapping proteins remains questionable, as it may result from a dual synthesis by the ovary/liver and the oviduct (responsible for IPL and OPL synthesis, respectively), or from incomplete mechanical separation due to the tight association and strong adhesions between the two sublayers. The first hypothesis was investigated by exploring the expression of genes coding the top ten abundant proteins (Table 2, Fig. 5). Results showed that these genes are specifically expressed by the oviduct or the liver/ovary, except TIMP3 (expressed in infundibulum and granulosa) and to a lesser extent OLFML3 (infundibulum-magnum junction and theca). Thus, from these results, we conclude that each sublayer is characterized by specific proteins that are synthesized and secreted by distinct tissues. Thus, the detection of some proteins in both sublayers is likely due to residual protein fibers of one layer remaining attached to the other during the separation process. This second hypothesis was further corroborated by analyzing each separated sublayer by electron microscopy, where small pieces of one or the other sublayer are clearly visible (Fig. 6). We assume that the proteins identified in both sublayers contribute to the physical interaction of OPL and IPL. Interestingly, a number of abundant proteins expected to be OPL-specific but identified in both sublayers (LYZ, VMO1, AvBD11, PTN, LOC101750704, OVAL X and OLFML3), possess glycosaminoglycan-binding properties [31]. These carbohydrate-binding proteins may possibly interact with glycosylated ZP proteins (IPL-specific), as demonstrated for AvBD11, in quail [21].

Intracellular and transmembrane proteins reflect the presence of cell remnants or cell debris and are mainly associated with IPL

The PL proteome contains both extracellular secreted proteins and cell-associated proteins. However, the proportion of cell-associated proteins is unexpectedly high for an extracellular structure like PL that is supposed to essentially contain proteins. They represent 65.8%

(271/412) of the total number of identified PL proteins, reaching even 85.5% (148/173) in the IPL-specific proteome. The presence of these cellular proteins probably originates from inclusions of live/dead cells (granulosa cells, blood cells, and desquamated oviductal cells), extracellular vesicles or remnants of oocyte plasma membrane in the PL. Indeed, it was previously showed that a number of live and dead maternal cells (granulosa cells, more than 3.2×10^5 per PL) are detected within the PL of laid unfertilized eggs [40]. An evidence for the presence of granulosa cells in our sample is provided by the identification of HSD3B1, which is produced by granulosa cells in preovulatory follicles and that is key enzyme of progesterone biosynthesis [41]. Blood or meat spots can also be found on or included in the PL as a result of a hemorrhage of small blood vessels or a desquamation of small pieces of tissue in the ovary during ovulation, or in the oviduct. Although we carefully verified that PL samples were devoid of large blood spots, traces (invisible to the naked eye) of blood clots or epithelial desquamations might still be present in/on the PL. The identification of components associated with "blood homeostasis", especially hemoglobin (HBA1, HBBA), indeed suggests the presence of blood derived molecules in PL. At last, the identification of VLDLR/LR8 receptor strongly supports that parts of the oocyte plasma membrane remained attached to PL. This receptor plays a crucial role in vitellogenesis during follicular development as it mediates the endocytosis of yolk components [42]. Taken together, these elements suggest that a number of these cell-associated proteins have no significant biological role within the PL but may rather reflects passive association of blood/cell-derived components during the process of ovulation and PL formation. Therefore, these cellular proteins will not be further discussed.

The major functions associated with secreted proteins of PL encompass immunity, fertilization, and regulation of embryo development

Functional annotation of IPL and OPL proteomes resulted in the classification of proteins into 9 functional terms, including “immunity”, “fertilization”, “regulation of development”, “stress response”, “nutrient supply”, “proteolysis and regulation”, “ovarian function”, “blood homeostasis”, and “unknown” function. With 412 proteins identified from the combination of OPL and IPL proteomic data enabled us to propose an exhaustive list of proteins composing the whole PL. Based on the number and relative amounts of related proteins, the three former functions (“immunity”, “fertilization”, “regulation of development”) have undoubtedly leading functions in PL compared to the others. “Ovarian function” (vitellogenesis, hormone regulation, tissue remodeling associated with follicular maturation and rupture) and “blood homeostasis” are functions identified for both cell-associated and secreted proteins. Regarding “ovarian function”, PL contains a number of major yolk proteins associated with vitellogenesis (APOA1, APOV1, APOB, APOD, VTG1, VTG2, VTG3) that probably result from the presence of yolk traces. However, we assumed that these proteins may also participate in the establishment of IPL ultrastructure. Except VTG3, all these proteins were previously identified in the PL proteome [28]. PL also contains proteins associated with hormone regulation (FST, CRIBP) or tissue remodeling (PLAT, PRSS23, SERPINE2, SERPINF2) that presumably derive from the ovary and that are inserted in the PL during folliculogenesis, or following follicular rupture during ovulation. The identification of proteins involved in “blood homeostasis” such as serum albumin (ALB), coagulation factor X (F10), complement system components (C3, C4A, C8G, C4BPA) and fibrinogen (FGB, FGG) confirms the presence of blood-derived proteins, as discussed above. PL contains proteins associated with “nutrient supply”, being involved in amino-acids supply (OVAL, that is essentially synthesized in the infundibulum-magnum junction and magnum, Fig. 3) or in vitamin transport and storage (AVD, RBP) for the developing embryo [39]. It remains unclear whether these proteins have an effective role in PL or whether they derive from the egg white,

which is in contact with OPL. Two proteins involved in "stress response" were identified in PL: CLU (clusterin) is a chaperone preventing stress-induced protein aggregation, while GPX3 (glutathione peroxidase 3) protects cells and enzymes from oxidative stress during their synthesis by reproductive tissues. Two proteases (MMP1, CTSEAL) and many antiproteases (CST3, SPINK7, A2ML4, CPAMD8, OVST, TIMP3, SPINK5, A2ML1…) have been identified. However, for most of them, their precise physiological function remains to be elucidated since they can potentially regulate many biological processes during folliculogenesis/ovulation or embryonic development. At last, it is noteworthy that the PL contains several proteins assigned to "unknown" function including some highly abundant molecules like VMO1, SLURP1/HEP21 and RARRES1/CCX32, whose genes are expressed in the upper oviduct but not in the liver or ovary.

OPL-proteome is characterized by a high number of abundant antimicrobials

PL is the last structure protecting the yolk from invading pathogens such as *Salmonella* and the first structure being modified in fertilized eggs during incubation [43]. The protective function of PL is attested by the diversity of proteins of high abundance involved in "immunity" essentially in both OPL and IPL, or specifically in OPL. This group includes antimicrobials, mucins and immunoglobulin-related proteins. The most abundant antimicrobials in our proteomic study are LYZ [43, 44], AvBD11 [24, 45], LOC101750704/MSMB3 [31, 32] and OVALX [46]. These proteins are among the top ten proteins in PL, OPL and IPL proteomes. In addition to AvBD11, two other members of the defensin family are also present in OPL but at a lower abundance, the ovodefensins OvoDA1 and OVODB1, for which antibacterial activities have previously been reported [47, 48]. Although present in IPL proteome, all these antibacterial compounds are specifically deposited in OPL by specialized segments of the oviduct (infundibulum, infundibulum-

magnum junction, magnum) while no or very weak expression of their corresponding gene is detected in liver nor in follicular tissues (Fig. 3). As mentioned above, the presence of these compounds in IPL proteome probably means that they are in the vicinity of IPL or might be involved in OPL/IPL association. Although some antibacterial properties were demonstrated for VMO1 and PTN [31], these two proteins were not classified in "immunity" as we believe that their primary function is rather related to "unknown" or "regulation of development", respectively. Other less abundant antibacterial proteins are also present in the OPL, such as TF, EXFABP [49, 50], the C-type lectin OC-17 [51], and several members of the LBP/BPI/Plunc family (LBP/BPI, BPIFB3/OCX36, BPIFB2/TENP, BPIFCB) [52]. The OPL contains four secreted gel-forming mucin proteins (LOC107053416/MUC5AC, MUC6, LOC395381/MUC5B, MUC2) (Table S3) that are high-molecular weight glycoproteins with important role in immunity, through their ability to form gel-like mucus and to bind a variety of microbes [53]. Ovomucin, which is composed of the two subunits alpha (MUC5B) and beta (MUC6), is known for its role in the gel-like properties of egg white and possesses antiviral and antibacterial activities [54]. The viscosity provided by MUC5B, MUC6, MUC5AC, MUC2 might trap bacteria and inhibit their motility towards the egg yolk and the developing embryo. Similarly to the major antibacterial proteins and peptides described above (LYZ, AvBD11, LOC101750704/MSMB3, OVALX, OvoDA1, OVODB1), ovomucin subunits MUC5B and MUC6, and MUC5AC are also predominantly synthesized in the upper oviduct and secreted in OPL (Fig. 5), which strengthens the function of OPL as an antimicrobial barrier. The OPL also contains immunoglobulins, immunoglobulin-like and immunoglobulin-binding proteins (VH26L10, IGLL1, Ig Mu chain region, JCHAIN) that might participate in the recognition of microbes. Nevertheless, their exact immune roles within the PL remains to be clarified.

The IPL proteome is characterized by proteins involved in fertilization

The IPL proteome contains a number of proteins involved in the structural organization of IPL, as well as in sperm-egg binding and in the induction of acrosome reaction (initial steps in egg fertilization) or in the regulation of these events. Among the nine ZP-coding genes identified in the chicken genome [55], eight ZP proteins were found in IPL proteome: ZP1, ZP3, UMOD/ZPD, LINC00954 (ZPAX2/LOC421956), ZP2, ZP4, ZP3L1 (LOC415305) and ZP3L2 (LOC415314). The gene coding for ZPAX1/LOC421952, which has not been identified in our study nor in Mann's study [28], is mostly expressed in early developmental stages of oocyte similarly to ZP2 and ZP4 [56], suggesting that it might be present at trace levels in PL. The three most abundant ZP proteins (ZP1, ZP3, UMOD/ZPD) form the structural scaffold of IPL [14]. Although detected in OPL proteome, these three compounds are primarily deposited in IPL as their gene expression is observed in the liver or in the ovary, but not in the upper oviduct (Fig. 5). In accordance with previous studies [36, 57], we found that ZP1 and LINC00954 genes are mainly expressed in the liver. In contrast, ZP2, ZP3, ZP4, UMOD/ZPD genes are expressed by the oocyte or the granulosa cells or both [56]. Besides their structural role, ZP proteins seem to have pivotal functions during fertilization by binding to the tip of sperm head (ZP1 and ZP3) [58] or by inducing acrosome reaction of sperm cells (ZP1, UMOD/ZPD) [15]. MFGE8 and OVCH1 that are mainly produced by the liver [36] and that we identified in OPL and IPL proteomes, might also be involved in fertilization as previously reported [59-61].

In addition, although OPL does not contain as many proteins involved in fertilization compared to IPL, and considering that OPL is deposited on IPL after ovulation and fertilization, OPL is likely to have a regulatory role in fertilization by trapping sperm cells in excess and decreasing their mobility. Although polyspermy occurs in birds, OPL is thought to limit abnormal polyspermy [27].

Some OPL and IPL proteins are predicted to orchestrate cell processes associated with the early stages of embryonic development

The rapid growth and functionalization of the yolk sac during the early phase of incubation involves the orchestration of important biological processes, such as cell adhesion, migration, proliferation, and differentiation. The inner surface of PL functions as a cell substratum for the adhesion and expansion of the chick blastoderm during the initial four days of incubation. It spreads from animal to vegetal pole to form the vascularized yolk sac responsible for forthcoming uptake and digestion of the yolk nutrients required for the embryo. The adhesion to the inner surface, which is required for blastoderm expansion, is mediated by cells of the ectoderm located at the edge of blastoderm [62, 63]. Interestingly, no expansion occurs when the blastoderm is cultured on the outer surface of PL, and adhering cells migrate across the PL [64], which suggests that the protein composition of the IPL is specifically adapted to the adhesion, migration and proliferation of the chick blastoderm. The cell adhesion properties of PL are attested by the identification of numerous ECM proteins involved in cell attachment and that may regulate blastoderm expansion potentially through the presence of RGD motifs (fibronectin, vitronectin, laminins, collagens, fibrillins, fibulins, thrombospondins and tenascins). Interestingly, the major IPL component ZP3 also contains an RGD motif in its N-terminal extremity; however, to our knowledge, its role in the cell adhesion and migration of blastoderm has never been investigated.

Cell proliferation and differentiation during embryonic development are known to be regulated by specific signaling pathways including Wnt, TGF-beta family, Notch and Hedgehog [65-68]. Both OPL and IPL contain potential modulators of TGF-beta family ligands, such as LTBP1 and fibrillins (FBN1, FBN2, FBN3), FST, OLFML3/ONT1, fibulins (FBLN2, FBLN5), heparan sulfate proteoglycans (HSPG2, Agrin), alpha-2-macroglobulin

(A2ML1, A2ML4), collagens (COL4A3, COL4A5, COL4A6, COL18A1) and TNX [69]. Several other proteins previously described to play a role in embryogenesis were specifically identified in OPL. These include antagonists of Wnt signaling (FRZB, LOC395991/crescent, and possibly DKK3) [70-72], and regulators of Hedgehog (SCUBE2) [65] and Notch1 (CCN3/NOV) [73, 74]. Besides the modulators of these important signaling pathways, OPL also contains growth factors involved in cell proliferation and migration, such as PTN [75] and VEGFA [76], and several proteins reported to be involved in angiogenesis or in its regulation (VEGFA, PTN, EDIL3, HSPG2, ANGPTL3, ENPP2, TMSD7A, SCUBE1, SCUBE2, ECM1, CCN3/NOV). OPL contains also an important antiapoptotic factor, TNFRSF6B (OPL-specific, identified in the top 30 abundant proteins [77]).

PL encloses a number of proteins potentially involved in morphogenesis and organogenesis during embryonic development. Among the major proteins of PL is OLFML3 (top ten, Table 1), which may have a role in the dorsoventral patterning during early development [78]. Although it is detected in both OPL and IPL, the analysis of tissue distribution revealed that OLFML3 gene is almost exclusively expressed in the upper oviduct, while a very weak expression is observed in theca (Fig. 5). These data suggest that OLFML3 is mainly deposited in OPL. Intriguingly, 10 proteins identified in both OPL/IPL (PTN, SLIT2) or specifically in OPL (CRTAC1, HAPLN2, NTN1, NTN3, NRG1, OLFM3, SEMA3B, SEMA3C) potentially regulates neural development, while NPNT (OPL-specific) has potential roles in kidney development (Table S6). Knowing that all these PL constituents are exogenous to the developing embryo and extra-embryonic tissues, their mode of action remains unclear.

PL is progressively degraded during yolk sac development [79]. Signs of IPL digestion that usually accompany cell migration, are particularly visible in the IPL in the so-called area vasculosa, and the existence of a “hatching enzyme” produced by yolk sac ectoderm possibly at the area vitellina was suggested by Yoshizaki et al.[80]. A tight control of this degradation

process is likely to be ensured by the significant number of PL proteases and antiproteases. We hypothesize that these proteins may regulate the proteolytic degradation of ECM proteins and the potential release of anti-angiogenic peptides (from COL18A1 (endostatin), COL4A3 (tumstatin) and HSPG2 (endorepellin), for example [81-83].

CONCLUSIONS

To our knowledge, this work is the most integrative study on the protein composition of chicken PL and its respective IPL and OPL sublayers. It combines proteomics, functional annotation, structural and expressional studies while integrating knowledge on avian physiology. This study gives significant insights on the structure and biochemistry of the avian PL, and highlights the multifaceted biological functions of this structure in reproduction including fertilization, early embryonic development, and antimicrobial protection (Fig. 8). Regarding the perspectives of this work, it would be interesting to further assess the role of these proteins in the histological structure of both sublayers (fibers organization), in their physical association and to investigate their modification and activity during the first stages of embryonic development. Comparisons with PL of other birds where differences in structure can be observed [7, 84] will help to better appreciate the specificities of this peculiar structure that has acquired very original features during evolution, to accompany bird speciation.

Table 1. Primers of house-keeping and candidate genes. F, forward; R, reverse.

Gene	Gene ID	Primers
EIF3I	419653	F-5'GACATGTGCTCACTGGCTCT-3' R-5'-CACTGCTGAGCTGGTCTTCA-3'
TBP	395995	F-5'-GCGTTTTGCTGCTGTTATTATGAG-3'
		R-5'-TCCTTGCTGCCAGTCTGGAC-3'
GUSB	427823	F-5'-TGTGATTGGGGAACTCATCTGG-3'
		R-5'-AAGTTCAGCATAGTACCCAGC-3'
B2M	414830	F-5'-GATCCCGAGTTCTGAGCTGT-3'
		R-5'-GCTTGCTCTTTGCCGTCATAC-3'
SDHA	395758	F-5'-AGATACGGGAAGGAAGGGGT-3'
		R-5'-ACCGTAGGCAAAACGGGAAT-3'

GAPDH	374193	F-5'-AGGCGAGATGGTGAAAGTCGGAGT-3'
		R-5'-TGCCCTTGAAGTGTCCGTGTGT-3'
LYZ	396218	F-5'-GACATAACAGCGAGCGTGAA-3'
		R-5'-GGCGTTTGCGTATAGTCGTT-3'
PTN	418125	F-5'-TGAGTACCACTGAGGCTGGA-3'
		R-5'-ACACTCTGCTCCAAATTGCTTC-3'
VMO1	418974	F-5'-GATTTGCACTGAAGGTTGAGC-3'
		R-5'-AACTGGATGTTGTTGGCAGC-3'
AvBD11	414876	F-5'-GAAAACCTGCTGCGTAGACACT-3'
		R-5'-AAGTCCCAGCTGTTCTTCCAG-3'
ZP1	395418	F-5'-AGTACCATTACGACTGCGGG-3'
		R-5'-TAGCGGCCATCCTTCACCAA-3'
ZP3	378906	F-5'-CCAGACCCTCAGAACAAGGC-3'
		R-5'-CAACCTCTTTCCCGGCATCA-3'
UMOD	404754	F-5'-ACAAGAGTGAGCTGGTAAGCC-3'
		R-5'-CAGTACCACATCTTGGCAGAA-3'
LOC101750704	101750704	F-5'-TTCTCTAGGACCCTGTGCGAT-3'
		R-5'-GTCTTCCACACTGCACCAAA-3'
OVAL	396058	F-5'-AAGACAGCACCAGGACACAGA-3'
		R-5'-TTCTGGCAGATTGGGTATC-3'
OVALX	420898	F-5'-TCCGTGAACATCCACCTACTCT-3'
		R-5'-GGCTTGGTCTGATGCTGTTT-3'
OvoDA1	422030	F-5'-CTCCAGCCTCGCTCACAC-3'
		R-5'-TTGAGAGGAGGGGATGAGAC-3'
SLURP1	395192	F-5'-TTGTCCTGTGCGTTGGAGG-3'
		R-5'-TTCCCGAGGGAGGTTTGATG-3'
LINC00954	421956	F-5'-ACGGCGCAATCTCTGTTGTA-3'
		R-5'-AGTGGCAACTGCTCTGTACC-3'
TIMP3	396483	F-5'-TCCGTGCTAAAGTTCTGGGG-3'
		R-5'-GGCCAGTGTAAACCTTCCCT-3'
OVODB1	106780806	F-5'-GGGTATAGGAAGCGCAAGGG-3'
		R-5'-GGGAGAAGGCAGCAGTACAT-3'
MUC6	414878	F-5'-CAACATTCAGTTCCGCCGTG-3'
		R-5'-TGCTCCCATAAGGTGCTATT-3'
LOC395381	395381	F-5'-GTGCACGTCTCCACTGTTGG-3'
		R-5'-AGACGTATGTGCAGGTTCCG-3'
LOC107053416	107053416	F-5'-ATGTGGCACAACAGGAACCA-3'
		R-5'-TGCCCATGGAACGGATTTGA-3'
OLFML3	419882	F-5'-TCCCTCGTGTACTTCCCCAA-3'
		R-5'-ATGATCTGGTAGCCGTCGTC-3'
RARRES1	395209	F-5'-CACAGCAAATGAACGTCTGTCA-3'
		R-5'-GGCCTGTTACACAAAGGTTTCC-3'

Table 2. Top ten abundant proteins found in OPL and IPL using GeLC-MS / MS approach. Quantitative values are expressed as the % NSAF of the total OPL or IPL protein content. The asterisk (*) indicates that the corresponding proteins are not in the top ten abundant proteins.

Gene Symbol	**Protein name**	**Gene ID**	**OPL**		**IPL**	
			%NSAF	**Rank**	**%NSAF**	**Rank**
LYZ	lysozyme	396218	35.39	1	32.54	1
VMO1	vitelline membrane outer layer 1	418974	14.06	2	4.29	5

AvBD11	avian beta-defensin 11	414876	10.42	3	3.75	6
PTN	pleiotrophin	418125	5.82	4	1.62	8
LOC101750704	beta-microseminoprotein-like (MSMB3 protein)	101750704	4.85	5	1.59	9
OLFML3	olfactomedin like 3	419882	3.94	6	*	*
RARRES1	retinoic acid receptor responder 1 (ovocalyxin-32)	395209	2.00	7	*	*
OVAL	ovalbumin	396058	1.99	8	1.91	7
TIMP3	TIMP metallopeptidase inhibitor 3	396483	1.95	9	*	*
OVALX	ovalbumin-related protein X	420898	1.57	10	*	*
ZP3	zona pellucida sperm-dinding protein 3	378906	*	*	23.76	2
SLURP1	secreted LY6/PLAUR domain containing 1 (Hep21 protein)	395192	*	*	8.67	3
ZP1	zona pellucida sperm-binding protein 1	395418	*	*	7.57	4
UMOD	Uromodulin (zona pellucida protein D)	404754	*	*	1.24	10

Figure 1. Ultrastructure of chicken OPL and IPL from freshly laid eggs.

Transverse section through the perivitelline layer (PL) of a freshly laid egg observed by transmission electron microscopy. The PL consists of two distinct fibrous proteinaceous layers, the inner and outer perivitelline layers (IPL and OPL, respectively), separated by a thin continuous membrane (black arrowhead).

Figure 2. Diagram describing the various steps of the experimental design.

I., analysis of the protein profile and composition of OPL and IPL: A, preparation of biological samples and protein extraction of the six biological samples (picture of separated OPL and IPL); B, electrophoretic migration of proteins extracted from OPL and IPL by SDS-PAGE; C, proteomic analyses using nanoLC-MS/MS analyses, protein identification and functional annotation. II., analysis of gene expression: A, tissues collection on 8 laying hens at the same post-ovulation stage; B, RNA extraction; C, RT-qPCR on 20 candidate genes selected from protein identification using 6 housekeeping genes. III., scanning and transmission electron microscopy: A, Sampling ; B, Scanning Electron Microscopy (SEM) ; C: Transmission Electron Microscopy (TEM)**.** GF1, Granulosa cells of follicle F1; GF2, Granulosa cells of follicle F2; GF3, Granulosa cells of follicle F3; In, Infundibulum; IPL,

Inner perivitelline membrane; J, Junction between infundibulum and magnum; L, Liver; Ma, Magnum; OPL, Outer perivitelline membrane; PL, Perivitelline layer**;** TF1, Theca of follicle F1; TF2, Theca of follicle F2; TF3, Theca of follicle F3.

Figure 3. SDS-PAGE protein profile of OPL and IPL from freshly laid eggs.

Three OPLs and three IPLs were sampled from freshly laid eggs and analyzed onto a 4-20 % acrylamide gel followed by Coomassie brilliant blue staining to verify the homogeneity of the OPL and IPL biological replicates, prior to mass spectrometry analyses.

Figure 4. Protein composition of OPL and IPL.

(A) Venn diagram showing the distribution of the 412 proteins between the two layers (OPL and IPL) and heat map of 40 differential common proteins. Proteins are considered as differential between IPL and OPL when p_value<0.05 and Log2(FoldChange)>1 or <-1. Log2(FoldChange)>1: proteins more abundant in IPL. Log2(FoldChange)<-1: proteins more abundant in OPL. **(B)** Quantitative distribution of the proteins identified by nanoLC-MS/MS. The ten most abundant proteins recovered by NSAF analysis are framed in red.

Figure 5. Heatmap of candidate gene expression in hen reproductive tissues.

Z-score range was colored from blue (-2 Row Z-score, low expression) to white (0 Row Z-score, intermediate expression) and to red (2 Row Z-score, high expression). GF1, granulosa cells of follicle F1; GF2, Granulosa cells of follicle F2; GF3, Granulosa cells of follicle F3; In, Infundibulum; J, Junction between infundibulum and magnum; L, Liver; Ma, Magnum; TF1, Theca of follicle F1; TF2, Theca of follicle F2; TF3, Theca of follicle F3. Z-score was used and corresponds to a value's relationship to the mean (average) of a group of values (expression of a gene in each tissue here), measured in terms of standard deviations from the mean ($z = (X - \mu) / \sigma$, where z is the z-score, X is the value of the element, μ is the population

mean, and σ is the standard deviation). Consequently, the color indicates an intuitive idea of the relative variation of each gene in the different tissues with blue and red color representing low and high expression, respectively, relatively to the whole set of selected tissues.

Figure 6. Ultrastructure of the inner surface of OPL and the outer surface of IPL.
(A) Diagrams of the whole PL and separated layers observed by transmission and scanning electron microscopy (TEM and SEM, respectively). OPL, outer perivitelline layer; OPL_{in}, inner surface of the outer perivitelline layer; IPL, inner perivitelline layer; IPL_{out}, outer surface of the inner perivitelline layer**. (B)** Transverse sections through the isolated OPL and IPL observed by TEM. Following OPL/IPL separation, the CM (black arrows) remains associated with either OPL (upper left panel) or IPL (lower right panel). A rupture of the OPL-bound CM (asterisk) is observed in the upper left panel. The upper right panel shows an OPL with no apparent CM. In the lower left panel, only small pieces of CM (black arrows) are bound to IPL. **(C)** Inner surface of OPL (OPL_{in}) and outer surface of IPL (IPL_{out}) observed by SEM. In accordance with TEM images, CM can be detected either on OPL_{in} (upper left panel) or on IPL_{out} (lower right panel) after OPL/IPL separation. The OPL-bound CM contains numerous holes (asterisks, upper left panel) through which OPL fibers can be observed (black arrowheads in the inset). These holes may correspond to the ruptured CM previously observed in TEM. Some OPL fibers can also be detected on IPL-bound CM (black arrowheads, inset of the lower right panel). The upper right panel shows an OPL_{in} area mostly without CM (only small pieces are present) where OPL fibers are clearly visible. The lower left panel shows an IPL_{ou} area that is mainly CM-free. OPL and IPL fibers are indicated by black arrowheads and white arrowheads, respectively. Black arrows indicate the continuous membrane (CM). Insets show enlargements of boxed areas.

Figure 7. Distribution of cellular and secreted proteins between IPL and OPL samples and associated functions.

(A) Among the 412 proteins identified, 271 are cellular proteins (148 are specific to IPL, 54 to OPL and the others are common to both layers). After functional annotation (see Material and Methods), cellular proteins could be assigned to four major functions and seem to have major functions in cell metabolism and cell structure. Two proteins are still not documented.

(B) Among the 412 proteins identified, 141 are secreted proteins (25 are specific to IPL, 44 to OPL and the others are common to both layers). Secreted proteins could be assigned to eight main functions and seem to have major functions in immunity and regulation of development. Ten proteins are still not documented.

Figure 8. Schematic representation of the main biological functions of chicken IPL and OPL.

The following are the supplementary data related to this article.

Supplementary Table S1. List of keratins identified in PL (XLSX)

Supplementary Table S2. List of proteins identified in IPL and associated quantitative values (XLSX)

Supplementary Table S3. List of proteins identified in OPL and associated quantitative values (XLSX)

Supplementary Table S4. List of proteins identified in whole PL (combined OPL and IPL proteomes) (XLSX)

Supplementary Table S5. Differential analysis of proteins identified in both IPL and OPL (XLSX)

Supplementary Table S6. Functional annotation of PL proteins (XLSX)

Supplementary Table S7. List of proteins previously found in PL proteome in 2008 [28] but not identified in the present study (XLSX)

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Author contributions

Conceptualization, M.B., S.R.G. and N.G. ; Methodology, M.B., C.Z.G., V.L., S.R.G. and N.G. ; Technical Realization, M.B., D.T., B.B., S.G. ; Data Treatment, M.B., D.T., B.B., S.R.G., N.G. ; Validation, M.B., D.T., B.B., C.Z.G., V.L., S.R.G. and N.G. ; Writing – Original Draft, M.B., S.R.G. and N.G. ; Writing–Review & Editing, M.B., B.B., C.Z.G., S.G., V.L., S.R.G. and N.G. ; Supervision, S.R.G. and N.G. All authors have given approval to the final version of the manuscript.

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The authors declare no competing interests.

REFERENCES

[1] E. Menkhorst, L. Selwood, Vertebrate extracellular preovulatory and postovulatory egg coats, Biol. Reprod. 79(5) (2008) 790-797.

[2] L. Shu, M.J. Suter, K. Räsänen, Evolution of egg coats: linking molecular biology and ecology, Mol. Ecol. 24(16) (2015) 4052-4073.

[3] J.L. Wong, G.M. Wessel, Defending the zygote: search for the ancestral animal block to polyspermy, Curr. Top. Dev. Biol. 72 (2006) 1-51.

[4] M. Monné, L. Jovine, A structural view of egg coat architecture and function in fertilization, Biol. Reprod. 85(4) (2011) 661-669.

[5] R. Bellairs, M. Harkness, R.D. Harkness, The vitelline membrane of the hen's egg: a chemical and electron microscopical study, J. Ultra. Res. 8(3-4) (1963) 339-359.

[6] J.F. Back, J.M. Bain, D.V. Vadehra, R.W. Burley, Proteins of the outer layer of the vitelline membrane of hen's eggs, Biochim. Biophys. Acta 705(1) (1982) 12-19.

[7] W.H. Chung, K.M. Lai, K.C. Hsu, Comparative study on histological structures of the vitelline membrane of hen and duck egg observed by cryo-scanning electron microscopy, J. Agric. Food Chem. 58(3) (2010) 1794-1799.

[8] S. Kido, Y. Doi, Separation and Properties of the Inner and Outer Layers of the Vitelline Membrane of Hen's Eggs, Poult. Sci. 67(3) (1988) 476-486.

[9] S. Kido, M. Janado, H. Nunoura, Macromolecular components of the vitelline membrane of hen's egg. I. Membrane structure and its deterioration with age, J. Biochem. 78(2) (1975) 261-268.

[10] S. Kido, M. Janado, H. Nunoura, Macromolecular components of the vitelline membrane of hen's egg. II. Physicochemical properties of glycoprotein I, J. Biochem. 79(6) (1976) 1351-1356.

[11] S. Kido, M. Janado, H. Nunoura, Macromolecular components of the vitelline membrane of hen's egg. III. Physicochemical properties of glycoprotein II, J. Biochem. 81(5) (1977) 1543-1548.

[12] N. Bausek, M. Waclawek, W.J. Schneider, F. Wohlrab, The major chicken egg envelope protein ZP1 is different from ZPB and is synthesized in the liver, J. Biol. Chem. 275(37) (2000) 28866-28872.

[13] Y. Takeuchi, K. Nishimura, N. Aoki, T. Adachi, C. Sato, K. Kitajima, T. Matsuda, A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZP C family glycoproteins in mammalian Zona pellucida. Its first identification, cDNA cloning and granulosa cell-specific expression, Eur. J. Biochem. 260(3) (1999) 736-742.

[14] H. Okumura, T. Sato, R. Sakuma, H. Fukushima, T. Matsuda, M. Ujita, Identification of distinctive interdomain interactions among ZP-N, ZP-C and other domains of zona pellucida glycoproteins underlying association of chicken egg-coat matrix, FEBS Open Bio 5 (2015) 454-465.

[15] H. Okumura, Y. Kohno, Y. Iwata, H. Mori, N. Aoki, C. Sato, K. Kitajima, D. Nadano, T. Matsuda, A newly identified zona pellucida glycoprotein, ZPD, and dimeric ZP1 of chicken egg envelope are involved in sperm activation on sperm-egg interaction, Biochem. J. 384(Pt 1) (2004) 191-199.

[16] S. Nishio, Y. Kohno, Y. Iwata, M. Arai, H. Okumura, K. Oshima, D. Nadano, T. Matsuda, Glycosylated chicken ZP2 accumulates in the egg coat of immature oocytes and remains localized to the germinal disc region of mature eggs, Biol. Reprod. 91(5) (2014) 107.

[17] D. Rodler, T. Sasanami, F. Sinowatz, Assembly of the inner perivitelline layer, a homolog of the mammalian zona pellucida: an immunohistochemical and ultrastructural study, Cells Tissues Organs 195(4) (2012) 330-339.

[18] M. Kinoshita, D. Rodler, K. Sugiura, K. Matsushima, N. Kansaku, K. Tahara, A. Tsukada, H. Ono, T. Yoshimura, N. Yoshizaki, R. Tanaka, T. Kohsaka, T. Sasanami, Zona pellucida protein ZP2 is expressed in the oocyte of Japanese quail (Coturnix japonica), Reproduction (Cambridge, England) 139(2) (2010) 359-371.

[19] S. Kido, A. Morimoto, F. Kim, Y. Doi, Isolation of a novel protein from the outer layer of the vitelline membrane, Biochem. J. 286 (Pt 1)(Pt 1) (1992) 17-22.

[20] S. De Boeck, J. Stockx, Mode of interaction between lysozyme and the other proteins of the hen's egg vitelline membrane, Int. J. Biochem. 18(7) (1986) 623-628.

[21] M.A. Rahman, A. Moriyama, A. Iwasawa, N. Yoshizaki, VMO-II Mediates the Binding of the Chalaziferous Layer with the Vitelline Membrane in Quail Eggs, J. Poult. Sci. 46(3) (2009) 240-248.

[22] J.M. Bain, J.M. Hall, Observations on the development and structure of the vitelline membrane of the hen's egg: an electron microscope study, Aust. J. Biol. Sci. 22(3) (1969) 653-665.

[23] E. Bar-Shira, A. Friedman, Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick, Dev. Comp. Immunol. 30(10) (2006) 930-941.

[24] V. Hervé-Grépinet, S. Réhault-Godbert, V. Labas, T. Magallon, C. Derache, M. Lavergne, J. Gautron, A.C. Lalmanach, Y. Nys, Purification and characterization of avian beta-defensin 11, an antimicrobial peptide of the hen egg, Antimicrob. Agents Chemother. 54(10) (2010) 4401-4409.

[25] D. Nakimbugwe, B. Masschalck, D. Deckers, L. Callewaert, A. Aertsen, C.W. Michiels, Cell wall substrate specificity of six different lysozymes and lysozyme inhibitory activity of bacterial extracts, FEMS Microbiol. Lett. 259(1) (2006) 41-46

[26] M.R. Bakst, B. Howarth, Jr., The fine structure of the hen's ovum at ovulation, Biol. Reprod. 17(3) (1977) 361-369.

[27] M.R. Bakst, B. Howarth, Jr., Hydrolysis of the hen's perivitelline layer by cock sperm in vitro, Biol. Reprod. 17(3) (1977) 370-379.

[28] K. Mann, Proteomic analysis of the chicken egg vitelline membrane, Proteomics 8(11) (2008) 2322-2332.

[29] M. Bregeon, N. Guyot, S. Réhault-Godbert, Mechanical Separation and Protein Solubilization of the Outer and Inner Perivitelline Sublayers from Hen's Eggs, J. Vis. Exp. 167 (2021) e61739.

[30] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, Anal. Chem. 68(5) (1996) 850-858.

[31] N. Guyot, V. Labas, G. Harichaux, M. Chessé, J.C. Poirier, Y. Nys, S. Réhault-Godbert, Proteomic analysis of egg white heparin-binding proteins: towards the identification of natural antibacterial molecules, Sci. Rep. 6 (2016) 27974.

[32] F. Coste, T. Moreau, V. Labas, M. Chessé, M. Bregeon, H. Meudal, K. Loth, B. Castaing, N. Guyot, S. Réhault-Godbert, Three-dimensional structures of avian beta-microseminoproteins: insight from the chicken egg-specific beta-microseminoprotein 3 paralog, FEBS Open Bio 11(6) (2021) 1739-1756.

[33] A. Keller, A.I. Nesvizhskii, E. Kolker, R. Aebersold, Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search, Anal. Chem. 74(20) (2002) 5383-5392.

[34] A.I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, A statistical model for identifying proteins by tandem mass spectrometry, Anal. Chem. 75(17) (2003) 4646-4658.

[35] L. Florens, M.J. Carozza, S.K. Swanson, M. Fournier, M.K. Coleman, J.L. Workman, M.P. Washburn, Analyzing chromatin remodeling complexes using shotgun proteomics and normalized spectral abundance factors, Methods (San Diego, Calif.) 40(4) (2006) 303-311.

[36] A. Gloux, M.J. Duclos, A. Brionne, M. Bourin, Y. Nys, S. Réhault-Godbert, Integrative analysis of transcriptomic data related to the liver of laying hens: from physiological basics to newly identified functions, BMC Genomics 20(1) (2019) 821.

[37] B. Zybailov, A.L. Mosley, M.E. Sardiu, M.K. Coleman, L. Florens, M.P. Washburn, Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae, J. Proteome Res. 5(9) (2006) 2339-2347.

[38] H.J. Haas, N.T. Spratt, Jr., Contributions to an analysis of the avian vitelline membrane's potential to promote outgrowth of the yolk sac-serosal membrane, Anat. Rec. 184(2) (1976) 227-231.

[39] M. Da Silva, C. Dombre, A. Brionne, P. Monget, M. Chessé, M. De Pauw, M. Mills, L. Combes-Soia, V. Labas, N. Guyot, Y. Nys, S. Réhault-Godbert, The Unique Features of Proteins Depicting the Chicken Amniotic Fluid, Mol. Cell. Proteomics 18(Suppl 1) (2019) S174-S190.

[40] Q. Li, W. Li, X. Li, L. Liu, Y. Zhang, Y. Guo, X. Chen, G. Xu, J. Zheng, The Distribution Characteristics and Applications for Maternal Cells on Chicken Egg Vitelline Membrane, Sci. Rep. 7(1) (2017) 6626.

[41] H. Nitta, J.I. Mason, J.M. Bahr, Localization of 3 beta-hydroxysteroid dehydrogenase in the chicken ovarian follicle shifts from the theca layer to granulosa layer with follicular maturation, Biol. Reprod. 48(1) (1993) 110-116.

[42] J. Nimpf, W.J. Schneider, Receptor-mediated lipoprotein transport in laying hens, J. Nutr. 121(9) (1991) 1471-1474.

[43] M.T. Hincke, M. Da Silva, N. Guyot, J. Gautron, M.D. McKee, R. Guabiraba-Brito, S. Réhault-Godbert, Dynamics of Structural Barriers and Innate Immune Components during Incubation of the Avian Egg: Critical Interplay between Autonomous Embryonic Development and Maternal Anticipation, J. Innate Immun. 11(2) (2019) 111-124.

[44] F. Baron, S. Bonnassie, M. Alabdeh, M.F. Cochet, F. Nau, C. Guérin-Dubiard, M. Gautier, S.C. Andrews, S. Jan, Global Gene-expression Analysis of the Response of Salmonella Enteritidis to Egg White Exposure Reveals Multiple Egg White-imposed Stress Responses, Front. Microbiol. 8 (2017) 829.

[45] N. Guyot, H. Meudal, S. Trapp, S. Iochmann, A. Silvestre, G. Jousset, V. Labas, P. Reverdiau, K. Loth, V. Hervé, V. Aucagne, A.F. Delmas, S. Rehault-Godbert, C. Landon, Structure, function, and evolution of Gga-AvBD11, the archetype of the structural avian-double-β-defensin family, Proc. Natl. Acad. Sci. U. S. A. 117(1) (2020) 337-345.

[46] S. Réhault-Godbert, V. Labas, E. Helloin, V. Hervé-Grépinet, C. Slugocki, M. Berges, M.C. Bourin, A. Brionne, J.C. Poirier, J. Gautron, F. Coste, Y. Nys, Ovalbumin-related protein X is a heparin-binding ov-serpin exhibiting antimicrobial activities, J. Biol. Chem. 288(24) (2013) 17285-17295.

[47] V. Hervé, H. Meudal, V. Labas, S. Réhault-Godbert, J. Gautron, M. Berges, N. Guyot, A.F. Delmas, Y. Nys, C. Landon, Three-dimensional NMR structure of Hen Egg Gallin (Chicken Ovodefensin) reveals a new variation of the β-defensin fold, J. Biol. Chem. 289(10) (2014) 7211-7220.

[48] N. Whenham, T.C. Lu, M.B. Maidin, P.W. Wilson, M.M. Bain, M.L. Stevenson, M.P. Stevens, M.R. Bedford, I.C. Dunn, Ovodefensins, an Oviduct-Specific Antimicrobial Gene Family, Have Evolved in Birds and Reptiles to Protect the Egg by Both Sequence and Intra-Six-Cysteine Sequence Motif Spacing, Biol. Reprod. 92(6) (2015) 154.

[49] F. Baron, S. Jan, F. Gonnet, M. Pasco, J. Jardin, B. Giudici, M. Gautier, C. Guérin-Dubiard, F. Nau, Ovotransferrin plays a major role in the strong bactericidal effect of egg white against the Bacillus cereus group, J. Food Prot. 77(6) (2014) 955-962.

[50] L.A. Julien, F. Baron, S. Bonnassie, F. Nau, C. Guérin, S. Jan, S.C. Andrews, The anti-bacterial iron-restriction defence mechanisms of egg white; the potential role of three lipocalin-like proteins in resistance against Salmonella, Biometals 32(3) (2019) 453-467.

[51] O. Wellman-Labadie, R. Lakshminarayanan, M.T. Hincke, Antimicrobial properties of avian eggshell-specific C-type lectin-like proteins, FEBS Lett. 582(3) (2008) 699-704.

[52] J. Gautron, S. Réhault-Godbert, G. Pascal, Y. Nys, M.T. Hincke, Ovocalyxin-36 and other LBP/BPI/PLUNC-like proteins as molecular actors of the mechanisms of the avian egg natural defences, Biochem. Soc. Trans. 39(4) (2011) 971-976.

[53] S.K. Linden, P. Sutton, N.G. Karlsson, V. Korolik, M.A. McGuckin, Mucins in the mucosal barrier to infection, Mucosal Immunol. 1(3) (2008) 183-197.

[54] D.A. Omana, J. Wang, J. Wu, Ovomucin - a glycoprotein with promising potential, Trends Food Sci. Technol. 21(9) (2010) 455-463.

[55] D.C. Hughes, ZP genes in avian species illustrate the dynamic evolution of the vertebrate egg envelope, Cytogenet. Genome Res. 117(1-4) (2007) 86-91.

[56] S. Nishio, H. Okumura, T. Matsuda, Egg-Coat and Zona Pellucida Proteins of Chicken as a Typical Species of Aves, Curr. Top. Dev. Biol. 130 (2018) 307-329.

[57] M. Bourin, J. Gautron, M. Berges, C. Hennequet-Antier, C. Cabau, Y. Nys, S. Réhault-Godbert, Transcriptomic profiling of proteases and antiproteases in the liver of sexually mature hens in relation to vitellogenesis, BMC Genomics 13 (2012) 457.

[58] N. Bausek, H.H. Ruckenbauer, S. Pfeifer, W.J. Schneider, F. Wohlrab, Interaction of sperm with purified native chicken ZP1 and ZPC proteins, Biol. Reprod. 71(2) (2004) 684-690.

[59] M. Ensslin, T. Vogel, J.J. Calvete, H.H. Thole, J. Schmidtke, T. Matsuda, E. Töpfer-Petersen, Molecular cloning and characterization of P47, a novel boar sperm-associated zona pellucida-binding protein homologous to a family of mammalian secretory proteins, Biol. Reprod. 58(4) (1998) 1057-1064.

[60] M.A. Ensslin, B.D. Shur, Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin-domain protein involved in sperm-egg binding, Cell 114(4) (2003) 405-417.

[61] L.L. Lindsay, J.C. Yang, J.L. Hedrick, Ovochymase, a Xenopus laevis egg extracellular protease, is translated as part of an unusual polyprotease, Proc. Natl. Acad. Sci. U. S. A. 96(20) (1999) 11253-8.

[62] J.R. Downie, S.M. Pegrum, Organization of the chick blastoderm edge, J. Embryol. Exp. Morphol. 26(3) (1971) 623-635.

[63] D.A. New, The adhesive properties and expansion of the chick blastoderm, J. Embryol. Exp. Morphol. 7 (1959) 146-164.

[64] E.A. Chernoff, J. Overton, Scanning electron microscopy of chick epiblast expansion on the vitelline membrane. Cell-substrate interactions, Dev. Biol. 57(1) (1977) 33-46.

[65] X. Qi, X. Li, Mechanistic Insights into the Generation and Transduction of Hedgehog Signaling, Trends Biochem. Sci. 45(5) (2020) 397-410.

[66] C. Siebel, U. Lendahl, Notch Signaling in Development, Tissue Homeostasis, and Disease, Physiol. Rev. 97(4) (2017) 1235-1294.

[67] Z. Steinhart, S. Angers, Wnt signaling in development and tissue homeostasis, Development (Cambridge, England) 145(11) (2018) dev146589.

[68] M.Y. Wu, C.S. Hill, Tgf-beta superfamily signaling in embryonic development and homeostasis, Dev. Cell 16(3) (2009) 329-343.

[69] C. Chang, Agonists and Antagonists of TGF-β Family Ligands, Cold Spring Harb. Perspect. Biol. 8(8) (2016) a021923.

[70] C.M. Cruciat, C. Niehrs, Secreted and transmembrane wnt inhibitors and activators, Cold Spring Harb. Perspect. Biol. 5(3) (2013) a015081.

[71] Y. Kawano, R. Kypta, Secreted antagonists of the Wnt signalling pathway, J. Cell Sci. 116(Pt 13) (2003) 2627-2634.

[72] Y. Mii, M. Taira, Secreted Wnt "inhibitors" are not just inhibitors: regulation of extracellular Wnt by secreted Frizzled-related proteins, Dev. Growth Differ. 53(8) (2011) 911-923.

[73] J.I. Jun, L.F. Lau, Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets, Nat. Rev. Drug Discov. 10(12) (2011) 945-963.

[74] K. Katsube, K. Sakamoto, Y. Tamamura, A. Yamaguchi, Role of CCN, a vertebrate specific gene family, in development, Dev. Growth Differ. 51(1) (2009) 55-67.

[75] C. Xu, S. Zhu, M. Wu, W. Han, Y. Yu, Functional receptors and intracellular signal pathways of midkine (MK) and pleiotrophin (PTN), Biol. Pharm. Bull. 37(4) (2014) 511-520.

[76] K. Matsumoto, M. Ema, Roles of VEGF-A signalling in development, regeneration, and tumours, J. Biochem. 156(1) (2014) 1-10.

[77] S.L. Hsieh, W.W. Lin, Decoy receptor 3: an endogenous immunomodulator in cancer growth and inflammatory reactions, J. Biomed. Sci. 24(1) (2017) 39.

[78] H. Inomata, T. Haraguchi, Y. Sasai, Robust stability of the embryonic axial pattern requires a secreted scaffold for chordin degradation, Cell 134(5) (2008) 854-865.

[79] C. Jensen, Ultrastructural changes in the avian vitelline membrane during embryonic development, J. Embryol. Exp. Morphol. 21(3) (1969) 467-484.

[80] N. Yoshizaki, W. Yamaguchi, S. Ito, C. Katagiri, On the Hatching Mechanism of Quail Embryos: Participation of Ectodermal Secretions in the Escape of Embryos from the Vitelline Membrane, Zool. Sci. 17(6) (2000) 751-758.

[81] Y. Maeshima, P.C. Colorado, A. Torre, K.A. Holthaus, J.A. Grunkemeyer, M.B. Ericksen, H. Hopfer, Y. Xiao, I.E. Stillman, R. Kalluri, Distinct antitumor properties of a type IV collagen domain derived from basement membrane, J. Biol. Chem. 275(28) (2000) 21340-21348.

[82] M. Mongiat, S.M. Sweeney, J.D. San Antonio, J. Fu, R.V. Iozzo, Endorepellin, a novel inhibitor of angiogenesis derived from the C terminus of perlecan, J. Biol. Chem. 278(6) (2003) 4238-4249.

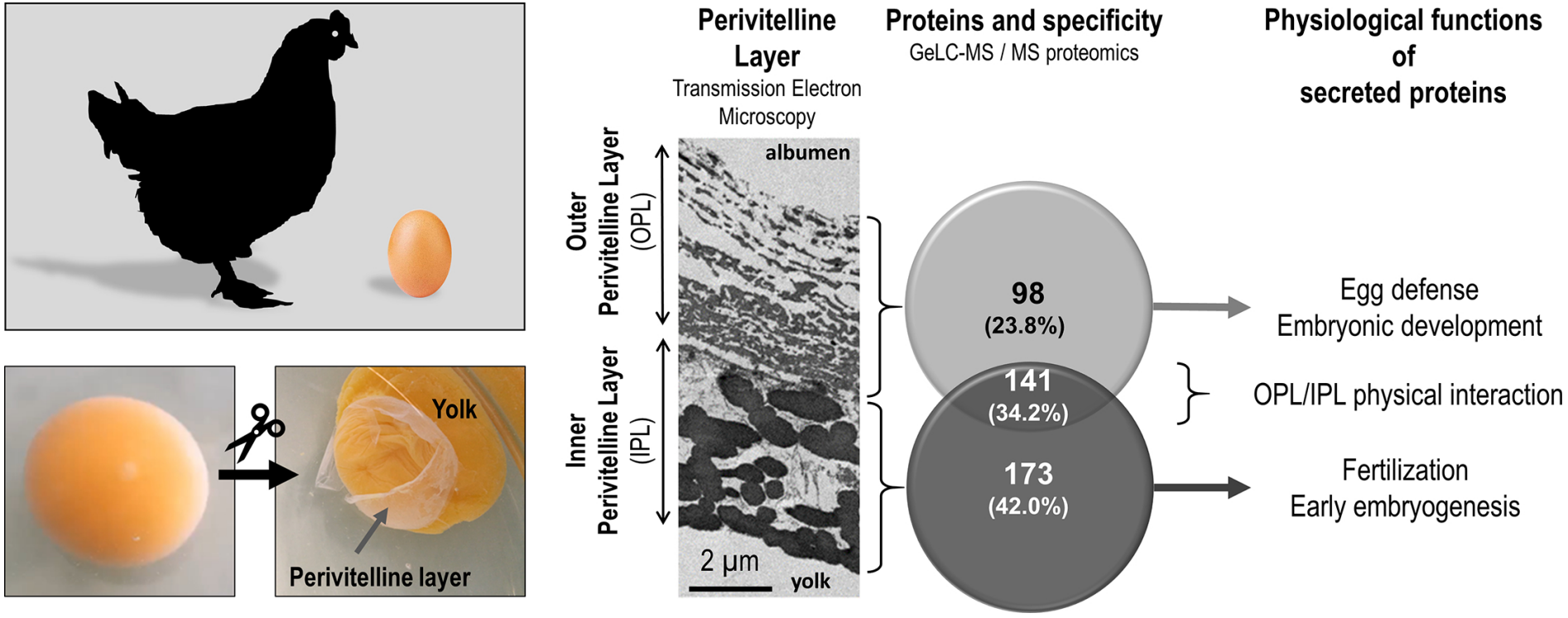
[83] M.S. O'Reilly, T. Boehm, Y. Shing, N. Fukai, G. Vasios, W.S. Lane, E. Flynn, J.R. Birkhead, B.R. Olsen, J. Folkman, Endostatin: an endogenous inhibitor of angiogenesis and tumor growth, Cell 88(2) (1997) 277-285.

[84] K. Damaziak, M. Kieliszek, D. Gozdowski, Structural and proteomic analyses of vitelline membrane proteins of blackbird (Turdus merula) and song thrush (Turdus philomelos), Sci. Rep. 10(1) (2020) 19344.

Graphical abstract

Highlights

- The present study used a multi-scale approach (proteomics, gene expression analysis, ultrastructural observations) to investigate structural and functional features of chicken PL and its two sublayers, namely IPL and OPL.
- Although identified in the two sublayers, the most abundant PL proteins are synthesized by tissues responsible for the formation of either IPL (liver, ovary) or OPL (oviduct), but rarely by both.
- Some proteins identified in both OPL and IPL are thought to be involved in the physical adhesion of OPL with IPL.
- IPL proteins are mainly associated with fertilization and early development, while OPL proteins are primarily involved in antimicrobial defense and embryogenesis.



Graphics Abstract

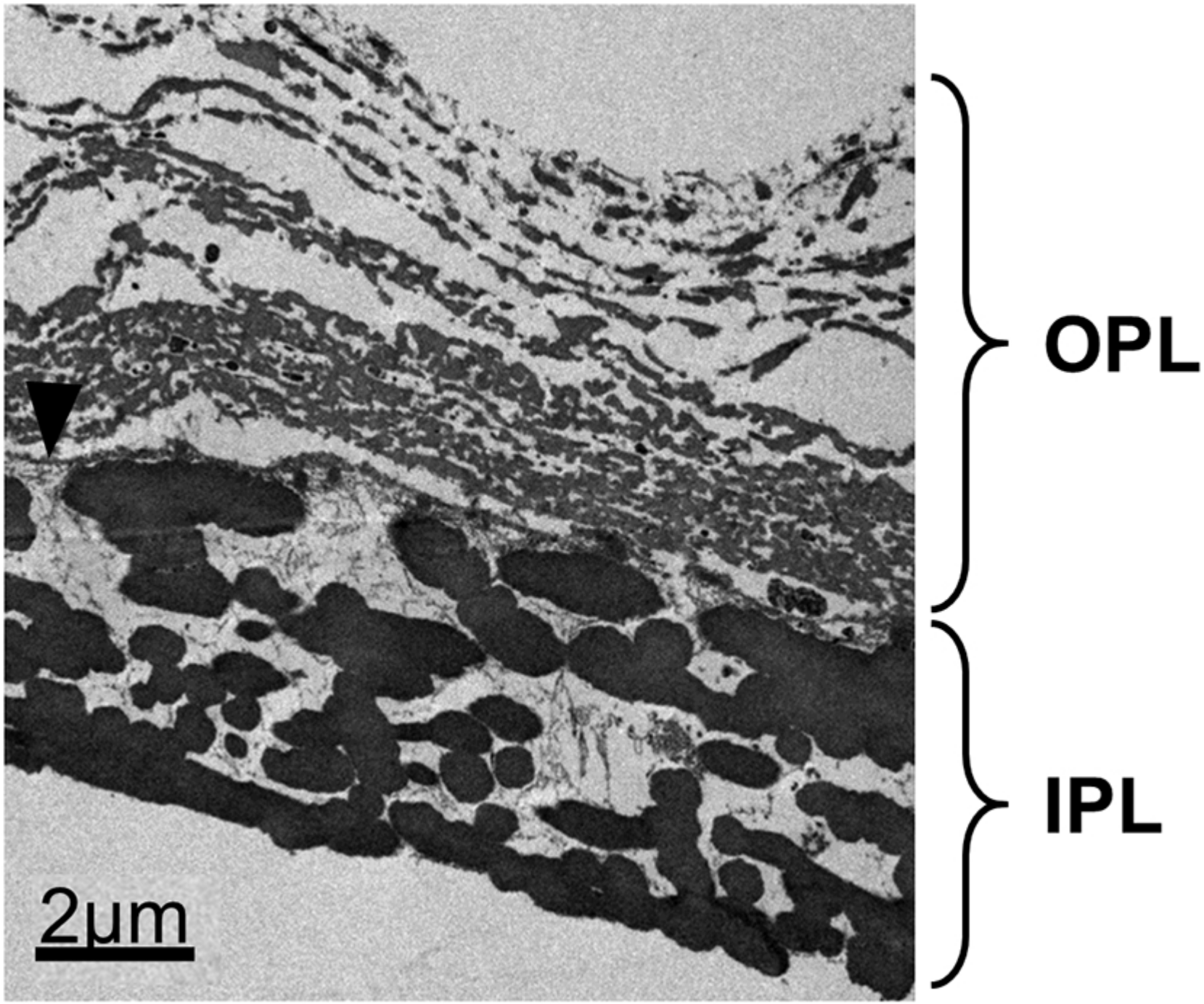


Figure 1

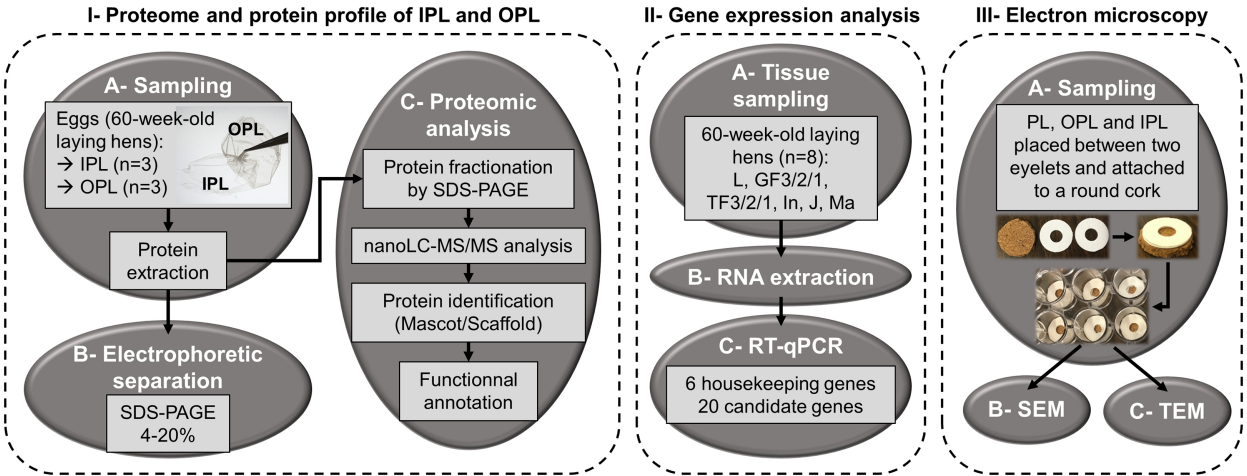

I- Proteome and protein profile of IPL and OPL
A- Sampling
Eggs (60-week-old laying hens):
→ IPL (n=3)
→ OPL (n=3)
OPL
IPL
Protein extraction
B- Electrophoretic separation
SDS-PAGE 4-20%
C- Proteomic analysis
Protein fractionation by SDS-PAGE
nanoLC-MS/MS analysis
Protein identification (Mascot/Scaffold)
Functionnal annotation
II- Gene expression analysis
A- Tissue sampling
60-week-old laying hens (n=8): L, GF3/2/1, TF3/2/1, In, J, Ma
B- RNA extraction
C- RT-qPCR
6 housekeeping genes
20 candidate genes
III- Electron microscopy
A- Sampling
PL, OPL and IPL placed between two eyelets and attached to a round cork
B- SEM
C- TEM

Figure 2

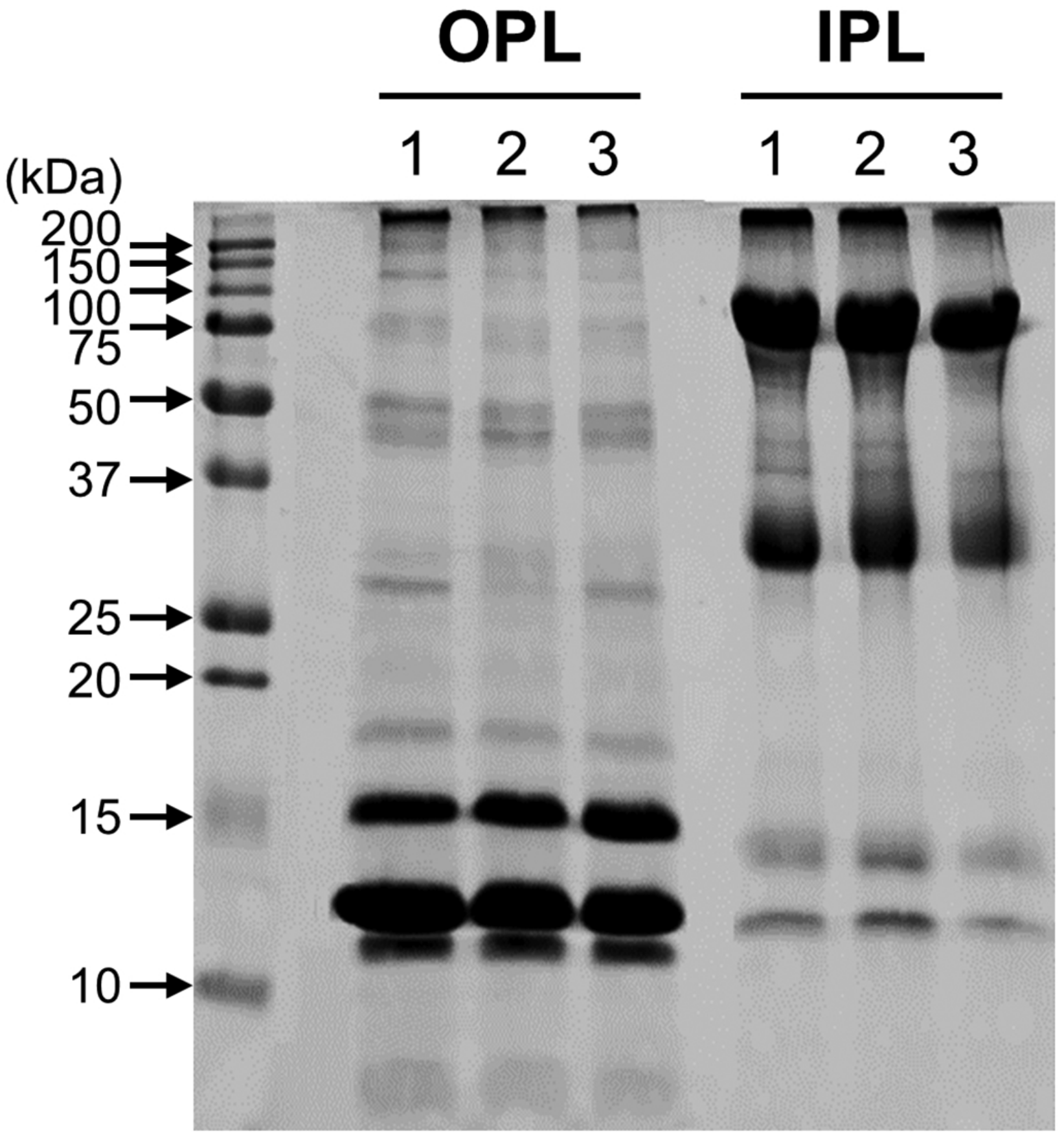


Figure 3

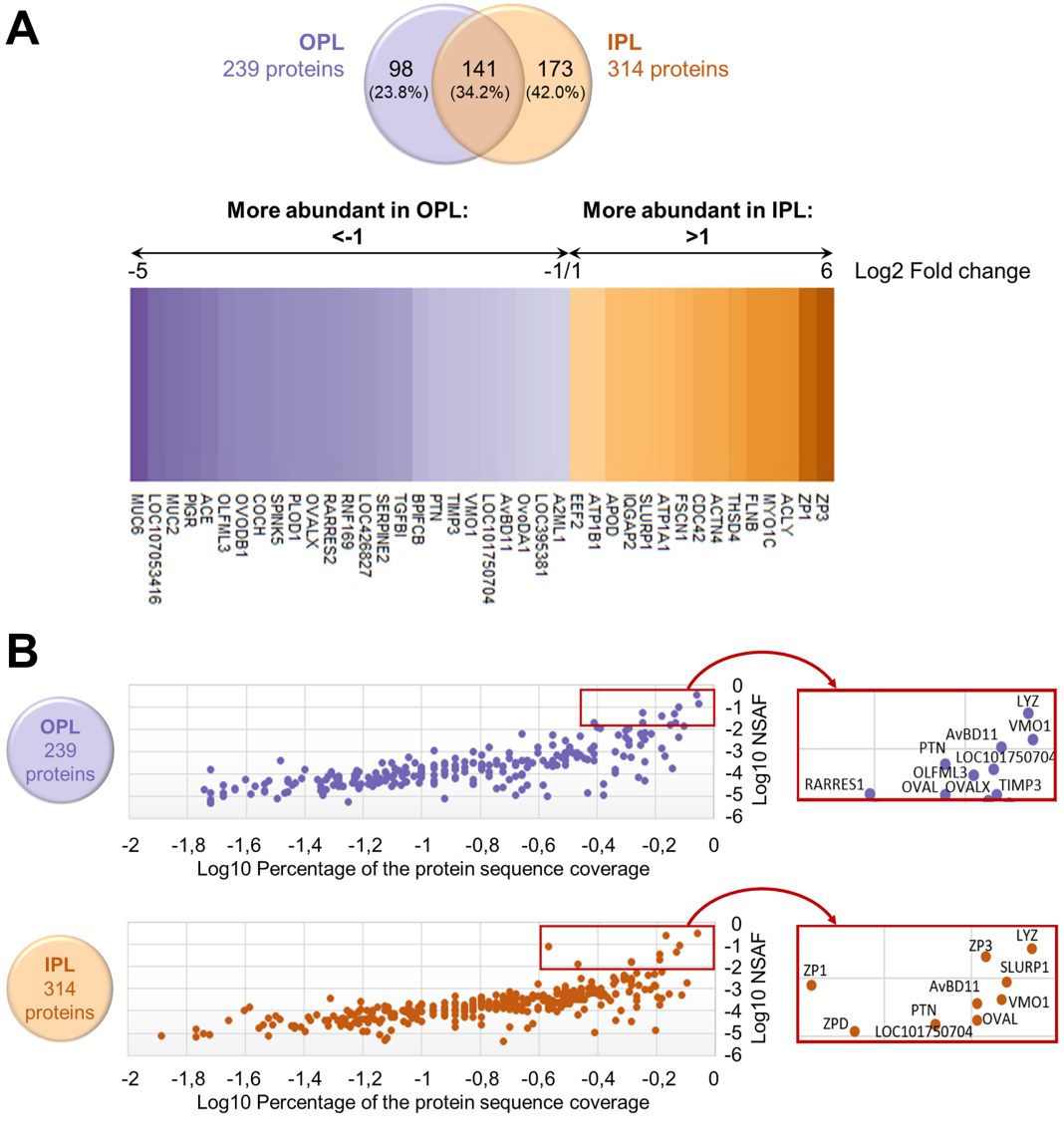
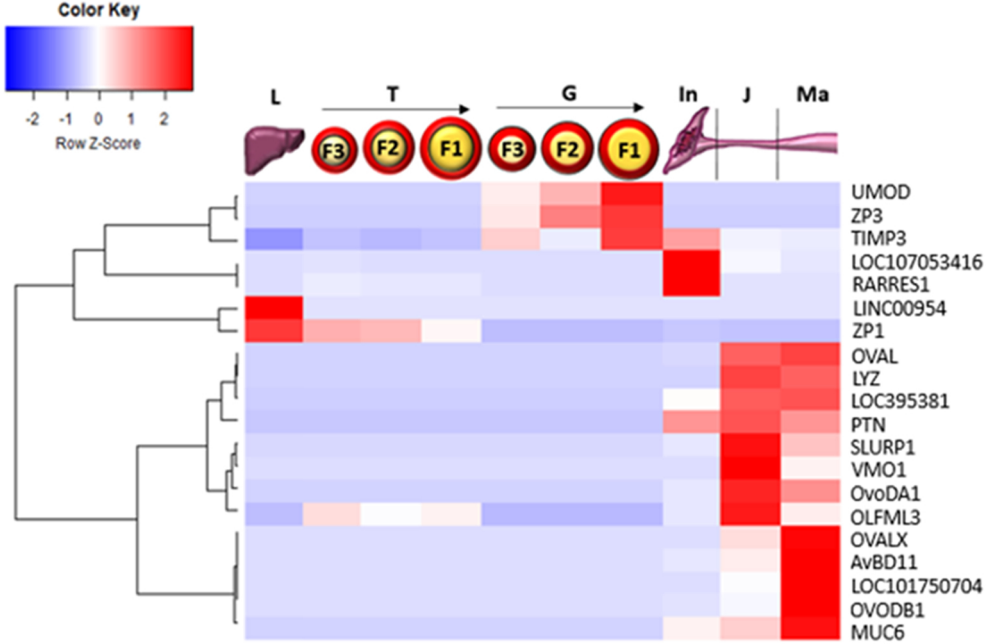

A
OPL
239 proteins
98
(23.8%)
141
(34.2%)
173
(42.0%)
IPL
314 proteins
More abundant in OPL:
<-1
More abundant in IPL:
>1
-5
-1/1
6
Log2 Fold change
MUC6
LOC107053416
MUC2
PIGR
ACE
OLFML3
OVODB1
COCH
SPINK5
PLOD1
OVALX
RARRES2
RNF169
LOC426827
SERPINE2
TGFBI
BPIFCB
PTN
TMP3
VMO1
AvBD11
LOC101750704
OvoDA1
LOC395381
A2ML1
EEF2
ATP1B1
APOD
IGGAP2
SLURP1
ATP1A1
FSCN1
CDC42
ACTN4
THSD4
FLNB
MYO1C
ACLY
ZP1
ZP3
B
OPL
239
proteins
Log10 NSAF
0
-1
-2
-3
-4
-5
-6
-2
-1,8
-1,6
-1,4
-1,2
-1
-0,8
-0,6
-0,4
-0,2
0
Log10 Percentage of the protein sequence coverage
LYZ
VMO1
AvBD11
PTN
LOC101750704
OLFML3
OVAL
OVALX
TIMP3
RARRES1
IPL
314
proteins
Log10 NSAF
Log10 Percentage of the protein sequence coverage
ZP3
LYZ
ZP1
SLURP1
AvBD11
VMO1
PTN
OVAL
ZPD
LOC101750704

Figure 4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Row Z-Score
L
T
G
In
J
Ma
F3
F2
F1
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F2
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UMOD
ZP3
TIMP3
LOC107053416
RARRES1
LINC00954
ZP1
OVAL
LYZ
LOC395381
PTN
SLURP1
VMO1
OvoDA1
OLFML3
OVALX
AvBD11
LOC101750704
OVODB1
MUC6

Figure 5

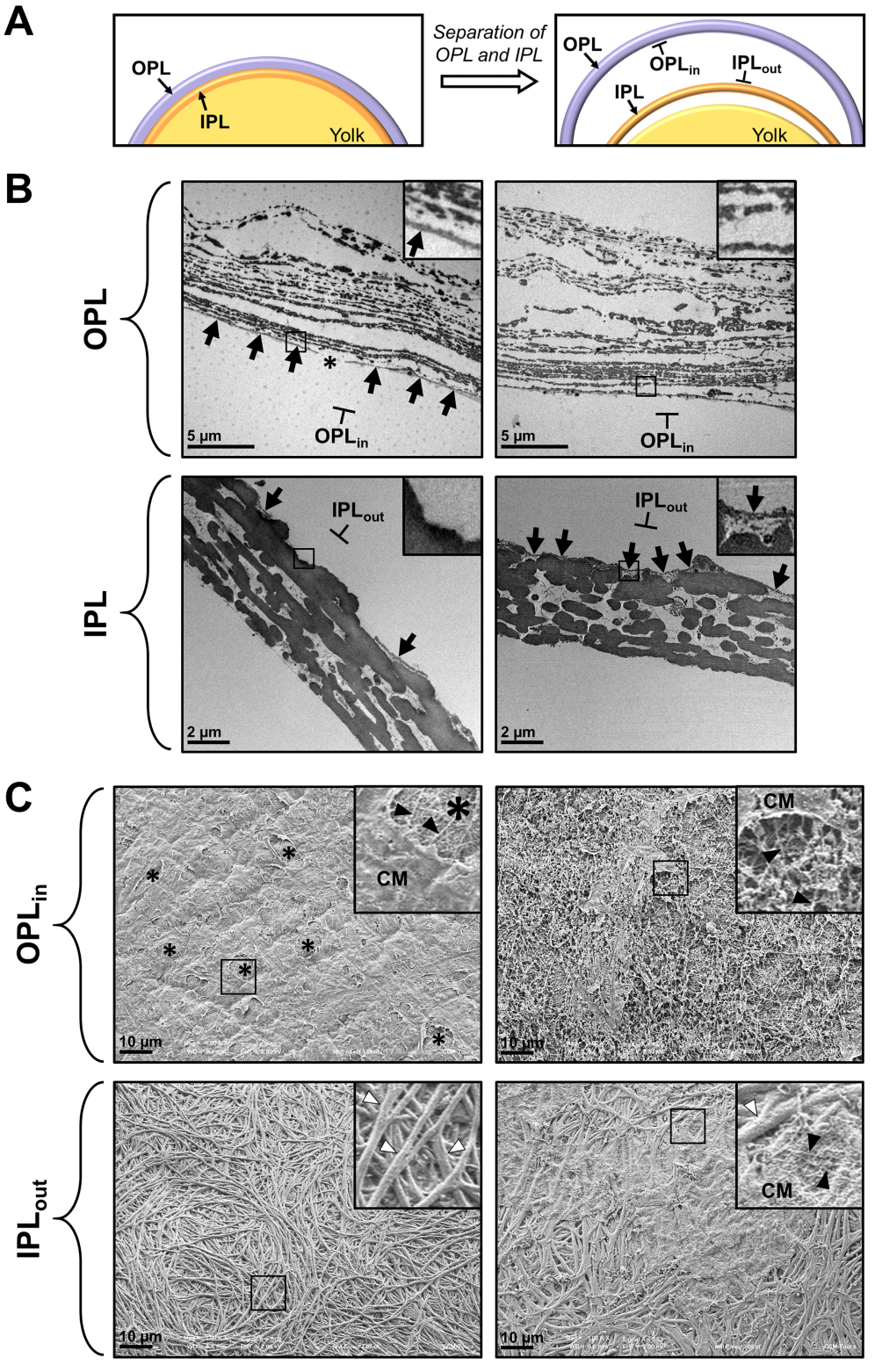
A
OPL
IPL
Yolk
Separation of OPL and IPL
OPL
OPL$_{in}$
IPL$_{out}$
IPL
Yolk
B
OPL
5 µm
OPL$_{in}$
5 µm
OPL$_{in}$
IPL
IPL$_{out}$
2 µm
IPL$_{out}$
2 µm
C
OPL$_{in}$
CM
10 µm
CM
10 µm
IPL$_{out}$
10 µm
CM
10 µm

Figure 6

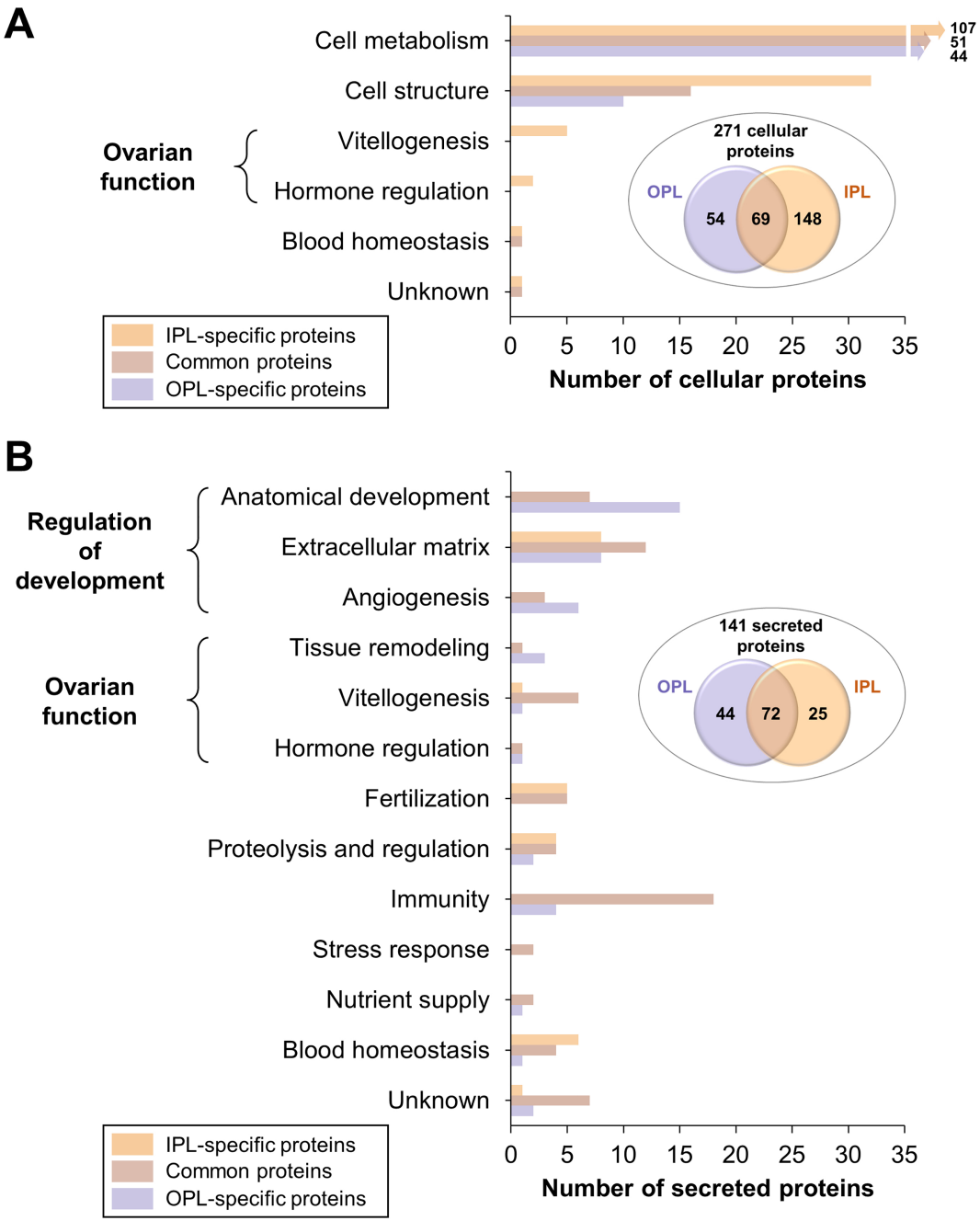
A
Cell metabolism
Cell structure
Vitellogenesis
Hormone regulation
Blood homeostasis
Unknown
Ovarian function
107
51
44
271 cellular proteins
OPL
IPL
54
69
148
IPL-specific proteins
Common proteins
OPL-specific proteins
0 5 10 15 20 25 30 35
Number of cellular proteins
B
Regulation of development
Anatomical development
Extracellular matrix
Angiogenesis
Ovarian function
Tissue remodeling
Vitellogenesis
Hormone regulation
Fertilization
Proteolysis and regulation
Immunity
Stress response
Nutrient supply
Blood homeostasis
Unknown
141 secreted proteins
OPL
IPL
44
72
25
IPL-specific proteins
Common proteins
OPL-specific proteins
0 5 10 15 20 25 30 35
Number of secreted proteins

Figure 7

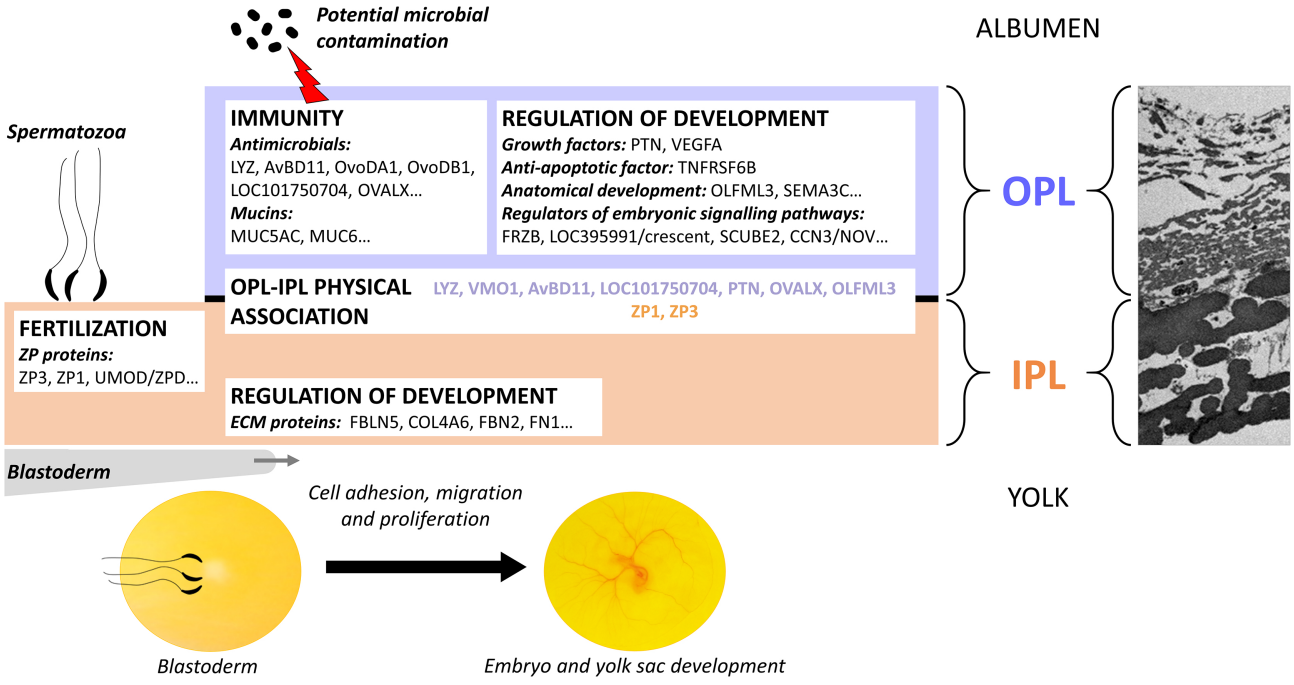
Potential microbial contamination
ALBUMEN
Spermatozoa
IMMUNITY
Antimicrobials:
LYZ, AvBD11, OvoDA1, OvoDB1, LOC101750704, OVALX...
Mucins:
MUC5AC, MUC6...
REGULATION OF DEVELOPMENT
Growth factors: PTN, VEGFA
Anti-apoptotic factor: TNFRSF6B
Anatomical development: OLFML3, SEMA3C...
Regulators of embryonic signalling pathways:
FRZB, LOC395991/crescent, SCUBE2, CCN3/NOV...
OPL
OPL-IPL PHYSICAL ASSOCIATION
LYZ, VMO1, AvBD11, LOC101750704, PTN, OVALX, OLFML3
ZP1, ZP3
FERTILIZATION
ZP proteins:
ZP3, ZP1, UMOD/ZPD...
IPL
REGULATION OF DEVELOPMENT
ECM proteins: FBLN5, COL4A6, FBN2, FN1...
Blastoderm
YOLK
Cell adhesion, migration and proliferation
Blastoderm
Embryo and yolk sac development

Figure 8