Regulation of the bovine oviductal fluid proteome

Julie Lamy¹, Valérie Labas^{1,2}, Grégoire Harichaux^{1,2}, Guillaume Tsikis¹,
Pascal Mermillod¹ and Marie Saint-Dizier^{1,3}

¹Physiologie de la Reproduction et des Comportements (PRC), INRA, CNRS, IFCE, Université de Tours, Nouzilly, France, ²INRA, Plateforme d'Analyse Intégrative des Biomolécules (PAIB), Nouzilly, France and ³Université François Rabelais de Tours, UFR Sciences et Techniques, Tours, France

Correspondence should be addressed to M Saint-Dizier; Email: marie.saint-dizier@univ-tours.fr

Abstract

Our objective was to investigate the regulation of the proteome in the bovine oviductal fluid according to the stage of the oestrous cycle, to the side relative to ovulation and to local concentrations of steroid hormones. Luminal fluid samples from both oviducts were collected at four stages of the oestrous cycle: pre-ovulatory (Pre-ov), post-ovulatory (Post-ov), and mid- and late luteal phases from adult cyclic cows (18–25 cows/stage). The proteomes were assessed by nanoLC–MS/MS and quantified by label-free method. Totally, 482 proteins were identified including a limited number of proteins specific to one stage or one side. Proportions of differentially abundant proteins fluctuated from 10 to 24% between sides at one stage and from 4 to 20% among stages in a given side of ovulation. In oviductal fluids ipsilateral to ovulation, Annexin A1 was the most abundant protein at Pre-ov compared with Post-ov while numerous heat shock proteins were more abundant at Post-ov compared with Pre-ov. Among differentially abundant proteins, seven tended to be correlated with intra-oviductal concentrations of progesterone. A wide range of biological processes was evidenced for differentially abundant proteins, of which metabolic and cellular processes were predominant. This work identifies numerous new candidate proteins potentially interacting with the oocyte, spermatozoa and embryo to modulate fertilization and early embryo development.

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Introduction

Crucial events leading to the establishment of pregnancy occur within the mammalian oviduct (Coy *et al.* 2012, Hunter 2012). Changes in the composition of the oviductal fluid (OF) across the oestrous cycle constitute a way to provide a suitable microenvironment for sperm storage and capacitation, final maturation of oocyte, gamete transport, fertilization and early embryo development (Coy *et al.* 2012, Hunter 2012). Indeed, during oestrus in the pre-ovulatory period, the caudal part of both oviducts are able to maintain the viability of spermatozoa in the so-called 'sperm reservoir', during an estimated period of 24–48 h in the cow (Hunter & Rodriguez-Martinez 2004, Suarez & Pacey 2006, Sostaric *et al.* 2008). After ovulation, several events occur in the oviduct ipsilateral to ovulation: the transport and final maturation of the oocyte from the infundibulum to the ampulla–isthmus junction, where fertilization takes place; the release of spermatozoa from the sperm reservoir; their hyperactivation and transport toward the oocyte; the fertilization and the early development of the embryo (Hunter 2012), which enters the uterus 4–5 days after fertilization, at the 8-cell or morula stage in the bovine.

The proteins in the OF may originate from three sources: *de novo* synthesis and secretion from the secretory cells in the oviductal epithelium, transudate from blood, and, in the post-ovulatory period, putative inputs from the ovulating follicle (Leese *et al.* 2001, 2008). Several proteins upregulated in the bovine oviductal epithelium around ovulation were reported to interact with the oocyte (Goncalves *et al.* 2008), spermatozoa (Grippio *et al.* 1995) and/or embryo (Killian 2004) and to modulate the events described previously. For instance, HSPA8, a heat shock protein (HSP), was reported to play a role in the maintenance of bull sperm survival (Elliott *et al.* 2009). GRP78 is another oviduct-derived HSP suspected to interact with bull sperm cells *in vivo* and probably be involved in sperm survival (Boilard *et al.* 2004) and in the modulation of sperm–zona pellucida interaction (Marin-Briggiler *et al.* 2010). However, to date, very few candidates interacting *in vivo* with gametes and embryo have been identified.

The above-mentioned oviductal events occur in the presence of fluctuating levels of steroid hormones in the circulating blood and locally in the oviduct. Sperm storage occurs at the end of the follicular phase of the

oestrous cycle in parallel with high concentrations of 17β -oestradiol (E_2) and low concentrations of progesterone (P_4) in blood (Glencross *et al.* 1973). By contrast, the post-ovulatory events occur when the luteal phase of the oestrous cycle begins, in parallel with low concentrations of E_2 and increasing concentrations of P_4 in blood (Glencross *et al.* 1973). In addition to systemic changes induced in the oviductal environment, local mechanisms may regulate the secretory activity of the oviductal epithelium. We reported important fluctuations in the topical concentrations in the bovine OF of steroid hormones between different stages of the oestrous cycle and sides relative to ovulation (Lamy *et al.* 2016). In particular, concentrations of P_4 measured in the oviduct ipsilateral to the corpus luteum were 20 times higher during the mid-luteal phase than just before ovulation and 4–16 times higher than in the contralateral oviduct across the oestrous cycle (Lamy *et al.* 2016). Previous studies on gene expression in bovine oviductal epithelial cells (OECs) detected important changes between the follicular and the luteal phases of the oestrous cycle (Bauersachs *et al.* 2004, Cerny *et al.* 2015) as well as between ipsi- and contralateral sides relative to ovulation during the post-ovulatory period (Bauersachs *et al.* 2003). However, there is no exhaustive proteomic profiling of the tubal fluid during the oestrous cycle in the cow and very little is currently known on the regulation of this oviductal physiological activity.

The aim of this study was to monitor the proteome of the bovine OF according to the stage of the oestrous cycle, to the side relative to ovulation and to the topical concentrations of E_2 and P_4 . These effects were studied on bovine ipsi- and contralateral OF samples collected previously and characterized for their steroid profiling (Lamy *et al.* 2016).

Materials and methods

Collection and preparation of samples

Bovine OF samples were collected and prepared as described previously (Lamy *et al.* 2016). Briefly, both oviducts and ovaries from individual adult cows were collected in a local slaughterhouse (Vendôme, France; less than 40 min from the laboratory), immediately placed on ice and transported to the laboratory. The oviducts were classified into one of four stages of the oestrous cycle according to the morphology of ovaries and corpus luteum, as described previously (Ireland *et al.* 1980): post-ovulatory (Post-ov, days 1–5), early-to-mid luteal phase (Mid-lut, days 6–12), late luteal phase (Late-lut, days 13–18) and pre-ovulatory (Pre-ov, days 19–21) phases. The oviducts were also separated into ipsilateral (to pre-ovulatory follicle, ovulation site or corpus luteum) and contralateral sides. The entire oviducts were cleaned of surrounding tissues and vessels and spread on a Petri dish. Then their content (OF + mucosa cells) was collected by evenly gentle squeezing (applying pressure) of the oviduct at one time with a glass slide. This content was aspirated with a pipette and put in a

Table 1 Pools of bovine oviductal fluid used in immunoblotting and mass spectrometry analyses.

Side relative to ovulation	Stage	Number of pools for immunoblot	Number of animals
Ipsilateral	Pre-ov	4	22 (6+6+5+5)
	Mid-lut	3	23 (8+8+7)
	Late-lut	3	25 (8+8+9)
	Post-ov	4	27 (7+10+3+7)
Contralateral	Pre-ov	4	18 (5+4+5+4)
	Mid-lut	3	23 (9+7+7)
	Late-lut	3	23 (7+7+9)
	Post-ov	4	23 (6+8+3+6)

conical 1.5-mL tube, and then oviductal cells were separated from the OF by centrifugation at 2000g for 5 min at 4°C. The supernatants were then centrifuged for 5 min at 6000g at 4°C. The remaining supernatants (20–100 µL/oviduct) were stored at –80°C until analysed.

The exclusion from the Pre-ov group of animals with ovarian cysts and atretic follicles was carried out as described previously (Lamy *et al.* 2016). Briefly, all oviducts attached to an ovary with a follicle larger than 20 mm in diameter were discarded at the time of oviduct collection. In order to exclude remaining animals with ovarian cysts or atretic follicles, and based on previous data obtained in the cow (Monniaux *et al.* 2008, Braw-Tal *et al.* 2009, Nishimoto *et al.* 2009), animals with intra-follicular concentrations of P_4 higher than 160 ng/mL, of E_2 lower than 40 ng/mL, and/or with a ratio of $E_2:P_4$ concentrations less than 1 were excluded from the Pre-ov group. Finally, the mean intra-follicular concentrations of P_4 and E_2 in the Pre-ov group ($n=22$) were 58.8 ± 9.6 ng/mL (12.0–160.0 ng/mL) and 1302.3 ± 212.0 ng/mL (76.0–3173.7 ng/mL) respectively.

Pools of 3–10 individual fluids were made to reach a final volume of 150–250 µL per sample (this volume was required for steroid assay, as described previously (Lamy *et al.* 2016)). A total of 3–4 pools per stage and side were obtained and a fraction was kept at –80°C for immunoblots (see Table 1 for details). For proteomic analysis, identical volumes of each pool were mixed to obtain a single sample/stage/side (18–25 animals/sample). In the following sections, the term ‘sample’ will refer to this secondary pool of OF. Protein concentrations in the samples were determined using the Uptima BC Assay kit (Interchim, Montluçon, France) according to manufacturer’s instructions and using bovine serum albumin as a standard. Each sample was migrated separately (72 µg per lane) on a 10% SDS-PAGE (50V, 30 min). The gel was stained with Coomassie (G-250) and each lane was cut horizontally in 3 bands for quantitative proteomic analysis.

Mass spectrometry analysis

NanoLC–MS/MS

After SDS-PAGE and cutting of the bands, each band was in-gel digested with bovine trypsin (Roche Diagnostics GmbH) as described previously (Labas *et al.* 2015).

All experiments were performed on triplicate using a dual linear ion trap Fourier Transform Mass Spectrometer (FT-MS) LTQ Orbitrap Velos (Thermo Fisher Scientific) coupled

to an UltiMate 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, The Netherlands). Five microliters of each sample were loaded on trap column for desalting and separated using nano-column as described previously (Labas *et al.* 2015). The gradient consisted of 4–55% B for 90 min at a flow rate of 300 nL/min. Mobile phases consisted of (A) 0.1% formic acid, 97.9% water, 2% acetonitrile (v/v/v) and (B) 0.1% formic acid, 15.9% water, 84% acetonitrile (v/v/v).

Data were acquired using Xcalibur software (version 2.1; Thermo Fisher Scientific). The instrument was operated in positive data-dependent mode. Resolution in the Orbitrap was set to $R=60,000$. In the scan range of m/z 300–1800, the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated and fragmented using Collision-Induced Dissociation (CID). The ion selection threshold was 500 counts for MS/MS, and the maximum allowed ion accumulation times were 200 ms for full scans and 50 ms for CID–MS/MS in the LTQ. 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 active during 30 s with a repeat count of 1. The lock mass was enabled for accurate mass measurements. Polydimethylcyclsiloxane (m/z , 445.120025, $(\text{Si}(\text{CH}_3)_2\text{O})_6$) ions were used for internal recalibration of the mass spectra.

Protein identification and data validation

Raw data files were converted into Mascot Generic Format (MGF) using Proteome Discoverer software (version 1.3; Thermo Fischer Scientific). Precursor mass range of 350–5000 kDa and signal-to-noise ratio of 1.5 were the criteria used for generation of peak lists. In order to identify the proteins, MS/MS ion searches were performed using the MASCOT search engine (version 2.2; Matrix Science, London, UK) via Proteome Discoverer 1.4 software (Thermo Fisher Scientific) against a local database (369,225 entries). From the NCBI nr database (the nr database is a ‘non-redundant’ protein database for Blast searches; download 07/08/15), a sub-database was generated using Proteome Discoverer 1.4 software from keywords targeting mammalian taxonomy. The parameters used for database searches included trypsin as protease with two missed cleavages allowed, carbamidomethylcysteine (+57 Da), oxidation of methionine (+16) and N-terminal protein acetylation (+42) as variable modifications. The tolerance of the ions was set at 5 ppm for parent and 0.8 kDa for fragment ion matches. Mascot results from the target and decoy databases were incorporated to Scaffold software (version 4.4.4, Proteome Software, Portland, USA). Peptide identifications were accepted if they could be established at a probability greater than 95.0% as specified by the PeptideProphet algorithm (Keller *et al.* 2002). Peptides were considered distinct if they differed in sequence. Protein identifications were accepted if they could be established at a probability of greater than 95.0% as specified by the ProteinProphet algorithm (Nesvizhskii *et al.* 2003) and contained at least two identified peptides (false discovery rate (FDR) <0.01 %).

Label-free protein quantification

Scaffold software was employed (version 4.4.4, Proteome Software) using a spectral count quantitative module. All proteins with greater than two peptides identified in nr database with high confidence were considered for protein quantification. To eliminate quantitative ambiguity into protein groups, we ignored all the spectra matching any peptide which was shared between proteins. To allow comparisons, we normalized the MS/MS data using the Total Spectra option. Thereby, quantification was carried out on distinct proteins (normalized spectral counts). Analysis of variance and, if significant, Student *t*-tests were done on technical replicates to identify changes between sides at a given stage and stages in a given side. Proteins were considered differentially abundant between stages of the oestrous cycle or sides relative to ovulation if the *P* value in the Student *t*-test was <0.05 and the ratio of normalized spectral counts >2 or <0.5. Lists of the most differentially abundant proteins were the quantitatively major proteins (based on spectral counting quantitative method) at one stage or side among proteins.

Immunoblotting

Primary antibodies used in immunoblotting are presented in [Supplementary Table 1](#) (see section on supplementary data given at the end of this article). All antibodies were diluted in Tris-buffered saline supplemented with 0.5% Tween 20 (TBST) and supplemented with lyophilized low-fat milk (5% w/v; TBST–milk). Secondary antibodies were goat anti-mouse conjugated to horseradish peroxidase (HRP; 1:5000, A4416, Sigma Aldrich, Saint-Quentin Fallavier, France) or goat anti-rabbit HRP (1:5000, A6154, Sigma Aldrich). Pools of OF from each stage or side were migrated in triplicate (20 µg of proteins per lane) on an 8–16% gradient SDS-PAGE. Liquid transfer was performed overnight at 4°C. The western blots were blocked in TBST–milk. Ponceau red staining was used to check homogeneous loading among lanes in each blot and to normalize the data, as described previously (Romero-Calvo *et al.* 2010). Ponceau staining was quantified by densitometry for analysis of the whole lane using an Image Scanner (Amersham Biosciences, GE Healthcare Life Sciences) and analysed using the TotalLab Quant software (version 11.4, TotalLab, Newcastle upon Tyne, UK). Then, membranes were incubated with primary antibodies under mild agitation at 37°C for 1.5 h or overnight at 4°C, and then washed and incubated with secondary antibodies for 1 h at 37°C. The peroxidase was revealed with chemiluminescent substrates (SuperSignal West Pico and West Femto Chemiluminescent Substrates, Thermo Scientific, Waltham, MA, USA) and the images were digitized with a cooled CCD Camera (ImageMaster VDS-CL, Amersham Biosciences). The intensity of the signal was quantified using the TotalLab Quant software (version 11.4, TotalLab).

Correlations between differentially abundant proteins and local concentrations of steroid hormones

Correlations between local concentrations of P_4 and E_2 and normalized spectral counts of differentially abundant proteins

among stages in ipsilateral OF were analysed by Pearson tests using the R software (version 3.2.2, R foundation for Statistical Computing, Vienna, Austria). A *P* value lower than 0.1 was regarded as a trend.

Analysis of molecular functions, biological process and networks of differentially abundant proteins

Gene names were determined from the protein GI accession numbers using UniProt Knowledgebase (UniProtKB). The Gene Ontology analysis and pie graphs were realized using the Protein Analysis Through Evolutionary Relationships (PANTHER) database. Finally, networks of differentially abundant proteins between stages and sides were built using Ingenuity Pathway Analysis (IPA) software. The IPA was restrained to differentially abundant proteins between Pre-ov and Post-ov in the ipsilateral side. These datasets contained the respective gene symbols and Pre-ov:Post-ov ratios of normalized spectral counts of differentially abundant proteins. Networks of these genes were generated based on information contained in the Ingenuity Knowledge Base, considering direct and indirect relationships (as general settings), experimentally observed, highly and moderately predicted interaction networks in mammals, with no restriction for tissues/cell lines or mutations. IPA networks with a score of 4 or greater (*P* value < 0.001) were reported.

Results

A total of 482 proteins were identified in bovine OF throughout the oestrous cycle (Supplementary Table 2). A high proportion (>83%) of these proteins was common between sides relative to ovulation at a given stage (totals of 401, 425, 431 and 422 proteins in common between sides at Pre-ov, Post-ov, Mid-lut and Late-lut respectively). Also, a high proportion (>72%) of proteins was found at all four stages in a given side

Figure 1 Distribution of proteins identified in the ipsilateral bovine oviductal fluid throughout the oestrous cycle. Proteins shared between Post-ov and Late-lut stages (*n*=6) and between Pre-ov and Mid-lut stages (*n*=3) were not represented.

relative to ovulation (Fig. 1 for the ipsilateral OF). As a consequence, a limited number of proteins were found to be specific to one stage in a given side: 22 proteins were found to be specific to the Post-ov stage vs less than 6 for the other stages.

Changes in the oviductal proteome according to the side of ovulation

The proportion of differentially abundant proteins between ipsi- and contralateral OF was highest at Post-ov (24%, 115/482) and varied between 10 and 15% for the other stages (Fig. 2A). The relative abundance of the top-20 differentially abundant proteins between sides at Pre-ov and Post-ov is shown in Fig. 2B. The lists of the top-40 differentially abundant proteins with their respective ipsi:contra ratios and known biological functions at Pre-ov and Post-ov are shown in Tables 2 and 3 respectively (see all differentially abundant proteins relative to the ovulation side at the four stages of the oestrous cycle in Supplementary Table 3). Differentially abundant proteins between ipsilateral and contralateral OF were globally more abundant at Post-ov than at Pre-ov (Fig. 2B). The most abundant proteins in the ipsilateral side before ovulation included CD109 antigen (CD109) and several members of the T-complex protein family (CCT5, CCT8, CCT6A). At the Post-ov stage numerous proteins involved in the cell response to stress, such as proteins of the heat shock protein family (HSPA8, HSP90, GRP78, HSPA6), were among the most abundant proteins in the ipsilateral OF (Fig. 2B).

Changes in the oviductal proteome related to the stage of the oestrous cycle

When comparing stages of the oestrous cycle in a given side, percentages of differentially abundant proteins fluctuated from 4 to 20% according to the side (Fig. 3A and B). In particular, 81 proteins (17%) were differentially abundant between Pre-ov and Post-ov in ipsilateral OF, of which 51 were more abundant at Post-ov and 30 more abundant at Pre-ov. The list of the top-40 differentially abundant proteins between Pre-ov and Post-ov in ipsilateral OF with the respective Pre-ov:Post-ov ratios and biological functions is shown in Table 4 (see all differentially abundant proteins among stages in ipsi- and contralateral OF in Supplementary Tables 4 and 5 respectively). The patterns of main differentially abundant proteins according to the stage of the oestrous cycle in ipsilateral OF are shown in Fig. 3C. Annexin A1 (ANXA1) was by far the most abundant protein at Pre-ov compared with Post-ov in the side of ovulation. By contrast, the most abundant proteins at Post-ov compared with Pre-ov, but also with Mid-lut and Late-lut, included numerous HSP (GRP78, HSPA8, HSPA6, HSP90 AA1, AB1 and B1)

at equivalent levels of detection. Another pattern of variation included proteins more abundant at Pre-ov compared with Mid-lut and Late-lut, among which phosphatidylethanolamine-binding protein 1 (PEBP1), CD109 and peroxiredoxin-2 (PRDX2) were found. The oviduct-specific glycoprotein 1 (OVGP1), also named oviductin, was more abundant at both Pre-ov and Post-ov compared with Mid-lut, although the ratios were below the fixed threshold of 2 (Pre-ov:Mid-lut and Post-ov:Mid-lut for normalized spectral counts (NSC) of 1.5 and 1.7 respectively, the difference was significant ($P < 0.05$)).

In order to identify variability in the abundance of proteins among individual pools of OF (within a given stage), four proteins found to be differentially abundant (GRP78, HSPA8, HSP90AA1 and ANXA1) and one protein (HSP70) with no significant fluctuation among stages were analysed by immunoblotting. The signal intensities from western blotting were similar among pools in a given stage and ratios among stages were in agreement with results from proteomic analyses (Fig. 4).

Changes in the oviductal proteome related to local concentrations of progesterone and 17 β -oestradiol

Based on concentrations of P_4 and E_2 in the same OF samples (Lamy *et al.* 2016), correlations between normalized levels of differentially abundant proteins among stages and mean concentrations of P_4 or E_2 were investigated. There was a positive ($P = 0.08$) association between P_4 and seven proteins (phosphatidylethanolamine-binding protein 1 (PEBP1), peroxiredoxin-2 (PRDX2), beta-actin (ACTB), high mobility group protein B1 (HMGB1) and ribosomal protein (RP) L18 (RPL18)), whereas there was a negative association between P_4 and septin-9 (SEPT9) and RPS19 proteins (Supplementary Fig. 1).

Functional analysis of differentially abundant proteins

A Gene Ontology (GO) analysis was conducted on differentially abundant proteins according to the side or the stage. One-third (28–44% across all comparisons) of differentially abundant proteins were classified as

Figure 2 (A) Percentages of differentially abundant proteins between ipsi- and contralateral oviductal fluids throughout the oestrous cycle (black bars; $n = 482$) and percentages of proteins more abundant in ipsilateral compared with contralateral fluids (white bars; numbers of differentially abundant proteins are indicated with brackets).

(B) Variation of top-20 differentially abundant proteins according to the side of ovulation at Pre-ov and Post-ov. AHCY, adenosylhomocysteinase; ALDH9A1, 4-trimethylaminobutylaldehyde dehydrogenase; ATIC, bifunctional purine biosynthesis protein; CCT5, T-complex protein subunit epsilon; CCT6A, T-complex protein subunit zeta; CCT8, T-complex protein subunit theta; CD109, CD109 antigen; CFL1, cofilin-1; CLTC, clathrin heavy chain 1; CNDP2, cytosolic non-specific dipeptidase; DYNC1H1, cytoplasmic dynein heavy chain 1; EPRS, bifunctional glutamate/proline-tRNA ligase; FKBP4, peptidyl-prolyl cis-trans isomerase; GRP78, 78 kDa glucose-regulated protein; HSP90AB1, heat shock protein 90 beta; HSP90B1, endoplasmic, MYO6, myosin 6; HSPA4, heat shock protein 4; HSPA6, heat shock protein 6; HSPA8/Hsc71, heat shock cognate 71 kDa protein; IQGAP1, ras GTPase-activating-like protein 1; LOC781156, proteasome alpha subunit 2; MYH9/14, myosin 9 or 14; P4HB, protein disulphide isomerase; PA2G4, proliferation-associated protein 2G4; PDIA6, protein disulphide isomerase 6; PFKL, ATP-dependent 6-phosphofructokinase; PGK1, phosphoglycerate kinase; RPL19, 60S ribosomal protein L19; TKT, transketolase; TPM3, tropomyosin-alpha-3 chain; TUBB4A/BA1C, tubulin beta-4 chain or alpha-1C chain.

Table 2 Top-40 differentially abundant proteins between ipsi- and contralateral bovine oviductal fluids (OFs) at Pre-ov. Biological functions were retrieved from PANTHER database.

Protein name	Gene name	Ipsi	Contra	Ipsi: contra ratio	Biological functions
More abundant in ipsilateral OF					
Clathrin heavy chain 1	CLTC	58.9	21.0	2.8	Synaptic transmission, neurotransmitter secretion, intracellular protein transport.
Ras GTPase-activating-like protein 1	IQGAP1	41.6	2.2	19	Metabolic process, cell cycle, cell communication.
CD109 antigen	CD109	41.4	9.9	4.2	–
Cytoplasmic dynein 1 heavy chain 1	DYNC1H1	35.0	9.3	3.8	Metabolic process, cellular component movement, mitosis, chromosome segregation.
Transketolase	TKT	32.9	12.1	2.7	Vitamin biosynthetic process, pentose-phosphate shunt, cellular amino acid catabolic process.
Bifunctional purine biosynthesis protein	ATIC	29.8	11.9	2.5	Purine nucleobase metabolic process.
T-complex protein 1 subunit theta	CCT8	27.7	13.1	2.1	Protein folding, protein complex assembly, protein complex biogenesis.
T-complex protein 1 subunit epsilon	CCT5	23.1	4.1	5.6	Protein folding, protein complex assembly, protein complex biogenesis.
T-complex protein 1 subunit zeta	CCT6A	22.5	6.1	3.7	Protein folding, protein complex assembly, protein complex biogenesis.
Bifunctional glutamate/proline – tRNA ligase	EPRS	21.3	1.3	17	tRNA metabolic process, protein metabolic.
Uncharacterized protein LOC105084588	LOC105084588	10.6	0.3	33	–
Proteasome subunit beta type-5	PSMB5	7.4	0.3	23	Proteasome-mediated ubiquitin-dependent catabolic process, response to oxidative stress.
26S protease regulatory subunit 4	PRS4	6.6	2.5	2.6	Metabolic process.
IQ motif containing GTPase activating protein 2	IQGAP2	6.4	0.0	0.0	Metabolic process, cellular component movement, mitosis.
Serine/threonine protein phosphatase	CPPED1	6.0	0.9	6.5	Phosphate-containing compound metabolic process.
Talin 1	TLN1	5.3	2.5	2.1	Cellular process, cellular component morphogenesis, cellular component organization.
Alpha-2-macroglobulin	A2M	3.9	0.3	12	Complement activation, proteolysis, cellular process.
Fructose 1,6-bisphosphate aldolase	HmN_000669700	3.9	1.6	2.4	–
Nucleoside diphosphate kinase B	NME2	3.3	0.7	4.9	Apoptotic process, phosphate-containing compound metabolic process, nitrogen compound metabolic process.
Phosphoglucosyltransferase-2	PGM2	2.6	0.3	8.1	Glycosyltransferase.
More abundant in contralateral OF					
Protein disulphide isomerase 6	PDIA6	35.0	85.2	0.4	Protein folding, cellular process, response to stress.
Endoplasmic	HSP90B1	34.5	67.9	0.5	Immune system, protein folding, response to stress.
Myosin 6	MYO6	0.0	18.1	#	Metabolic process, cytokinesis, cellular component movement.
Protein disulphide isomerase	P4HB	2.3	9.6	0.2	Apoptotic process, protein folding, cell communication.
Heat shock 70kDa protein 4	HSPA4	4.3	8.9	0.5	Immune system, protein folding, protein complex assembly.
Proteasome alpha 2 subunit	LOC781156	3.3	7.3	0.4	Proteolysis.
Proliferation-associated protein 2G4	PA2G4	1.6	7.3	0.2	Translation, cellular protein modification, proteolysis.
Tropomyosin-alpha-3 chain	TPM3	3.2	7.1	0.5	Metabolic process, cellular component movement, muscle contraction.
4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	3.3	7.0	0.5	Metabolic process.
60S ribosomal protein L19	RPL19	2.9	6.4	0.5	Translation.
Poly(rC)-binding protein 2	PCBP2	0.0	6.0	#	Induction of apoptosis, RNA splicing via transesterification reactions, transcription from RNA polymerase II promoter.
LIM and SH3 domain protein 1	LASP1	2.9	5.4	0.5	Ion transport.
60S ribosomal protein L14	RPL14	0.4	5.1	0.07	Translation.

(Continued)

Table 2 Continued.

Protein name	Gene name	Ipsi	Contra	Ipsi: contra ratio	Biological functions
Calcium/calmodulin-dependent protein kinase type II subunit α	EH28_09084	0.4	4.7	0.08	Translation.
Cullin-associated NEDD8-dissociated protein 1	CAND1	1.3	3.8	0.3	Protein complex assembly, cellular protein modification, cellular process.
Stathmin 1/oncoprotein 18	STMN1	1.6	3.5	0.5	Regulation of microtubule polymerization or depolymerization.
Membrane-associated progesterone receptor component 1	PGRMC1	0.0	3.5	#	Meiotic cell cycle process involved in oocyte maturation.
Proteasome subunit beta type-6 precursor	PSMB6	0.3	3.5	0.09	Proteolysis involved in cellular catabolic process.
Dihydropyrimidine dehydrogenase [NADP(+)]	DPYD	0.0	2.6	#	Nitrogen compound metabolic, catabolic, pyrimidine nucleobase metabolic.
Protein transport protein Sec31A	SEC31A	0.0	2.6	#	Intracellular protein transport, exocytosis.

Contra, NSC in contralateral OF; Ipsi, normalized spectral counts (NSC) in ipsilateral OF. # indicates proteins specific to the contralateral side of ovulation.

catalytic proteins, around another third (25–30%) were binding proteins and between 11 and 23% were structural proteins (Supplementary Figs 2, 3, 4, 5 and 6). A wide range of biological processes was evidenced, among which metabolism and cellular processes were the main categories and included 32–38% and 15–18% of differentially abundant proteins respectively (Supplementary Figs 2, 3, 4, 5 and 6).

Finally, in order to integrate differentially abundant proteins between Pre-ov and Post-ov in ipsilateral OF in a more general model, functional interactions among these proteins were investigated using Ingenuity Pathway Analysis. One network involved in protein synthesis, cell cycle and cell survival integrated 28 differentially abundant proteins (out of 81: significant score of 52; Fig. 5A). A second network was implicated in post-translational modification, protein folding and cellular compromise and integrated 27 differentially abundant proteins (significant score of 50), including numerous HSP (Fig. 5B).

Discussion

Our objective was to investigate the effects of systemic and local regulatory mechanisms on the bovine oviductal proteome. We have reported here for the first time the proteomic content of the bovine oviductal fluid and have identified differentially abundant proteins according to the side of ovulation, the stage in the oestrous cycle and local concentrations of progesterone.

Very few proteins were found to be specific to a given side or stage. The relatively high number of proteins specific to the ipsilateral Post-ov OF compared with other stages (22 vs less than 6 specific proteins) may be linked to the important roles of the oviduct at these stages and sides. However, given the very low abundance (less than 4 normalized spectral counts) of these proteins detected at one stage and not at the three others, it is difficult to know whether these proteins are actually absent or below the detection limit in the other stages.

Functional classification of the proteins identified in the bovine OF revealed their main involvement in metabolism and cellular processes. Furthermore, most of the regulated proteins were involved in catalytic, binding activities or in protein folding and response to cell stress. A high proportion of identified proteins was thus classically known as non-secreted intracellular proteins and it is unclear how these proteins were exported from epithelial oviductal cells to localize in the OF. This may indicate a limitation of such analysis, mixing secreted proteins and proteins released in the milieu after natural cell death and induced unwitting cell damage. However, the proportions of proteins involved in different intracellular processes were in keeping with previously reported OF proteomes collected by flushing the oviducts of sows (Georgiou *et al.* 2005), ewes (Soleilhavoup *et al.* 2016) and mares (Smits *et al.* 2016). It is possible that unconventional ways of protein export, also known as ER/Golgi-independent secretion (Nickel 2003), and new extra-cellular functions of these intracellular proteins have yet to be discovered. Furthermore, the presence of extracellular vesicles, including exosomes, has been reported for bovine OF (Alminana *et al.* 2015). Exosomes are known to contain proteins and may also contain elements from the cytoplasm and cytoskeleton (Gyorgy *et al.* 2011). This could explain the presence of numerous ribosomal proteins, actin and other intracellular proteins in this study since the whole fluid, including extracellular vesicles, was analysed. Further research is needed to elucidate the mechanisms leading to the release of non-classically secreted proteins in the OF and to identify proteins entrapped in microvesicles in OF.

During oestrus in inseminated cows, a limited population of spermatozoa bind to the ciliated cells in the caudal isthmus of both oviducts, creating a local sperm reservoir to maintain the viability of spermatozoa before ovulation (Suarez & Pacey 2006, Suarez 2007). The number of bound spermatozoa was reported to be similar in ipsi- and contralateral oviducts after artificial insemination in cows (Sostaric *et al.* 2008). In addition

Table 3 Top-40 differentially abundant proteins between ipsi- and contralateral bovine oviductal fluids (OFs) at Post-ov. Biological functions were retrieved from PANTHER database.

Protein name	Gene name	Ipsi	Contra	Ipsi: contra ratio	Biological functions
More abundant in ipsilateral OF:					
78 kDa glucose-regulated protein	GRP78	250.5	52.1	4.8	Protein folding, protein complex assembly, response to stress.
Heat shock cognate 71 kDa protein	HSPA8	156.3	67.8	2.3	Immune system, protein folding, response to stress.
Heat shock protein 90 beta	HSP90AB1	140.4	66.7	2.1	Immune system, protein folding, response to stress.
Cytoplasmic dynein 1 heavy chain 1	DYNC1H1	119.8	27.5	4.4	Immune system, protein folding, response to stress.
Endoplasmic	HSP90B1	107.1	44.7	2.4	Metabolic process, cellular component movement, mitosis.
Myosin-9	MYH9	101.4	23.1	4.4	Metabolic process, cytokinesis, cellular component movement.
Myosin-14	MYH14	96.2	26.8	3.6	Metabolic process, cytokinesis, cellular component movement.
ATP-dependent 6-phosphofructokinase	PFKL	49.6	9.8	5.0	Immune system, protein folding, response to a stress.
Heat shock 70 kDa protein 6	HSPA6	43.9	18.6	2.4	Immune system, protein folding, response to stress.
Cofilin-1	CFL1	42.8	19.5	2.2	Protein complex assembly, cellular process, component morphogenesis.
Protein S100-B	S100B	35.4	9.9	3.4	Macrophage activation, DNA replication, cell cycle.
Protein disulphide isomerase A4	PDIA4	33.8	9.9	25	Protein folding, cellular process, response to stress.
Serotransferrin	TF	32.8	1.3	11	Metabolic process, transport.
Aminopeptidase B	RNPEP	25.1	2.3	2.3	Proteolysis.
Talin 1	TLN1	21.9	9.7	2.7	Cellular process, cellular component morphogenesis, cellular component organization.
Threonine-tRNA ligase	TARS	20.3	7.5	9.6	tRNA metabolic process, protein metabolic.
60S ribosomal protein L12	RPL12	19.3	2.0	2.5	Translation.
Micromolar calcium-activated neutral protease 1	CAPN1	17.2	6.8	11	Protein metabolic process.
Peroxisome oxidin-5	PRDX5	13.4	1.2	2.0	Cellular response to reactive oxygen species, hydrogen peroxide catabolic process, NADPH oxidation.
Aldehyde dehydrogenase family 16 member A1	ALDH16A1	12.5	6.1	4.6	Metabolic process.
More abundant in contralateral OF:					
Tubulin beta-4B chain	TUBB4A	149.3	329.3	0.5	Cellular component movement, mitosis, chromosome segregation.
Tubulin alpha-1C chain	TUBA1C	119.6	248.4	0.5	Cellular component movement, mitosis, chromosome segregation.
Pyruvate kinase	PKM	26.8	101.7	0.3	Glycolysis.
Epoxide hydrolase 2	EPXH2	26.7	85.2	0.3	Metabolic process.
Bifunctional purine biosynthesis protein	ATIC	13.3	55.4	0.2	Purine nucleobase metabolic process.
Adenosylhomocysteinase	AHCY	24.1	51.3	0.5	Coenzyme metabolic process, sulphur compound metabolic process, nitrogen compound metabolic process.
Peptidyl-prolyl cis-trans isomerase	FKBP4	20.5	43.3	0.5	Protein folding, cellular protein modification process, cellular process.
Cytosolic non-specific dipeptidase	CNDP2	13.2	38.1	0.3	Cellular amino acid biosynthetic process, protein phosphorylation, proteolysis.
Phosphoglycerate kinase 1	PGK1	19.3	36.7	0.5	Glycolysis.
T-complex protein 1 subunit theta	CCT8	9.6	34.5	0.3	Protein folding, protein complex assembly, protein complex biogenesis.
Glucose-6-phosphate isomerase	GPI	9.4	32.6	0.3	Angiogenesis, gluconeogenesis, glycolysis.
Na(+)/H(+) exchange regulatory cofactor	SLC9A3R1	13.4	29.9	0.4	Actin cytoskeleton organization, adenylate cyclase-activating domain receptor signalling pathway.
Tryptophan-tRNA ligase	WARS	6.5	27.4	0.2	Translation.
Adenylyl cyclase-associated protein 1	CAP1	9.3	27.3	0.3	Cell communication.
T-complex protein 1 subunit zeta	CCT6A	4.0	26.1	0.2	Protein folding, protein complex assembly, protein complex biogenesis.

(Continued)

Table 3 Continued.

Protein name	Gene name	Ipsi	Contra	Ipsi: contra ratio	Biological functions
T-complex protein 1 subunit epsilon	CCT5	7.0	19.9	0.4	Protein folding, protein complex assembly, protein complex biogenesis.
Retinal dehydrogenase 2	ALDH1A2	4.1	17.7	0.2	Metabolic process.
Eukaryotic translation elongation factor 1 epsilon-1	EEF1E1	8.7	17.5	0.5	Immune system, translation, cell communication.
RuvB-like 2	RUVBL2	7.1	16.5	0.4	DNA recombination, DNA repair, histone H2A acetylation.
D-3-Phosphoglycerate dehydrogenase	PHGDH	5.2	14.7	0.4	Glycolysis, gluconeogenesis.

Contra, NSC in contralateral OF; Ipsi, normalized spectral counts (NSC) in ipsilateral OF.

Figure 3 (A and B) Percentages of differentially abundant proteins between stages (black bars, $n=482$), and percentages of proteins more abundant at one stage (white bars; the stage in which most abundant proteins were evidenced and numbers of differentially abundant proteins are indicated) in ipsi- (A) and contralateral (B) bovine oviductal fluids. Stages are symbolized by boxes in the pictograms below each bar. ov, ovulation. (C) Variation of main differentially abundant proteins according to the stage of the oestrous cycle in ipsilateral oviductal fluid. ANXA1/A2, annexins A1/A2; CCT6A, T-complex subunit zeta; CCT8, T-complex subunit theta; CD109, CD109 antigen; DYNC1H1, cytoplasmic dynein 1 heavy chain 1; EPXH2, epoxide hydrolase 2; GRP78, 78 kDa glucose-regulated protein; GSTP1, glutathione S-transferase P; HSP, heat shock protein; OVGP1, oviduct-specific protein 1; PDIA4, protein disulphide isomerase 4; PEBP1, phosphatidylethanolamine-binding protein 1; PFKL, ATP-dependent 6-phosphofructokinase; PKM, pyruvate kinase; PRDX2, peroxiredoxin 2; TARS, threonine-tRNA ligase; TCPI, T-complex protein 1; TF, Serotransferrin; TXN, thioredoxin.

Table 4 Top-40 differentially abundant proteins between Pre-ov and Post-ov in bovine ipsilateral oviductal fluids (OFs). Biological functions were retrieved from PANTHER database.

Protein names	Gene symbol	Pre-ov	Post-ov	Pre: post-ov ratio	Biological functions
More abundant at Post-ov					
78kDa glucose-regulated protein	GRP78	37.0	250.5	0.1	Protein folding, protein complex assembly, response to stress.
Heat shock protein 90 alpha	HSP90AA1	109.5	213.9	0.5	Immune system, protein folding, response to a stress.
Heat shock cognate 71 kDa protein	HSPA8	62.8	156.3	0.4	Immune system, protein folding, response to a stress.
Heat shock protein 90 beta	HSP90AB1	64.1	140.4	0.5	Immune system, protein folding, response to a stress.
Cytoplasmic dynein 1 heavy chain 1	DYNC1H1	53.2	119.8	0.3	Metabolic process, cellular component movement, mitosis.
Endoplasmic	HSP90B1	34.5	107.1	0.3	Immune system, protein folding, response to a stress.
ATP-dependant 6-phosphofructokinase	PFKL	7.6	49.6	0.2	Glycolysis.
Heat shock 70kDa protein 6	HSPA6	20.2	43.9	0.5	Immune system, protein folding, response to stress.
Protein disulphide isomerase 4	PDIA4	0.4	32.8	0.01	Protein folding, cellular process, response to a stress.
Serotransferrin	TF	7.5	25.1	0.3	Metabolic process, transport.
Talin 1	TLN1	5.3	20.3	0.3	Cellular process, cellular component morphogenesis, cellular component organization.
Threonine-tRNA ligase	TARS	2.3	19.3	0.1	tRNA metabolic process, protein metabolic.
Ribonuclease inhibitor	RNH1	3.0	17.7	0.2	Antigen processing, nucleobase-containing compound metabolic process, cellular defence response.
3'(2'),5'-Bisphosphate nucleotidase 1	BPNT1	8.0	15.0	0.5	Sulphur compound metabolic process, phospholipid metabolic process, nucleobase-containing compound metabolic process.
Micromolar calcium-activated neutral protease 1	CAPN1	0.0	13.4	#	Metabolic process.
Fatty acid synthase	FASN	0.7	11.6	0.06	Cellular amino acid metabolic process, fatty acid biosynthetic process.
Septin-9	SEPT9	5.6	11.1	0.5	Metabolic process, cytokinesis, mitosis.
Filamin-B	FLNB	1.0	11.0	0.09	Cellular component movement, cellular component morphogenesis, cellular component organization.
Septin-2	SEPT2	1.3	8.7	0.2	Metabolic process, cytokinesis, mitosis.
Sorting nexin-2	SNX2	0.7	8.4	0.08	Cellular process, vesicle-mediated transport, organelle organization.
More abundant at Pre-ov					
Annexin A1	ANXA1	325.0	99.3	3.4	Fatty acid metabolic process, cell communication.
Pyruvate kinase	PKM	84.5	26.8	3.2	Glycolysis.
Epoxide hydrolase	EPXH2	78.2	26.7	2.9	Metabolic process.
Annexin A2	ANXA2	69.0	33.8	2.0	Fatty acid metabolic process, mesoderm development.
Clathrin heavy chain 1	CLTC	58.9	19.7	3.0	Synaptic transmission, neurotransmitter secretion, Intracellular protein transport.
Aflatoxin B1 aldehyde reductase member 2	AKR7A2	40.1	9.8	4.1	Metabolic process, cation transport.
Cytosolic non-specific dipeptidase	CNDP2	33.5	13.2	2.5	Cellular amino acid biosynthetic process, protein phosphorylation, proteolysis.
Bifunctional purine biosynthesis protein	ATIC	29.8	13.3	2.2	'de novo' IMP biosynthetic process.
T-complex protein 1 subunit theta	CCT8	27.7	9.6	2.9	Protein folding, protein complex assembly, protein complex biogenesis.
T-complex protein 1 subunit zeta	CCT6A	22.5	4.0	5.6	Protein folding, protein complex assembly, protein complex biogenesis.
Bifunctional glutamate/proline-tRNA ligase	EPRS	21.3	6.6	3.2	tRNA metabolic process, protein metabolic process.
Tryptophan-tRNA ligase	WARS	16.9	6.5	2.6	Translation.
Retinal dehydrogenase 2	ALDH1A2	14.4	4.1	3.5	Metabolic process.

(Continued)

Table 4 Continued.

Protein names	Gene symbol	Pre-ov	Post-ov	Pre: post-ov ratio	Biological functions
D-3-phosphoglycerate dehydrogenase	PHGDH	14.3	5.2	2.8	Carbohydrate metabolic process, cellular amino acid biosynthetic process.
Delta-aminolevulinic acid dehydratase	ALAD	10.6	3.9	3.7	Porphyrin-containing compound metabolic process.
Uncharacterized protein LOC105084588	–	10.6	0.6	17	–
60S ribosomal protein L4	RPL4	10.0	4.2	2.5	Translation.
T-complex protein 1 subunit alpha	TCP1	9.5	1.9	5.3	Protein folding.
EF-hand domain-containing protein D2	EFHD2	7.5	2.0	4.7	Mesoderm development, muscle organ development.
Thioredoxin	TXN	7.5	1.5	4.9	Cell redox homeostasis.

Post-ov, NSC during the post-ovulatory stage; Pre-ov, normalized spectral counts (NSC) during the pre-ovulatory stage. The symbol # indicates proteins specific to the Post-ov stage.

to sperm-cell interactions, associations of spermatozoa with oviductal proteins secreted before ovulation may contribute to sperm survival and their ability to fertilize the oocyte (Rodriguez & Killian 1998, Boilard *et al.* 2004, Elliott *et al.* 2009, Marin-Briggiler *et al.* 2010). However, to date, very few candidate proteins interacting with spermatozoa in the bovine oviduct have been identified. In this study, a total of 30 and 24 proteins were found to be more abundant at Pre-ov compared with Post-ov in ipsi- and contralateral oviducts respectively. Annexins A1 (ANXA1) and A2 (ANXA2), previously recognized as membrane proteins expressed at the apical side of oviductal epithelial cells in the bovine (Teijeiro *et al.* 2016), were among the most abundant proteins before ovulation. Antibodies directed against annexins A1 and A2 were shown to inhibit the binding of bull sperm to explants of oviductal epithelium, placing these proteins as strong candidates for interaction with spermatozoa *in vivo* (Ignotz *et al.* 2007). Several subunits of the T-complex protein 1 (subunit alpha or TCP1, theta or CCT8 and zeta or CCT6A), classically implicated in protein folding and complex assembly, were also among the most abundant proteins at Pre-ov compared with Post-ov OF samples. High-molecular-weight protein complexes including TCP1 were found on the surface of human, murine and bovine spermatozoa (Dun *et al.* 2011, Redgrove *et al.* 2011, Byrne *et al.* 2012) and have been implicated in sperm–oocyte interaction at the time of fertilization (Dun *et al.* 2011, Redgrove *et al.* 2011). Moreover, in a study analysing the proteomic response of porcine OF to the presence of gametes *ex vivo*, CCT8 was upregulated in the presence of spermatozoa (Georgiou *et al.* 2005). However, the potential roles of secreted T-complex proteins on gametes in the oviductal lumen are currently unknown.

Proteins involved in cell redox homeostasis were also upregulated at Pre-ov compared with other stages. For instance, peroxiredoxin-2 (PRDX2) was more abundant at Pre-ov than at Mid-lut and Late-lut in ipsilateral OF. Furthermore, PRDX2 tended to be negatively correlated with topical concentrations of P_4 in ipsilateral OF. Likewise, thioredoxin (TXN) was significantly more

abundant at Pre-ov than at Post-ov and Late-lut. Accordingly, the expression of thioredoxin increased in oviductal epithelia during oestrus compared with the luteal phase in mice (Osborne *et al.* 2001) and after E_2 administration in prepubertal female lambs (Sahlin *et al.* 2001). Interestingly, thioredoxin was upregulated in response to spermatozoa but not oocytes in porcine OF *ex vivo*, whereas peroxiredoxin-2 was upregulated in response to oocytes but not spermatozoa (Georgiou *et al.* 2005), suggesting the possible specific antioxidant effects on gametes around the time of ovulation. Supporting this hypothesis, the preincubation of spermatozoa with thioredoxin increased the rate of blastocyst formation obtained after *in vitro* fertilization in mice (Kuribayashi & Gagnon 1996). Thioredoxin added in the culture medium of mouse (Nonogaki *et al.* 1991) and bovine (Bing *et al.* 2003) embryos increased their rate of development to the blastocyst stage, suggesting the possible beneficial effect of this secreted protein even after fertilization.

After ovulation, oocyte maturation, fertilization and early embryo development take place only in the oviduct ipsilateral to the side of ovulation. To our knowledge, this is the first study investigating the effects of the side of ovulation on the oviductal proteome in mammals. The bovine, as a mono-ovular species, offers

Figure 4 Western blotting of four differentially abundant proteins (GRP78, HSPA8, HSP90AA1, ANXA1) and one protein with no significant variation (HSP70) between stages of the oestrous cycle in pools of bovine oviductal fluids. Mean ratios of western signal intensities between stages are indicated on the right. Ratio values for which significant differences between stages were found in the proteomic analysis are in bold.

Figure 5 Ingenuity Pathway Analysis networks integrated differentially abundant proteins between Pre-ov and Post-ov in bovine ipsilateral oviductal fluids. Entire lines represent direct and evidence interactions, dash lines represent presumed interactions between proteins. Proteins in green were upregulated at Post-ov whereas proteins in red were upregulated at Pre-ov, with the significance of that regulation represented by colour intensity. A: network integrating 28 differentially abundant proteins involved in protein synthesis, cell cycle and cell survival; B: network integrating 27 differentially abundant proteins involved in post-translational modification, protein folding and cellular compromise. The inset on the right represents a simplified representation of interactions between main regulated proteins.

a good model to study these local *in vivo* regulatory mechanisms. Several members of the heat shock protein (HSP) family, which are generally implicated in cellular response to stress and protein folding, were among the most abundant proteins in ipsilateral OF at Post-ov compared with other stages and sides. Similarly, in sheep, HSPA8 is more abundant in OF at oestrus compared with the luteal phase (Soleilhavoup *et al.* 2016). GRP78 followed the same pattern of expression in porcine oviduct epithelial cells (Seytanoglu *et al.* 2008). Furthermore, the gene expression of GRP78 was upregulated during oestrus compared with Day 12 post-oestrus in bovine oviductal epithelial cells (Bauersachs *et al.* 2004). Both HSPA8 and GRP78 were previously identified as membrane proteins localized on the apical plasma membranes of bovine oviductal epithelial cells and were shown to bind spermatozoa *in vitro* in several mammalian species, including the bull (Boilard *et al.* 2004, Lachance *et al.* 2007, Elliott *et al.* 2009, Marin-Briggiler *et al.* 2010). Furthermore, HSPA8 was shown to enhance bull sperm viability *in vitro* (Elliott *et al.* 2009). Thus, the overabundance of HSPA8 in ipsilateral OF after ovulation may play a role in sperm storage, although such a role would be expected before ovulation. Human sperm incubated with recombinant GRP78 exhibited an enhanced P_4 -induced increase in intracellular calcium, which is a crucial step for capacitation (Lachance *et al.* 2007). *In vitro*, fewer GRP78-treated sperm cells bound to the oocyte zona pellucida compared with non-incubated spermatozoa (Marin-Briggiler *et al.* 2010), suggesting that GRP78 may modulate the ability of spermatozoa to fertilize the oocyte.

Heat shock protein 90 (alpha subunit HSP90AA1 and beta subunit HSPAB1) was another HSP protein more abundant in ipsilateral OF at Post-ov compared with other stages and side. Another study revealed greater gene expression of HSP90AA1 in the summer than in the winter in bovine oviductal tissues and after heat stress in cultured oviductal epithelial cells (Kobayashi *et al.* 2013). As HSP90 is known to stimulate prostaglandin (PG) E_2 production in fibroblasts, it was hypothesized that heat stress may deregulate the HSP90-dependent balance between PGE_2 and $PGF_{2\alpha}$ synthesis and thus disrupt oviduct motility and gamete/embryo transport in cattle (Kobayashi *et al.* 2013). HSP90 was identified among oviductal apical plasma membrane proteins that bound to boar spermatozoa *in vitro* (Elliott *et al.* 2009). However, the potential role played by oviductal HSP90 on sperm function remains unknown.

Disulphide isomerase A4 (PDIA4), involved in cellular response to stress, and the threonine-tRNA ligase (TARS), implicated in tRNA and protein metabolism, were also among the most upregulated proteins in ipsilateral post-ovulatory OF. Both proteins were previously identified in the bovine OF as potentially responsible for hardening the zona pellucida of the oocyte after ovulation

(Mondejar *et al.* 2013). PDIA4 and TARS may thus play an important role in the maintenance of the high rate of monospermy at the time of fertilization *in vivo*. The protein whose role in zona pellucida hardening before fertilization has been well-described, at least in the cow and pig, is the oviduct-specific glycoprotein 1 (OVGP1; Coy *et al.* 2008, Coy *et al.* 2012). OVGP1 has been implicated in numerous other periovulatory events, such as the maintenance of viability and motility of bull spermatozoa (Abe *et al.* 1995) and embryo development in the pig (Kouba *et al.* 2000) and goat (Pradeep *et al.* 2011). Myosin 9, which followed the same pattern of abundance as that of proteins described previously, was suggested to be a binding partner to OVGP1 on both gametes (Kadam *et al.* 2006) and, as such, may also play a role in gamete maturation around the time of ovulation within the oviduct.

The relative proportion of secretory cells in the bovine oviductal epithelium is stable between the ipsi- and contralateral oviducts around the time of ovulation in the cow (Sostaric *et al.* 2008). Therefore, differences between sides in the oviductal fluid proteome may be due to the local regulation of the oviductal secretory activity. Important fluctuations in topical concentrations of P_4 and E_2 in the OF were previously reported among stages considered in this study (Lamy *et al.* 2016). These changes were mainly recorded in ipsilateral OF, in which P_4 levels increased from Post-ov (mean concentration of 56.9 ± 13.4 ng/mL) to Mid-lut (120.3 ± 34.3 ng/mL), then decreased from Late-lut (76.7 ± 1.8 ng/mL) to Pre-ov (6.3 ± 1.7 ng/mL) and were 4–16 times higher than in contralateral OF. Among the differentially abundant proteins between stages in ipsilateral OF, seven tended to be correlated with P_4 . Similarly, numerous ions, amino acids and energy substrates secreted in the bovine OF were reported to vary depending on circulating concentrations of P_4 (Hugentobler *et al.* 2010). Due to the limited number of points measured across the reproductive cycle (4), the present correlations were not significant but are to be regarded as preliminary data on hormonal regulatory mechanisms of the oviductal proteome. Surprisingly, two ribosomal protein (RPL18 and RPS19) and beta-actin, all three proteins often considered as stable proteins, were found among these regulated proteins. Similarly, the protein expression of actin was higher during the follicular phase than during the luteal phase of the reproductive cycle in porcine oviduct epithelial cells (Seytanoglu *et al.* 2008).

In addition to actin and RPL18, three other proteins tended to be negatively correlated with local P_4 levels: peroxiredoxin-2 (PRDX2), already evoked as one of the most abundant protein at Pre-ov, high mobility group box 1 protein (HMGB1), also known as amphoterin, and phosphatidylethanolamine-binding protein 1 (PEBP1). PEBP1 has been identified among potential

'decapacitation factors' that were able, when added *in vitro* to suspensions of mouse spermatozoa, to inhibit sperm ability to undergo P₄-induced acrosome reaction and to bind to the zona pellucida (Gibbons *et al.* 2005, Nixon *et al.* 2006). Thus, the increase in P₄ in the oviduct at the time of ovulation, in addition to triggering sperm hyperactivation (Fujinoki *et al.* 2016), may allow the inhibition of such decapacitation factor and finally contribute to the presence of spermatozoa able to fertilize around the oocyte.

Two networks were generated from differentially abundant proteins around the time of ovulation using Ingenuity Pathway Analysis. These networks revealed numerous potential interactions among proteins of the HSP family and between HSP and proteins of the T-complex family as well as septins 2 and 9. Indirect interactions were also found between HSP or ANXA2 and PDIA4. It is possible that proteins with direct interactions in ingenuity nets form proteins complexes in the OF around the time of ovulation to become functionally active, as recently hypothesized for PDIA4 and HSP90B1 in promoting the hardening of the oocyte zona pellucida in the porcine OF (Mondejar *et al.* 2013). As shown in Fig. 5B, it is possible that HSPA8, interacts with HSP90 and/or septin-2, all three proteins upregulated after ovulation, and that the resulting complexes play roles in post-ovulatory events. However, networks built by IPA are based on the current knowledge on molecular interactions. As these interactions were mostly studied in the intracellular and membrane compartments, such interactions among soluble proteins in the OF or inside extracellular microvesicles remain speculative and require further investigation.

Conclusion

In summary, this study is the first to monitor the proteome of the bovine oviductal fluid across the oestrous cycle and in both sides relative to ovulation. We identified a number of secreted proteins potentially regulated by endocrine and local mechanisms. These results provide a basis for a better understanding of the regulation of oviduct physiology and the oviductal environment and suggest new candidate proteins that may interact with gametes and embryo to modulate the reproductive events around the time of fertilization. Further studies will be required to analyse and understand differences among soluble proteins in the soluble fraction and those in extracellular vesicles in OF and to determine their function in early reproductive events.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0397>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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