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Charles Banliat, Daniel Tomas, Ana-Paula Teixeira-Gomes, Svetlana Uzbekova, Benoît Guyonnet, Valérie Labas, Marie Saint-Dizier

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1 **Title: Stage-dependent changes in oviductal phospholipid profiles throughout the**  
2 **estrous cycle in cattle**

3

4 **Authors' names and affiliations:** Charles Banliat<sup>a,b</sup>, Daniel Tomas<sup>a,c</sup>, Ana-Paula Teixeira-  
5 Gomes<sup>c,d</sup>, Svetlana Uzbekova<sup>a</sup>, Benoît Guyonnet<sup>b</sup>, Valérie Labas<sup>a,c</sup> and Marie Saint-Dizier<sup>a,e</sup>

6

7 <sup>a</sup>UMR PRC, INRA 85, CNRS 7247, University of Tours, IFCE, 37380 Nouzilly, France

8 <sup>b</sup>Union Evolution, rue Eric Tabarly CS10040, 35538 Noyal-Sur-Vilaine, France

9 <sup>c</sup>Plate-forme de Chirurgie et d'Imagerie pour la Recherche et l'Enseignement (CIRE), Pôle  
10 d'Analyse et d'Imagerie des Biomolécules (PAIB), INRA, CHRU of Tours, University of  
11 Tours, 37380 Nouzilly, France.

12 <sup>d</sup>UMR ISP, INRA 1282, University of Tours, 37380 Nouzilly, France

13 <sup>e</sup>University of Tours, Faculty of Sciences and Techniques, 37200 Tours, France

14 Corresponding author: Marie Saint-Dizier, University of Tours, Faculty of Sciences and  
15 Techniques, Parc de Grandmont, 37200 Tours, France; Tel : +33 247367014; Fax : +33  
16 247367094; Email: marie.saint-dizier@univ-tours.fr

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**Abstract**

Sperm capacitation, fertilization and embryo development take place in the oviduct during the periovulatory period of the estrous cycle. Phospholipids are crucial metabolites for sperm capacitation and early embryo development. The aim of this study was to monitor the abundance of phospholipids in the bovine oviductal fluid (OF) according to the stage of the estrous cycle and the side relative to ovulation. Pairs of bovine oviducts were collected in a slaughterhouse and classified into four stages of the estrous cycle: post-ovulatory (Post-ov), mid-luteal (Mid-lut), late-luteal (Late-lut) and pre-ovulatory (Pre-ov) phases (n = 17 cows/stage). Cell-free OF from oviducts ipsilateral and contralateral to the site of ovulation were analyzed using MALDI-TOF mass spectrometry. Lipid identification was achieved by high resolution mass spectrometry. A total of 274 lipid masses were detected in the mass range of 400-1000 Da, corresponding mostly to phosphatidylcholines (PC), lysoPC, phosphatidylethanolamine (PE), lysoPE and sphingomyelins (SM). Ipsilateral and contralateral OF did not differ in their lipid profiles at any stage of the cycle. However, 127 and 96 masses were differentially abundant between stages in ipsilateral and contralateral OF, respectively. Highest differences in lipid profiles were observed in the Pre-ov *vs.* Mid-lut and Pre-ov *vs.* Late-lut comparisons in both sides relative to ovulation. Differential abundance of specific molecules of PC, PE, SM and L-carnitine were observed at Pre-ov and Post-ov compared with the luteal phase. This work proposes new candidates potentially able to regulate sperm capacitation and early embryo development.

**Key words:** Bovine, oviduct fluid, estrus cycle, phospholipids, sphingolipids; MALDI-TOF mass spectrometry.

44

## 45 **1. Introduction**

46           The mammalian oviductal fluid (OF) is a complex and changing fluid resulting from  
47 the secretion of oviduct luminal epithelial cells, transudate from the circulating blood and  
48 presumably compounds from the follicular fluid at the time of ovulation [1, 2]. Gamete final  
49 maturation, fertilization and early embryo development take place in this dynamic fluid  
50 environment. However, the composition and physiological factors regulating the OF have  
51 been poorly investigated [3]. The OF contains a variety of lipids including fatty acids,  
52 triglycerides, cholesterol and phospholipids [4-6]. Glycerophospholipids and sphingolipids  
53 are vital energy substrates acting as structural and regulatory components of cell membranes  
54 and extracellular microvesicles [7-9]. In addition, a number of phosphatidylcholines (PC) and  
55 sphingomyelins (SM) are lipid mediators implied in many cell signaling pathways and act as  
56 crucial precursors of many biomolecules such as lysophospholipids (LPC) and prostaglandins  
57 [7].

58           After entering the oviduct, spermatozoa must accomplish capacitation to fertilize.  
59 Exogenous phospholipids and cholesterol have been reported to be taken up by spermatozoa,  
60 influencing sperm capacitation and ability to undergo acrosome reaction and fertilize the  
61 oocyte [10, 11]. After fertilization, developing embryos up to the blastocyst stage undergo  
62 important changes in their phospholipid composition [12]. There is evidence from *in vivo* and  
63 *in vitro* studies that the lipid environment to which early bovine embryos are exposed has a  
64 significant impact on their quality in terms of morphology, post-cryopreservation survival,  
65 lipidomic and transcriptomic signatures [13-16].

66           It is assumed that the oviductal secretions provide an optimal environment for sperm  
67 capacitation, fertilization and embryo development leading to the establishment of pregnancy.  
68 However, the specific requirements in lipid metabolism of gametes and embryo imply  
69 significant regulation in oviductal secretions between the periovulatory period and the luteal

70 phase of the cycle. In mono-ovular species, the side of ovulation may also affect the  
71 molecular composition of the proximal OF by topical hormonal regulations and putative input  
72 of the ovulatory follicle. Previously, we reported important fluctuations in the levels of steroid  
73 hormones, metabolites and proteins in the bovine OF according to the stage of estrous cycle  
74 and the side relative to ovulation [17-19]. The ovarian steroid hormones progesterone (P4)  
75 and 17 $\beta$ -estradiol (E2) play important regulatory roles in the secretory activity of the oviduct  
76 epithelium [17, 18, 20]. However, information on the lipid content of the oviduct is scarce.  
77 Only four studies on material from no more than four animals per study reported changes in  
78 the phospholipid content of the bovine OF across the cycle with no distinction between sides  
79 of ovulation nor between lipid species [4, 5, 21, 22]. A recent study reported the phospholipid  
80 profiles in uterine and oviductal tissues from zebuine females [23]. However, this study  
81 focused only on the early luteal (or post-ovulatory) phase of the estrous cycle in the ipsilateral  
82 oviduct.

83         The objectives of this study was thus to monitor the diversity and abundance of lipids  
84 in the bovine OF according to the stage of the estrous cycle and the side relative to ovulation.  
85 The MALDI-TOF (Matrix assisted laser desorption ionization - Time of flight) mass  
86 spectrometry (MALDI-MS) was used because it is largely recognized as a powerful, rapid and  
87 sensitive way to obtain lipid profiles in a relatively large range of molecular weights without  
88 requiring prior lipid extraction. For precise lipid identification, complementary tandem high-  
89 resolution mass spectrometry (HR-MS/MS) was applied.

90

## 91 **2. Materials and Methods**

### 92 ***2.1. Collection of bovine oviductal fluids***

93 Both oviducts and ovaries from adult *Bos Taurus* cows were collected at a local  
94 slaughterhouse (Vendôme, France), placed on ice within 15 min after death and transported to  
95 the laboratory (40-min transportation). Oviducts were classified into four stages in the estrous  
96 cycle according to the morphology of ovaries, as previously described (Ireland et al 1980):  
97 post-ovulatory (Post-ov), mid luteal (Mid-lut), late luteal (Late-lut) and pre-ovulatory (Pre-ov)  
98 stages of the estrous cycle (N=17 cows/stage). To avoid the inclusion of cows with cystic  
99 follicles in the Pre-ov group, animals with a Pre-ov follicle larger than 20 mm in diameter  
100 (>2.4 mL of follicular fluid) and/or with intra-follicular P4 concentrations higher than 160  
101 ng/mL (as measured by a competitive enzyme-linked immunosorbent assay [24]) were not  
102 included. The OF and epithelial cells were collected from the whole ipsilateral (to corpus  
103 luteum or Pre-ov follicle) and contralateral oviducts by gentle squeezing with a glass slide.  
104 Mixtures were stored on ice then OF was separated from the cells and cellular debris by two  
105 centrifugations (2000 g, 15 min then 12 000 g, 10 min) at 4°C. The OF collected (20-100 µL  
106 per oviduct) was immediately stored in liquid nitrogen to preclude any lipid degradation  
107 before analysis. The whole processing time from animal death up to sample storage was less  
108 than 4 hours.

## 109 **2.2. Lipid profiling by MALDI-TOF mass spectrometry (MALDI-MS)**

110 Lipid profiling of OF samples was performed as previously described for bovine follicular  
111 fluid [25] with slight modifications. Briefly, the samples were thawed and sonicated on ice for  
112 10 min. Then, 0.5 µL of OF was spotted on the TP Ground Steel 384 MALDI plate (Bruker  
113 Daltonics, Bremen, Germany), dried at room temperature for 30 minutes then overlaid with 2  
114 µL of 2,4,6-trihydroxyacetophenone (THAP) matrix at 10 mg/mL solubilized in 90%  
115 methanol and 0.2% (w/v) trifluoroacetic acid (TFA) and containing 0.001 mg/mL of  
116 phosphatidylcholine (PC 20:0, m/z 604.3375; Sigma P7081) as internal standard (not initially  
117 present in the OF). The sample/matrix mixtures were then dried at room temperature for 30

118 min. For each sample, five technical replicates were spotted. Spectra were acquired with an  
119 UltrafleXtreme MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped  
120 with a Smartbeam laser at 2 kHz laser repetition controlled by flexControl 3.4 software  
121 (Bruker Daltonics, Bremen, Germany). A total of 10 spectra per sample (2 spectra per  
122 technical replicate, 5000 shots per spectra) were acquired in the positive reflectron ion mode  
123 in the mass/charge ( $m/z$ ) range of 100-1200. External calibration was performed using a  
124 mixture of caffeine, MRFA peptide, leu-enkephalin, bradykinine 2-9 and glu1-fibrinopeptide at  
125 1 mM each, 1.64 mM of reserpine, 942  $\mu\text{M}$  of bradykinine and 771  $\mu\text{M}$  of angiotensine in 1  
126  $\mu\text{L}$  of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA, 20 mg/mL) solubilized in 50% acetonitrile  
127 and 0.1% (w/v) TFA. To increase the mass accuracy (mass error  $<0.05\%$ ), an internal  
128 calibration using a lock mass at  $m/z$  604.3375 corresponding to PC 20:0 was subsequently  
129 applied to all spectra by flexAnalysis 3.4 software (Bruker Daltonics), as previously described  
130 [25]. The data were integrated in the R software (version 3.5.0) and treated for baseline  
131 subtraction using the Statistics-sensitive Non-linear Iterative Peak-clipping algorithm (SNIP,  
132 minimum 15 iterations), smoothing (Savitzky-Golay algorithm,  $m/z$  range = 0.1), spectra  
133 alignment using prominent peaks (difference between spectra  $< 0.002\%$ ) and normalization  
134 on intensity using total ion count (TIC).

135 Peaks were detected using a total average spectrum with a signal/background noise  $> 2$  in the  
136 mass range of 400-1000  $m/z$ . The different isotopes of a given mass were not considered for  
137 analysis. The coefficient of variation (CV%) of intensity values (normalized peak height,  
138 NPH) was calculated for all the peaks in 10 technical replicates per sample, from 17 different  
139 OF for each stage and side. The mean CV% of the normalized peak intensity values was less  
140 than 25 %.

### 141 **2.3. Analysis of MALDI-MS data**

142 Statistical analyses were performed using the MALDIquant and MALDIquantForeign  
143 packages (v 1.17 & v0.11.1) of the R software (version 3.5.0) [26]. MS data did not pass the  
144 Kolmogorov-Smirnov's test of normality. All lipidomic data without preselection were  
145 submitted to the non-parametric Wilcoxon test to identify changes between sides of ovulation  
146 and to the Kruskal-Wallis test followed by Tukey's post-tests to identify changes between  
147 stages of the estrous cycle. Masses were considered differentially abundant between groups  
148 with a P-value < 0.05. Hierarchical clustering of differential masses was performed using the  
149 gplot (v 3.0.1) and RcolorBrewer (v 1.1-2) packages of the R software. Principal Component  
150 Analysis (PCA) of differential masses was performed using the FactoMineR package (v1.41)  
151 of the R software. The fold-changes were calculated for each mass as ratios between the mean  
152 normalized intensity values at two stages.

#### 153 ***2.4. Lipid identification by high-resolution tandem mass spectrometry (HR-MS/MS)***

154 Total lipids were extracted from OF according to the 'Bligh and Dyer' method. A pool of 160  
155  $\mu\text{L}$  of OF (made with an equivalent volume (20  $\mu\text{L}$ ) of each stage and side) was mixed with  
156 50  $\mu\text{L}$  of methanol (MetOH), vortexed during 60 s then put on ice for 2 h. Chloroform (210  
157  $\mu\text{L}$ ) was added then the samples were mixed, centrifuged (2000 g, 20 s), sonicated on ice for  
158 10 min, mixed with 35  $\mu\text{L}$  of water (Optima LC/MS grade, Fisher Scientific, Illkirch, France)  
159 and put on ice for 10 min. The samples were then centrifuged at 2500 g for 10 min, allowing  
160 to separate the aqueous phase (polarized lipids) from the organic phase (non-polarized lipids).  
161 The aqueous phase was then dried by a SpeedVac system (SPD 1010-230, Thermo Savant  
162 Eletronic, France) and pellets were stored in liquid nitrogen before analysis. Extracts were re-  
163 solubilized in 10  $\mu\text{L}$  of methanol for the positive mode and in 10  $\mu\text{L}$  of ammonium acetate at  
164 20 mM in methanol for the negative mode, then analyzed on a dual linear ion trap Fourier  
165 Transform Mass Spectrometer (FT-MS) LTQ Orbitrap Velos (Thermo Fisher Bremen,  
166 Germany) using the Xcalibur software (version 2.1, Thermo Fisher Scientific, Bremen,



167 Germany), as previously described [25]. Briefly, FT-MS spectra were acquired using the  
168 profile mode in the 400–2000  $m/z$  mass range, and the FT-MS/MS spectrum using high-  
169 energy collisional dissociation (HCD) fragmentation ion mode. Target resolution was set at  
170 100,000 for FT-MS and FT-MS/MS analysis. The selected precursor width for fragmentation  
171 was 1.5  $m/z$ . FT-MS and FT-MS/MS spectra were acquired, over 10 min, in triplicate, using  
172 different collision energy levels (20 to 60 keV with a step of 5 keV for the positive mode, 30  
173 and 40 keV for the negative mode). Data were collected using spectral stitching technique as a  
174 series of 100- $m/z$  wide windows that overlap by 5  $m/z$ . Peaks were considered when >500  
175 counts.

## 176 **2.5. Validation and analysis of HR-MS/MS data**

177 FT-MS and FT-MS/MS data were converted to Mascot generic format (MGF) using the  
178 Proteome Discoverer (version 1.4, ThermoFisher Scientific, San Jose, USA) and the  
179 LipidMatch softwares [27]. Each automatic lipid identification performed by LipidMatch  
180 (mass accuracy of 0.05  $m/z$  for precursor and 50 ppm for fragment ions, score = level 1) was  
181 validated using LipidBlast 2.0 software [28]. For phospholipids and sphingolipids, all  
182 identifications with a Rev-Dot score >800 were accepted considering the presence of specific  
183 fragments ions error mass accuracy of 0.8 Da. For each identified lipid, theoretical mass (error  
184 mass accuracy < 0.05 Da) and formula were obtained through interrogation of the LIPID  
185 MAPS database (<http://www.lipidmaps.org>). Masses highlighted by the hierarchical clustering  
186 were annotated with the LIPID MAPS database using the same criteria.

187

## 188 **3. Results**

### 189 **3.1. Lipid species identified in the OF**

190 Using MALDI-MS, the lipid profile of the OF retrieved a total of 274 masses in the 400-1000  
191 m/z (ratio of mass to charge, equivalent to Da) range, annotated mainly as  
192 phosphatidylcholines (PC), lysoPC (LPC), phosphatidylethanolamine (PE), lysoPE (LPE) and  
193 sphingomyelins (SM). One representative spectrum is shown in Fig. 1 and all the annotations  
194 are listed in Suppl. Table 1. By HR-MS/MS, 63 lipid species were identified, corresponding  
195 to cholesterol, 16 species of PC, one LPC, 27 PE, 11 LPE, one phosphatidylinositol (PI), one  
196 phosphatidylserine (PS), one SM and four L-carnitines (CAR). The observed and theoretical  
197 masses, name and formula of all identified lipid molecules are in Suppl. table 2.

### 198 ***3.2. Changes in lipid profiles according to the stage of the estrous cycle***

199 For a given stage of the estrous cycle, the OF lipid profiles did not differ between ipsilateral  
200 and contralateral OF. However, the lipid profiles varied according to the stage of the estrous  
201 cycle: in total, 127 and 96 masses were differentially abundant between stages in ipsilateral  
202 and contralateral OF, respectively ( $P < 0.05$ ; see all differential masses with p-values and  
203 fold-changes in Supplementary table 2). The PCA on differential masses in the ipsilateral OF  
204 clearly separated the Pre-ov stage from the others (Fig. 2). The highest proportion of  
205 differential masses were identified in the Pre-ov vs. Mid-lut comparison (75.4% of differential  
206 masses; 70 masses more and 34 less abundant at Pre-ov), followed in decreasing order by Pre-  
207 ov vs. Late-lut (52.9%; 44 more and 29 less abundant at Pre-ov), Pre-ov vs. Post-ov (31.9%;  
208 21 more and 23 less abundant at Pre-ov), Post-ov vs. Mid-lut (13.8%; 17 more and 2 less  
209 abundant at Post-ov) and Post-ov vs. Late-lut (10.1%; 17 more and 2 less abundant at Post-ov;  
210 Fig. 3). The Mid-lut vs. Late-lut comparison showed no significant difference. The proportion  
211 and distribution of differential masses in the contralateral OF were similar (Suppl. Fig. 1).

### 212 ***3.3. Changes in lipid molecules according to the stage of the estrous cycle***

213 The hierarchical clustering of differential masses in the ipsilateral OF confirmed the  
214 specificity of the Pre-ov stage and outlined two clusters of differentially abundant masses at

215 Pre-ov compared with the other stages of the estrous cycle (Fig. 4). After identification or  
216 annotation of these particular masses, a first cluster with more abundant lipids at Pre-ov than  
217 at other stages included molecules of PE, SM and LPC while a second cluster of masses, less  
218 abundant at Pre-ov than at other stages, included molecules of CAR and LPC. This second  
219 cluster of less abundant masses at Pre-ov was the only one identified in the contralateral  
220 oviduct (Suppl. Fig. 2).

221 The differential masses identified by HR-MS/MS included three molecules of CAR (18:2,  
222 20:2 and 20:3), all decreased in abundance at Pre-ov compared with Post-ov, Mid-lut and  
223 Late-lut ( $P < 0.05$ ; Fig. 5). On the opposite, all identified molecules of PE (36:4, 36:5, 38:2,  
224 38:3, 38:4, 38:5, 39:6, 40:4 (that could be also attributed to PC(37:4), 40:5 and 40:6), PC  
225 (34:1, 36:3, 36:4, 38:3, 40:5) and SM (d42:1) were increased at Pre-ov compared with at least  
226 one of the other stages (Post-ov, Mid-lut and/or Late-lut). In addition, five molecules of PE  
227 (36:5, 38:3, 39:6, 40:4 and 40:5) and two of PC (34:1 and 36:3) were increased at Post-ov  
228 compared with Mid-lut and/or Late-lut ( $P < 0.05$ ; Fig. 5).

229

#### 230 **4. Discussion**

231 In order to better understand the regulation of lipid secretion within the tubal fluid, a  
232 global lipidomic approach using MALDI-MS and HR-MS/MS was applied. For the first time,  
233 a wide range of lipid compounds were identified by mass spectrometry in the bovine OF. The  
234 lipid profiles in the OF showed no variation between the sides relative to ovulation but  
235 changed significantly according to the stage of the estrous cycle, notably between the pre-  
236 ovulatory stage and the luteal phase.

237 Although they constitute crucial substrates for spermatozoa and developing embryos,  
238 lipids in the mammalian oviduct have not been studied in detail [3]. Most previous studies  
239 conducted on the bovine OF made no distinction between the lipid compounds that were

240 quantified [5, 21, 22]. In the present study, 63 lipid species, mostly phospholipids, were  
241 identified by HR-MS/MS. Our findings show that the tubal secretions contain a complex  
242 mixture of cholesterol, glycerophospholipids (PC, PE, PI and PS), lysophospholipids (LPC,  
243 LPE), sphingomyelins and carnitines. Carnitines ( $\beta$ -hydroxy- $\gamma$ -trimethylammonium butyrate)  
244 are quaternary amines required for the transfer of long-chain fatty acids to mitochondria for  
245 subsequent  $\beta$ -oxidation [29]. One previous study from Killian *et al.* identified various  
246 glycerophospholipids and lysophospholipids in the bovine OF after separation by liquid  
247 chromatography, digestion and analysis of inorganic phosphorus [4]. Using high resolution  
248 MS, a more recent study identified molecules of PC, SM and PE in oviductal sections from  
249 *Bos indicus* cows [23]. However, ceramides and diacylglycerols were also identified in the  
250 latter while PI, PS, LPC, LPE and CAR were identified only in the present study. These  
251 discrepancies in identified lipids could be due to differences in MS techniques but may also  
252 reflect difference between bovine species, oviduct compartment (whole tissue section *vs.* OF)  
253 and region (ampulla *vs.* whole oviduct) studied.

254         The source of phospholipids and mechanism of their accumulation in the OF remain  
255 uncertain. Epithelial cells and cellular debris were eliminated from the OF by centrifugation  
256 before analysis, however, part of epithelial cells collected together with the OF may have  
257 released their content at the time of collection. Furthermore, the OF contains microvesicles  
258 and exosomes, both known as extracellular vesicles (EV) [30], that may originate from the  
259 oviduct epithelium [31] as well as from the pre-ovulatory follicle [32]. A large part of  
260 microvesicles (100 to 1000 nm in diameter) initially present in the OF were probably  
261 eliminated in the 12 000 g pellet. However, all exosomes (30 to 150 nm in diameter), typically  
262 collected by ultracentrifugation [9], were included in the present analysis. The exact lipid  
263 composition of EVs in the OF is not known but these submicroscopic vesicles have a lipid  
264 composition rich in phospholipids, similar to those in the cell membrane [8] and exosomes are

265 generally enriched in cholesterol, SM, glycosphingolipids and PS [33]. Thus, the lipids  
266 identified in this study may originate from either the cytoplasm of oviduct epithelial cells  
267 and/or the EV released in the OF.

268         The MALDI-MS profiling of a total of 136 OF samples collected at four different  
269 stages of the estrous cycle and in both sides relative to ovulation (17 females per stage and  
270 side) allowed us to identify the stage in the estrous cycle as a major factor of regulation of the  
271 OF phospholipid content. By contrast, the side of ovulation had no significant effect on the  
272 lipid profiles through the estrous cycle. We cannot exclude variation in other OF lipid  
273 compounds (triglycerides, ceramides, diacylglycerols and phosphatidic acid) that were not  
274 detected by MALDI-MS. To our knowledge, this is the first study comparing the lipid  
275 compounds in the oviduct according to the side of ovulation in a mono-ovular species. Many  
276 studies comparing the OF composition between the two sides relative to ovulation, including  
277 those from our laboratory, reported differences in steroid hormones and protein content in the  
278 OF [18, 19]. However, similar to the present work, previous studies in the field of  
279 metabolomics failed to evidence differences in the concentrations of amino acids and energy  
280 substrates between ipsilateral and contralateral oviducts [34-37]. In accordance, in a recent  
281 study using nuclear magnetic resonance (NMR) spectroscopy, of the 26 metabolites  
282 quantified in the OF, the concentrations of 14 metabolites varied according to the stage of the  
283 cycle while only five were affected by the side of ovulation with low extent of changes  
284 between sides [17]. In the present study, the similarity in lipid profiles observed between the  
285 two sides of ovulation does not support the hypothesis of a contribution of the pre-ovulatory  
286 follicle to the lipid content of the OF, in particular at Post-ov.

287         Interestingly, the highest proportions of differentially abundant lipids were identified  
288 when comparing the Pre-ov and Mid-lut or Pre-ov and Late-lut stages in both sides relative to  
289 ovulation. In a previous study, we reported important fluctuations in the concentrations of the

290 ovarian steroid hormones P4 and E2 according to the stage of the estrous cycle in the bovine  
291 OF [19]. In accordance with fluctuations observed in the circulating plasma, oviductal levels  
292 of P4 were highest at Mid-lut and Late-lut stages and lowest at Pre-ov (means of 120.3 and  
293 76.7 vs. 6.3 ng/mL, respectively) whereas E2 reached maximal levels at Pre-ov and minimum  
294 levels at Mid- and Late-lut (means of 290.5 vs. 86.3 and 44.0 pg/ml, respectively) [19].  
295 Therefore, lipid masses more abundant at Pre-ov than at other stages paralleled the circulating  
296 and topical levels of E2 whereas more abundant masses at Mid-Lut and Late-lut paralleled  
297 those of P4, supporting the hypothesis of some endocrine regulation of the lipid composition  
298 of the OF. In accordance, the total quantity of phospholipids in the OF were reported to vary  
299 between luteal and non-luteal phases of the cycle in cows [4, 5] and buffalos [22].  
300 Furthermore, in a *Bos indicus* model with contrasted circulating levels of E2 before ovulation  
301 and of P4 after ovulation, variation in the phospholipid profiles monitored by MALDI-MS  
302 were reported in the oviduct at Day 4 and in the uterus at Days 4 and 7 post-ovulation [23].  
303 Some of the differential lipid masses identified in the latter (for example m/z 782.6 PC (36:4)  
304 and m/z 836.6 PC (40:5)) were also found differentially abundant between Pre-ov and Mid-lut  
305 or Late-lut in the present study, leading to the hypothesis that a specific phospholipid profile  
306 is generated in the bovine genital secretions according to the steroid hormonal environment.

307 Not much is known about the mechanisms by which P4 and E2 may regulate the lipid  
308 content in the OF. Nuclear receptors for P4 and E2 have been reported in the bovine oviduct  
309 epithelium [38]. Membrane receptors for P4 are also expressed in bovine oviduct epithelial  
310 cells [39], raising the possibility of a non-genomic action of this hormone in the oviduct  
311 epithelium. Steroid hormones might modulate the rate of release of EV by the oviduct  
312 epithelial cells and thus modulate the accumulation of vesicular phospholipids into the OF.  
313 However, in a recent study, no difference in the concentration and size of bovine oviduct EVs  
314 have been recorded between stages in the estrous cycle [40]. Alternatively, E2 and P4 may

315 modulate the synthesis of proteins involved in the production, transport and cellular export of  
316 lipids and fatty acids. Accordingly, number of genes involved in the lipid biosynthesis process  
317 (including for instance *LPIAT1*, *PIGW*, *CPT1B* and *LPCAT4* coding for  
318 lysophosphatidylinositol acyltransferase 1, phosphatidylinositol-glycan biosynthesis protein,  
319 carnitine O-palmitoyltransferase 1, and lysophospholipid acyltransferase, respectively) were  
320 differentially expressed in oviduct epithelial cells from zebuine females with contrasted  
321 periovulatory concentrations of ovarian steroid hormones [41]. Also, various proteins  
322 involved in lipid metabolism were reported to vary in abundance according to the stage of the  
323 cycle in the bovine OF [18]. In particular, fatty acid synthase (FASN) was among the most  
324 differentially abundant proteins between the Pre-ov and Post-ov stages. Nevertheless,  
325 mechanistic studies designed to understand the action of the endocrine environment on the  
326 lipid content of the oviduct are needed.

327         Spermatozoa enter the oviduct during the pre-ovulatory period and can be stored on  
328 site for hours to days before ovulation, at which time sperm capacitation and fertilization  
329 occur [42]. Spermatozoa are rich in very-long-chain polyunsaturated fatty acids and are highly  
330 sensitive to the lipid environment to which they are exposed [43]. Exogenous  
331 glycerophospholipids can be incorporated within the membrane of mammalian spermatozoa  
332 [10, 44]. Several phospholipids including identified PC and LPC as well as carnitine  
333 molecules were found modulated in abundance during the periovulatory period compared  
334 with the other stages of the estrous cycle. Exogenous PC and LPC have a destabilizing effect  
335 on sperm membrane and were able to induce bull sperm capacitation and acrosome reaction  
336 within minutes [11, 45, 46]. By contrast, exogenous L-carnitine was reported to inhibit sperm  
337 capacitation and acrosome reaction, possibly through a stabilization of sperm membrane  
338 phospholipids [47-49]. The abundance of several molecules of CAR in the OF was highly  
339 decreased at Pre-ov and increased just after ovulation. It is tempting to speculate that this

340 specific pre-ovulatory oviductal environment plays a role in the prevention of premature  
341 capacitation before ovulation. However, numerous phospholipids were also upregulated in  
342 abundance at Pre-ov and the exact role of the complex lipidomic milieu identified at this stage  
343 on spermatozoa remain largely unknown.

344         Among phospholipids found more abundant during the periovulatory period compared  
345 with the luteal phase, the PC 34:1 (m/z 760.6) was previously reported as one of the most  
346 abundant phospholipid identified by MALDI-MS in bovine blastocysts produced either *in*  
347 *vivo* or *in vitro* [13, 50]. Furthermore, the m/z 754.6, 756.6, 768.6 and 780.6, annotated as  
348 molecules of PC and PE and observed as more abundant at Pre-ov compared with Mid-lut  
349 and/or Late-lut, have been reported to increase in abundance in bovine embryos at stages of  
350 development that physiologically occur in the oviduct [12]. Thus, specific phospholipid  
351 profiles fitting with the needs of the developing embryo seemed to appear in the OF during  
352 the periovulatory period. However, functional roles of the phospholipids with regulated  
353 abundance during the periovulatory period deserve further studies.

## 354 **5. Conclusions**

355         The periovulatory period of the estrous cycle displayed a highly specific OF lipidomic  
356 profile compared with that in the luteal phase, pointing out a probable regulatory role of  
357 ovarian steroid hormones in the regulation of the oviduct lipid content. The mechanisms by  
358 which phospholipids accumulate in the oviductal lumen and their exact roles on gametes and  
359 embryo require further investigations.

360

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**373 Conflict of interest**

374 The authors declare they have no conflict of interest.

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512

513 **Figure legends**

514 **Fig. 1.** Representative lipid profile obtained by MALDI-MS in the positive ion-mode on  
515 bovine OF at the Pre-ov stage of the estrous cycle.

516 **Fig. 2.** Principal component analysis of differentially abundant lipid masses identified across  
517 the estrous cycle in the oviductal fluid ipsilateral to ovulation. Each spot represents one  
518 biological replicate (n=17 samples per stage). Each ellipse encloses 80 % of spots for each  
519 stage. The square in each ellipse represents the mean of data for a given stage. Green  
520 symbols: post-ovulatory stage; red: mid-luteal phase; black: late luteal stage; blue: pre-  
521 ovulatory stage of the estrous cycle.

522 **Fig. 3.** Distribution of differentially abundant lipid species when comparing Pre-ov (**A**) and  
523 Post-ov (**B**) with other stages of the estrous cycle in the OF ipsilateral to ovulation. Numbers  
524 of identified masses and molecular species are indicated for all subgroups. The not  
525 represented Mid-lut vs. Late-lut comparison retrieved no difference.

526 **Fig. 4.** Heatmap representation of hierarchical clustering of the 127 differentially abundant  
527 lipid masses identified across the estrous cycle in the OF ipsilateral to ovulation. Each line  
528 corresponds to one molecular mass. For a given stage and mass, green lines represent higher  
529 abundance while red lines represent lower abundance compared with other stages. Black lines  
530 represent is the median abundance values. The proximity between the stages and lipid profiles  
531 are shown by the hierarchical trees on the top and left, respectively. The Cluster 1 identified  
532 includes masses more abundant at the pre-ovulatory stage (Pre-ov) compared with the post-  
533 ovulatory (Post-ov), mid- (Mid-lut) and late luteal (Late-lut) phases of the estrous cycle while  
534 the Cluster 2 includes masses less abundant at Pre-ov than at other stages. NA: no annotation  
535 nor identification.



536 **Fig. 5.** Changes in relative abundance of identified lipid molecules according to the stage of  
537 the estrous cycle in the OF ipsilateral to ovulation. Different letters indicate significant  
538 differences ( $P < 0.05$ ).

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543 **Legends of supplementary materials**

544 **Supplementary Table 1.** List of all differentially abundant masses in paired comparisons  
545 between stages of the estrous cycle in the OF ipsilateral and contralateral to ovulation with  
546 related p-values and ratios. Annotations were obtained through LIPID MAPS database search.

547 **Supplementary Table 2.** List of all lipid species identified by HR-MS/MS in the OF and  
548 mode of identification. The theoretical mass and formula were obtained through LIPID MAPS  
549 database search. Right columns indicate differences in abundance between the stages when a  
550 significant effect of the stage was identified ( $P < 0.05$ ).

551 **Supplementary Figure 1.** Distribution of differentially abundant lipid species when  
552 comparing Pre-ov (**A**) and Post-ov (**B**) with other stages of the estrous cycle in the OF  
553 contralateral to ovulation. Numbers of identified masses are indicated for all subgroups. The  
554 not represented Mid-lut vs. Late-lut comparison retrieved no difference.

555 **Supplementary Figure 2.** Heatmap representation of hierarchical clustering of the 96  
556 differentially abundant lipid masses identified across the estrous cycle in the OF contralateral  
557 to ovulation. Each line corresponds to one molecular mass. For a given stage and mass, green  
558 lines represent higher abundance while red lines represent lower abundance compared with  
559 other stages. Black lines represent is the median abundance values. The proximity between  
560 the stages and lipid profiles are shown by the hierarchical trees on the top and left,  
561 respectively. The cluster identified includes masses less abundant at Pre-ov than at other  
562 stages. NA: no annotation nor identification.

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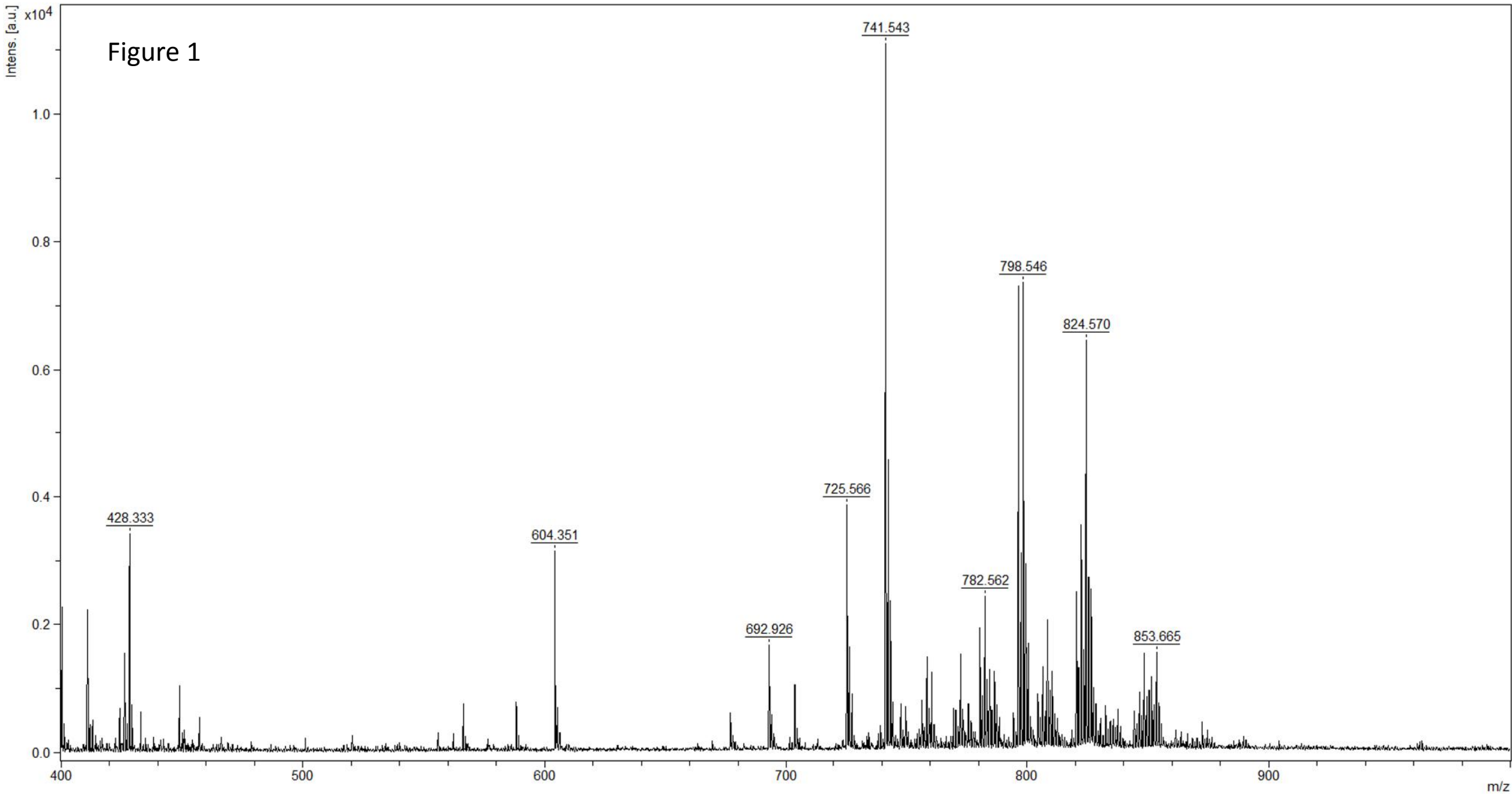


Figure 2

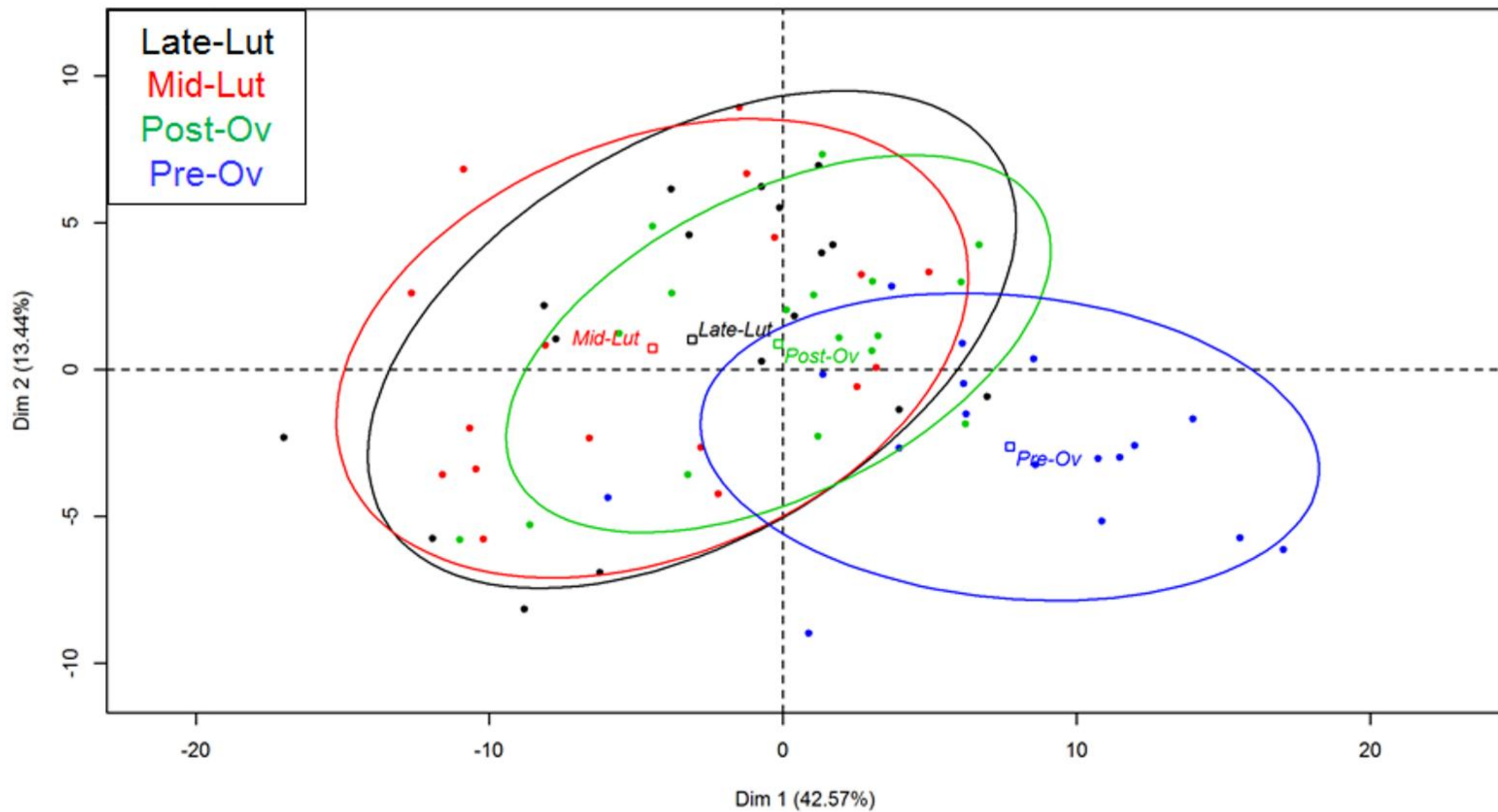


Figure 3

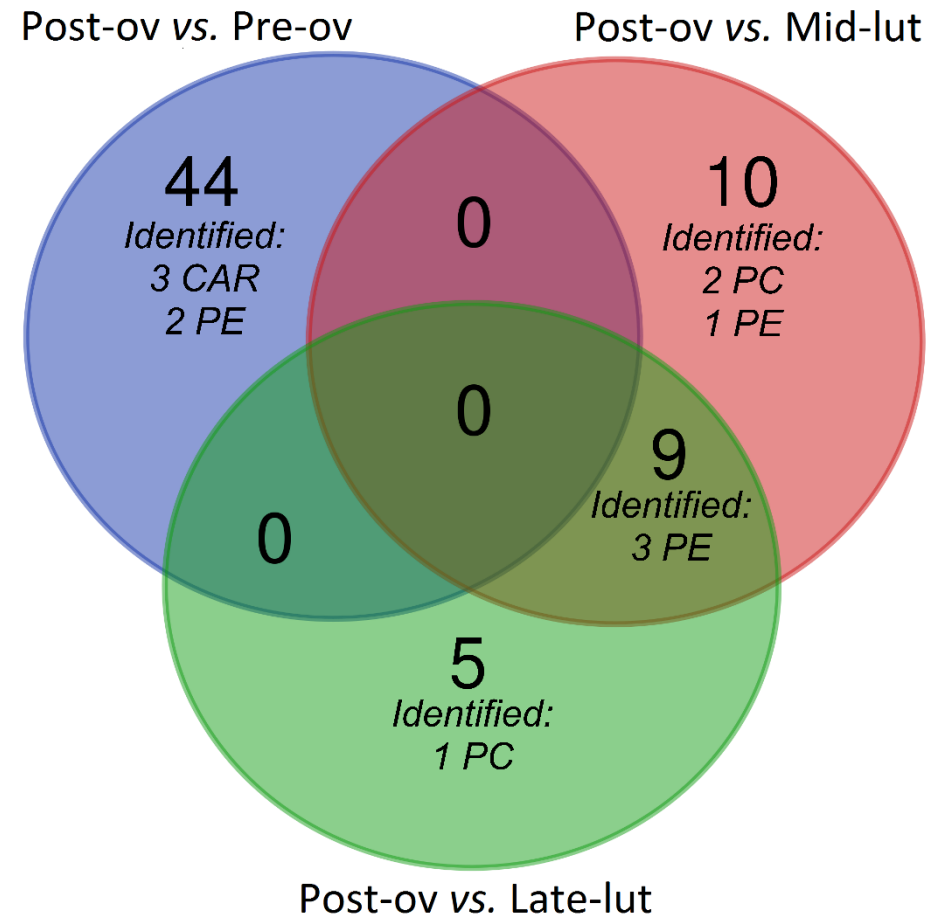
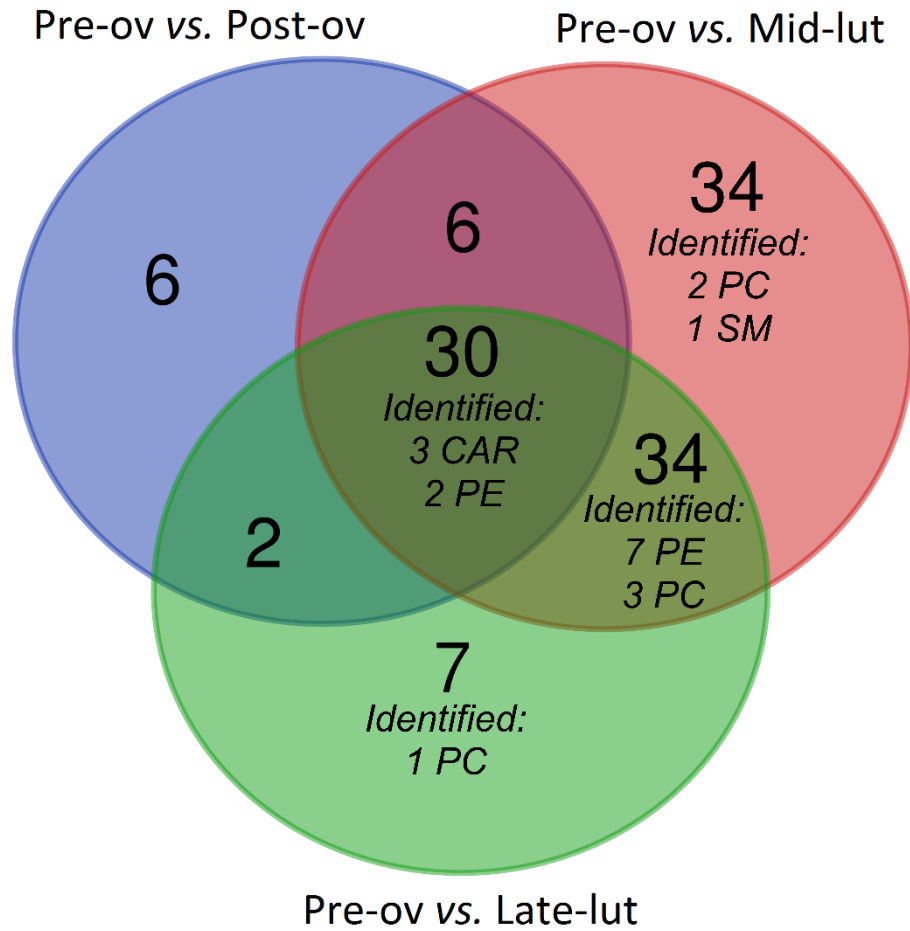
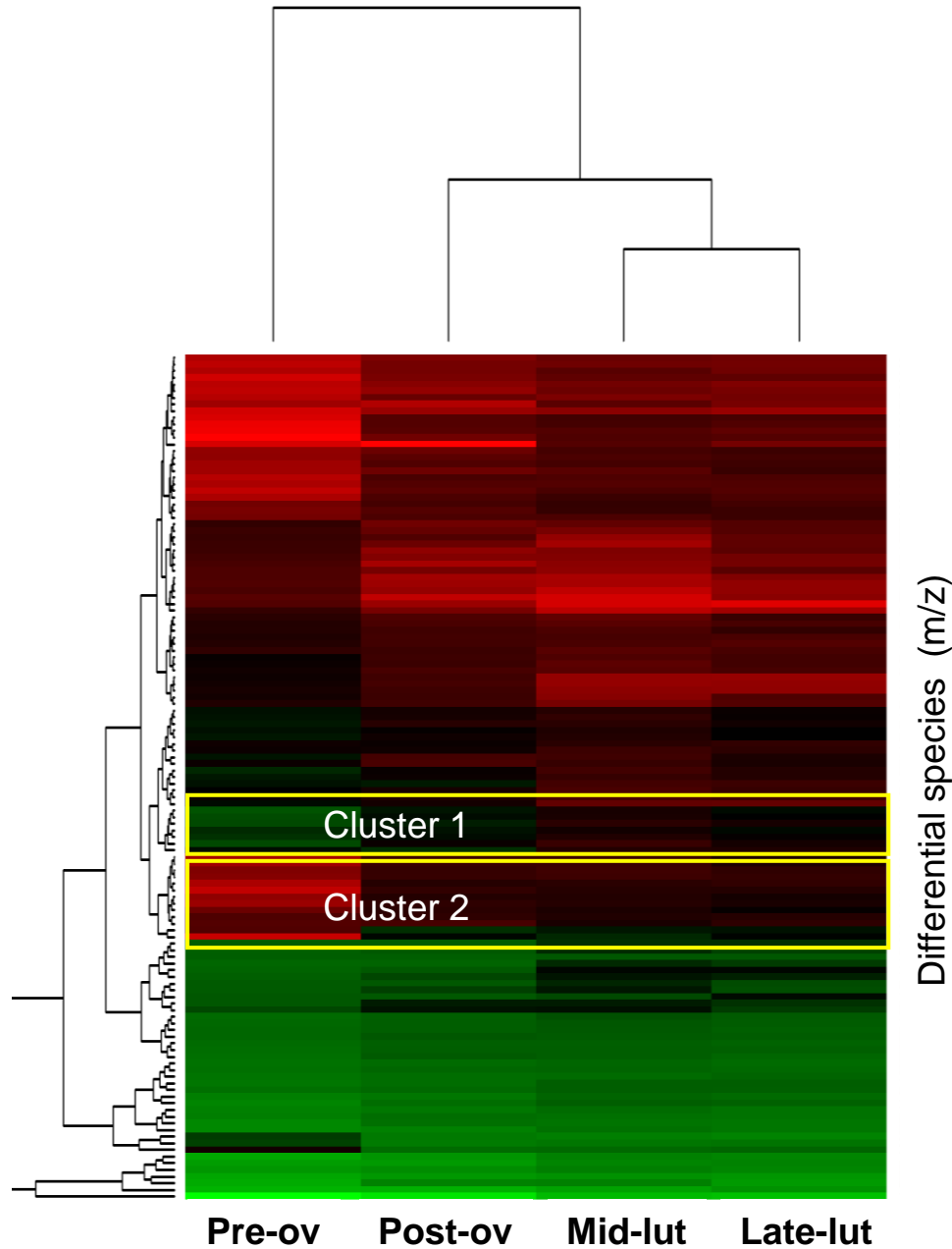


Figure 4



### Cluster 1

Differences between stages	Mass (m/z)	Lipid	Identification or annotation
Pre-ov > Post-ov, Mid-lut & Late-lut	534,33	LPC(19:2) [M+H] <sup>+</sup>	Annotated
	739,55	SM(35:1) [M+Na] <sup>+</sup>	Annotated
	740,55	PE(36:4) [M+H] <sup>+</sup>	Identified
	740,62	NA	NA
	757,63	SM(38:2) [M+H] <sup>+</sup>	Annotated
	768,56	PE(38:4) [M+H] <sup>+</sup>	Identified
	795,62	SM(38:2) [M+K] <sup>+</sup>	Annotated

### Cluster 2

Differences between stages	Mass (m/z)	Lipid	Identification or annotation
Post-ov, Mid-lut & Late-lut > Pre-ov	400,03	NA	NA
	401,32	NA	NA
	414,35	CAR(17:0)	Annotated
	416,27	NA	NA
	416,33	CAR(16-OH)+H	Annotated
	424,35	CAR(18:2)	Annotated
	438,31	CAR(18:3-OH)	Annotated
	441,33	NA	NA
	442,36	CAR(18:1-OH)	Annotated
	448,25	LPC(11:0) [M+Na] <sup>+</sup>	Annotated
	448,32	CAR(20:4)	Annotated
	450,35	CAR(20:3)	Identified
	466,35	LPC(14:1) [M+H] <sup>+</sup>	Annotated

Figure 5

