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► To cite this version:

Charles Banliat, Daniel Tomas, Ana-Paula Teixeira-Gomes, Svetlana Uzbekova, Benoît Guyonnet, et al.. Stage-dependent changes in oviductal phospholipid profiles throughout the estrous cycle in cattle. *Theriogenology*, 2019, 135, pp.65-72. 10.1016/j.theriogenology.2019.06.011 . hal-02627338

HAL Id: hal-02627338

<https://hal.inrae.fr/hal-02627338v1>

Submitted on 26 Oct 2021

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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^{a,b}, Daniel Tomas^{a,c}, Ana-Paula Teixeira-
5 Gomes^{c,d}, Svetlana Uzbekova^a, Benoît Guyonnet^b, Valérie Labas^{a,c} and Marie Saint-Dizier^{a,e}

6

7 ^aUMR PRC, INRA 85, CNRS 7247, University of Tours, IFCE, 37380 Nouzilly, France

8 ^bUnion Evolution, rue Eric Tabarly CS10040, 35538 Noyal-Sur-Vilaine, France

9 ^cPlate-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 ^dUMR ISP, INRA 1282, University of Tours, 37380 Nouzilly, France

13 ^eUniversity 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 β -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 μM of bradykinine and 771 μM of angiotensine in 1
126 μL of α -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 μL of OF (made with an equivalent volume (20 μL) of each stage and side) was mixed with
156 50 μL of methanol (MetOH), vortexed during 60 s then put on ice for 2 h. Chloroform (210
157 μL) was added then the samples were mixed, centrifuged (2000 g, 20 s), sonicated on ice for
158 10 min, mixed with 35 μL 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 μL of methanol for the positive mode and in 10 μL 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 (β -hydroxy- γ -trimethylammonium butyrate)
244 are quaternary amines required for the transfer of long-chain fatty acids to mitochondria for
245 subsequent β -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

361 **Acknowledgments**

362 The authors are grateful to Thierry Delpech, Albert Arnout and Justine Saulnier for their help
363 in the collection of biological samples, and to Marc Chodkiewicz for editing the manuscript.

364 Funding

365 This research was funded by INRA. CB was funded by the French cooperative UNION
366 EVOLUTION and the Association Nationale de la Recherche et de la Technologie (ANRT) as
367 recipient of the Convention Industrielle de Formation par la Recherche no 2017/0684. The
368 high resolution mass spectrometer was funded (SMHART project n°3069) by the European
369 Regional Development Fund (ERDF), the Conseil Régional du Centre, the French National
370 Institute for Agricultural Research (INRA) and the French National Institute of Health and
371 Medical Research (Inserm). The funding sources had no involvement in the study design,
372 analysis of data and publication.

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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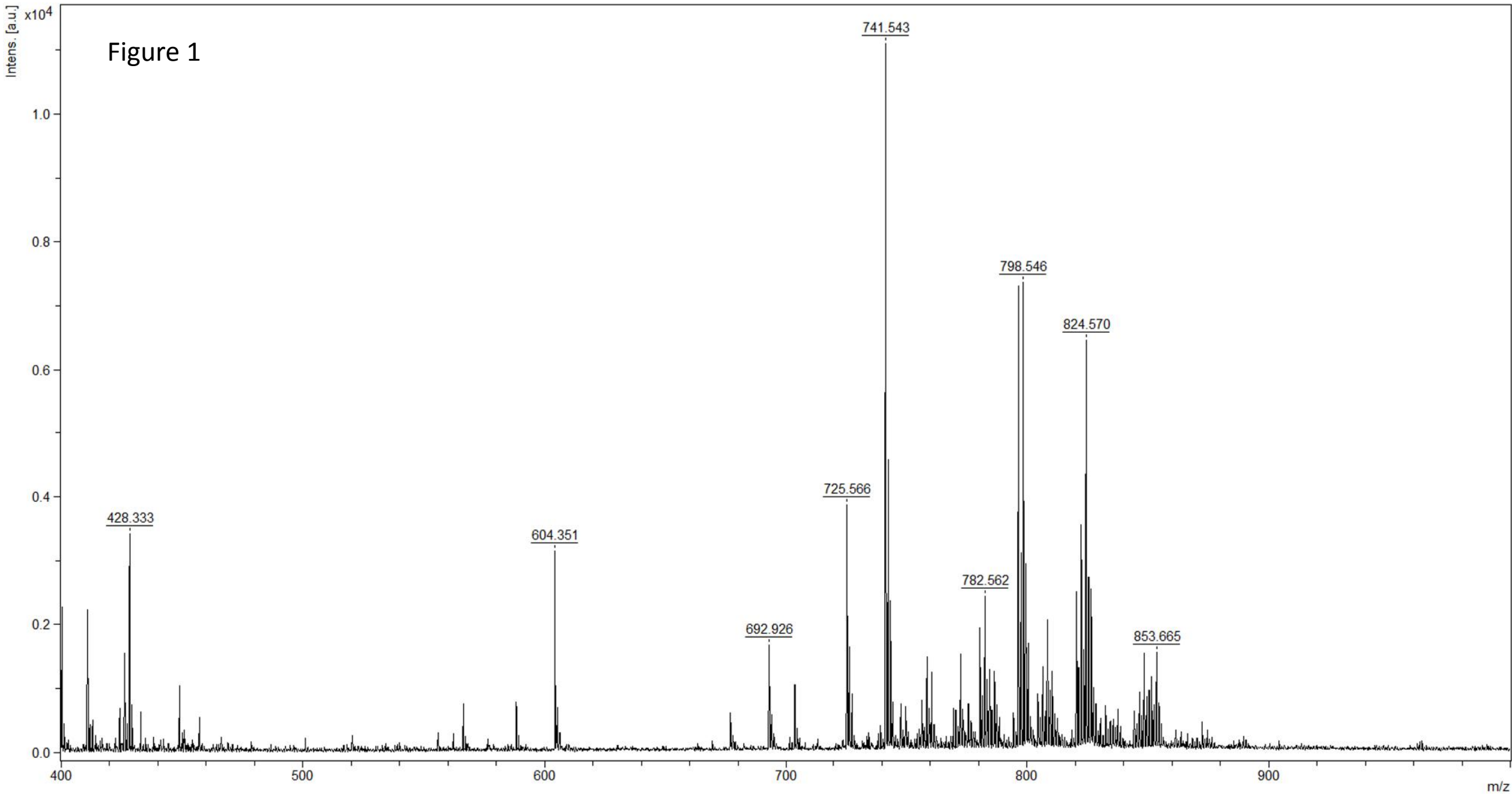


Figure 2

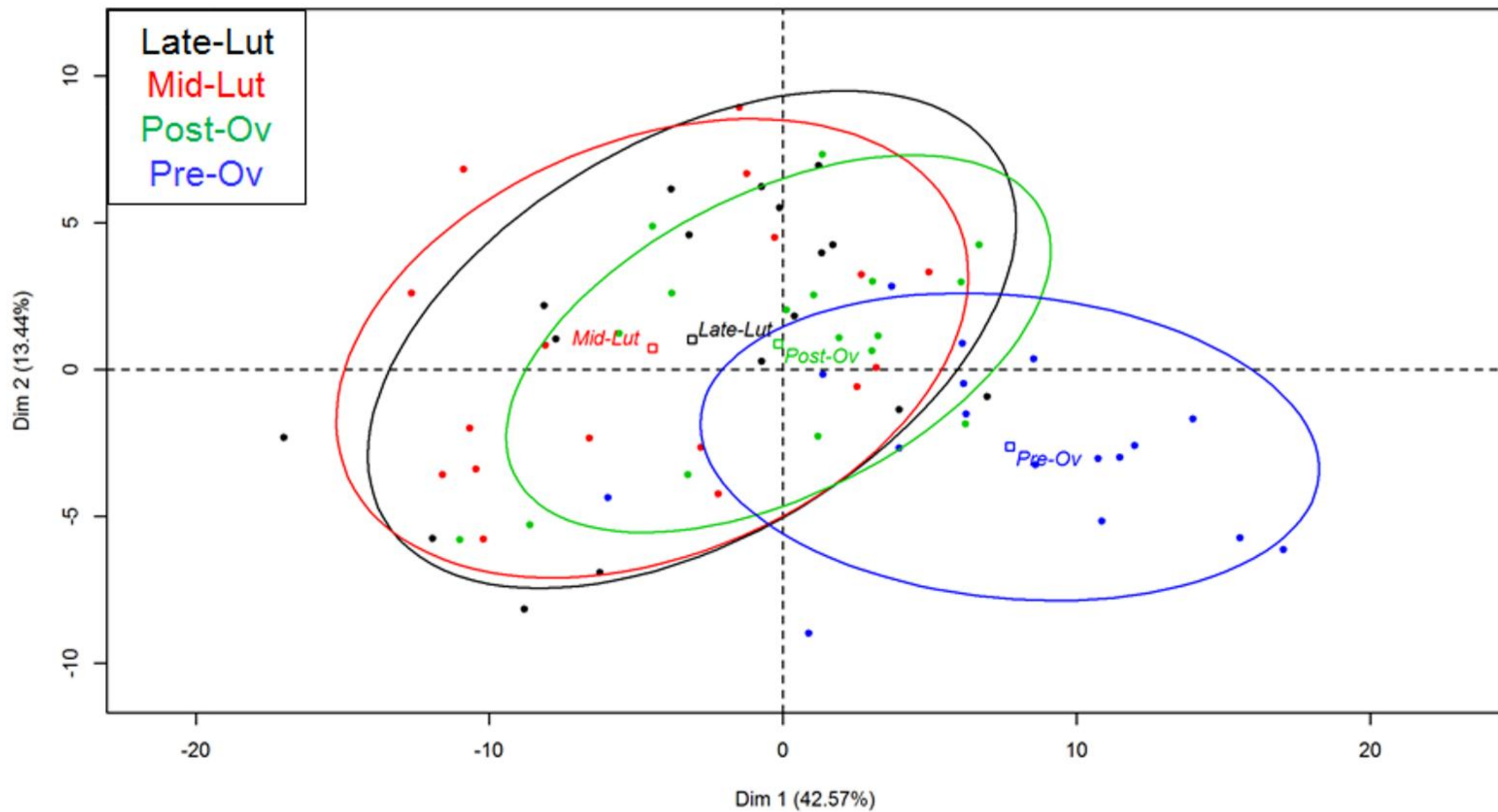


Figure 3

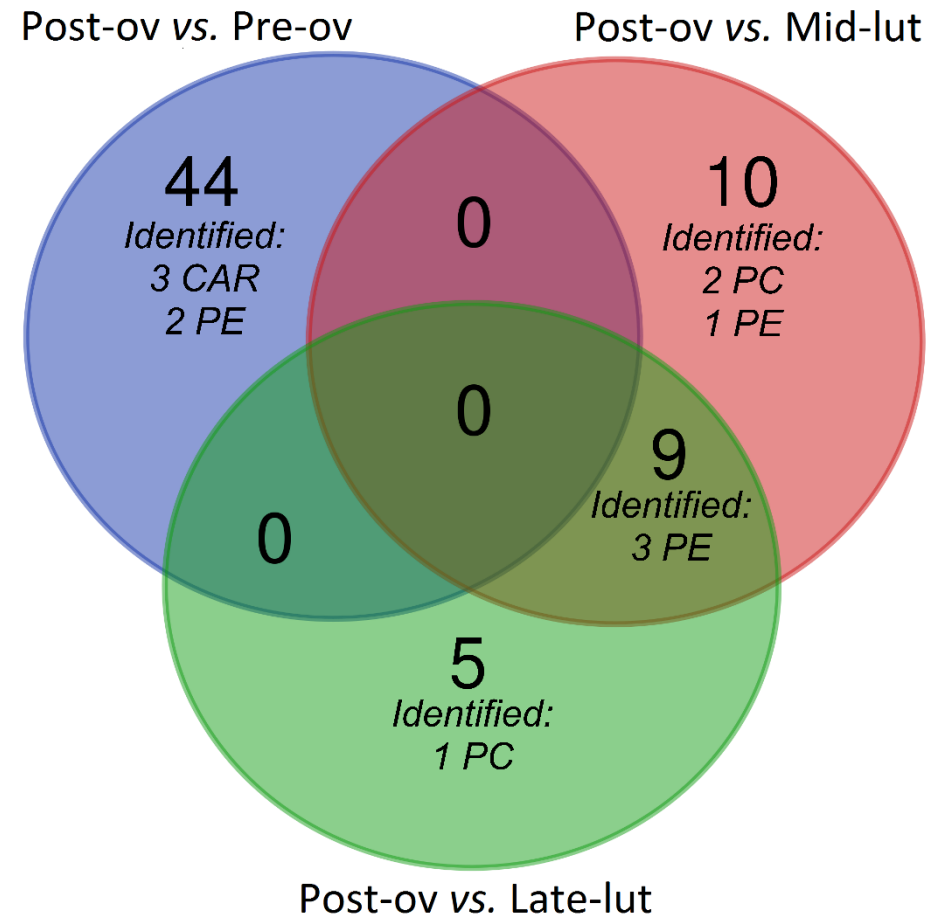
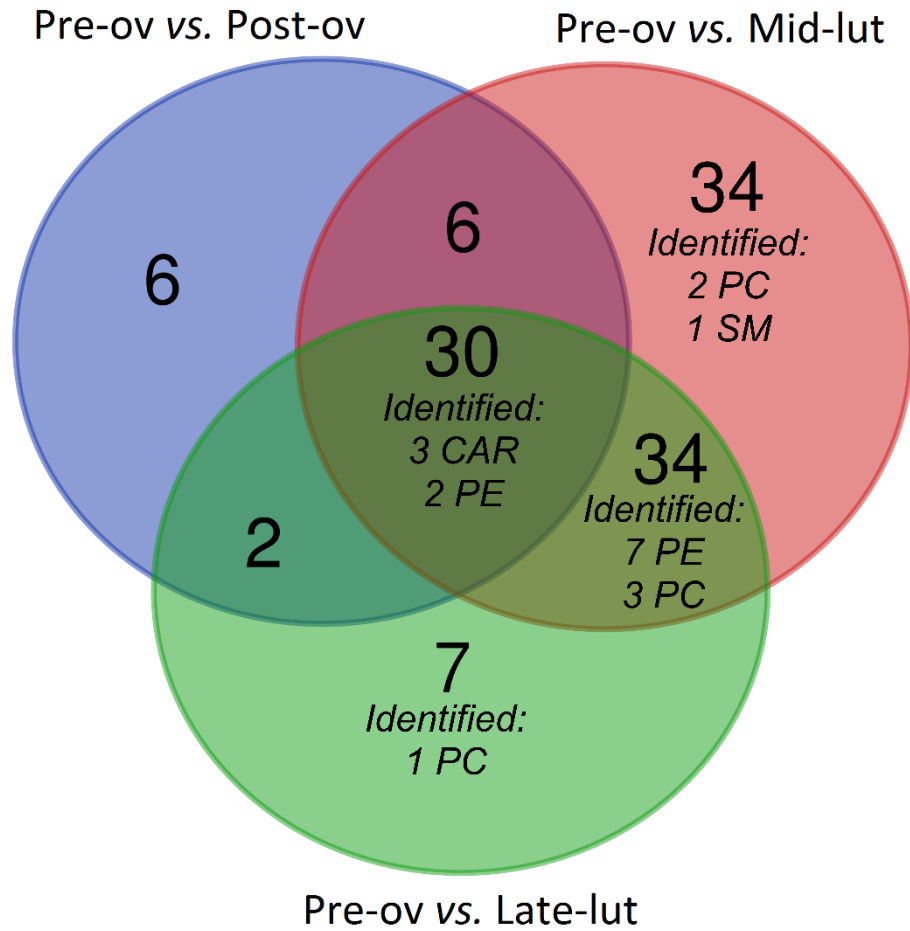
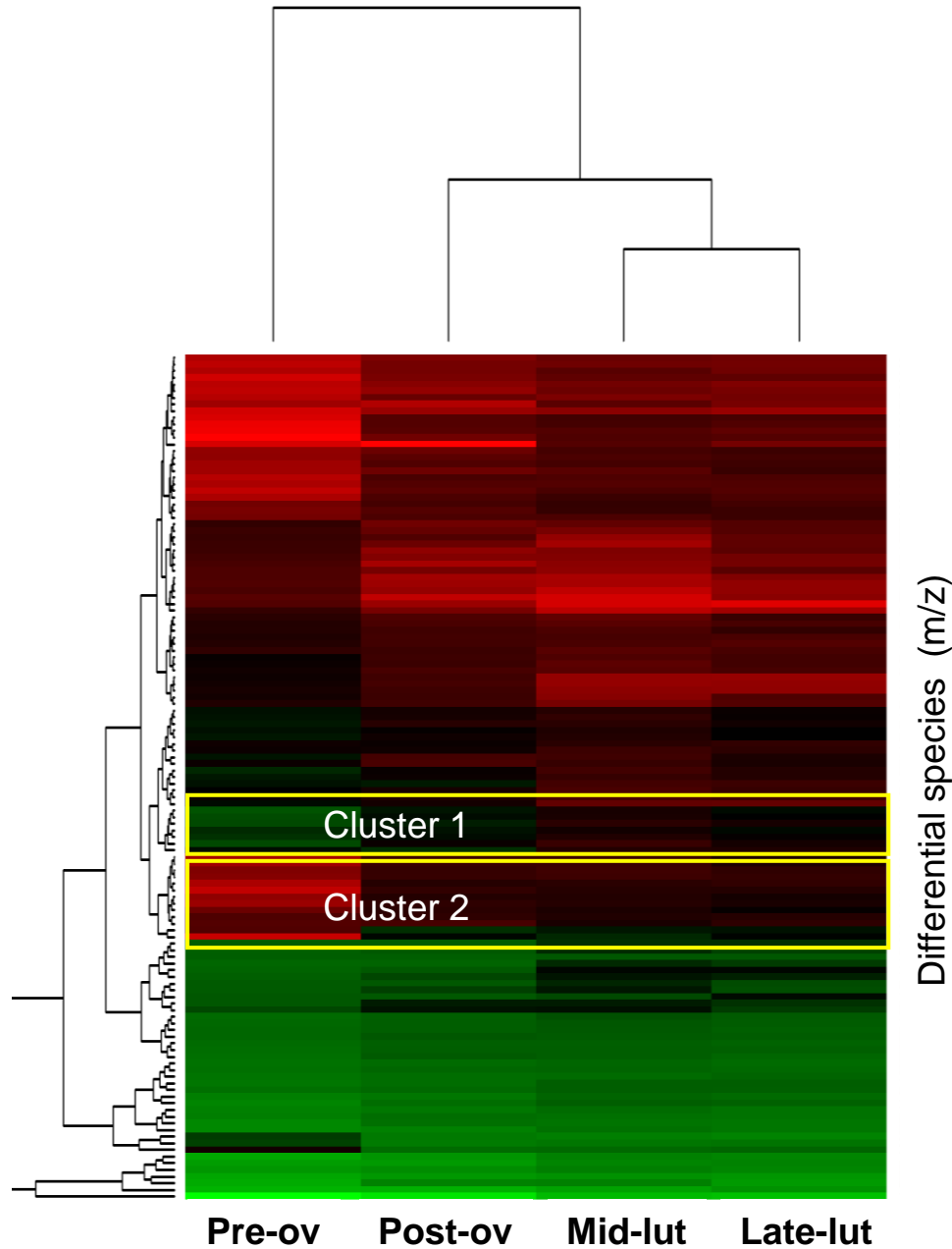


Figure 4



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] ⁺	Annotated
	739,55	SM(35:1) [M+Na] ⁺	Annotated
	740,55	PE(36:4) [M+H] ⁺	Identified
	740,62	NA	NA
	757,63	SM(38:2) [M+H] ⁺	Annotated
	768,56	PE(38:4) [M+H] ⁺	Identified
	795,62	SM(38:2) [M+K] ⁺	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] ⁺	Annotated
	448,32	CAR(20:4)	Annotated
	450,35	CAR(20:3)	Identified
	466,35	LPC(14:1) [M+H] ⁺	Annotated

Figure 5

