RESEARCH

Biochemical alterations in the follicular fluid of bovine peri-ovulatory follicles and their association with final oocyte maturation

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Abstract

Follicular fluid (FF), a product of vascular transudate and granulosa and thecal cell secretions, is the milieu that has evolved to support oocyte growth and maturation which plays a central role in oocyte quality determination. Therefore, a suboptimal FF composition may be reflected in compromised oocyte progression through maturation, fertilization, or embryo development. To date, the composition of bovine FF remains understudied. To address this, we comprehensively characterized the metabolomic constituency of bovine FF in the period during which the oocyte undergoes meiotic maturation. More specifically, FF from pre (−24 h) and peri (−2 h)-ovulatory follicles was profiled by high-throughput untargeted ultra-HPLC tandem mass spectroscopy. A total of 634 metabolites were identified, comprising lipids (37.1%), amino acids (30.0%), xenobiotics (11.5%), nucleotides (6.8%), carbohydrates (4.4%), cofactors and vitamins (4.4%), peptides (3.6%), and energy substrates (2.1%). The concentrations of 67 metabolites were significantly affected by the stage of follicle development, 33.3% ($n = 21$) were reduced ($P \leq 0.05$) by a mean of 9.0-fold, whereas 46 were elevated ($P \leq 0.05$) by a mean of 1.7-fold in peri- vs pre-ovulatory FF. The most pronounced individual metabolite concentration decreases were observed in hypoxanthine (98.9-fold), xanthine (65.7-fold), 17β-oestradiol (12.4-fold), and inosine (4.6-fold). In contrast, the greatest increases were in retinal (4.9-fold), 1-methyl-5-imidazoleacetate (2.7-fold), and isovalerylcarnitine (2.7-fold). This global metabolomic analysis of bovine FF temporal dynamics provides new information for understanding the environment supporting oocyte maturation and facilitating ovulation that has the potential for improving oocyte quality both *in vivo* and *in vitro*.

Lay Summary

The ovaries are part of the female reproductive system, and they produce and store eggs in structures known as ‘follicles’. Depending on the species, one or more follicles release an egg from the ovary during ovulation. FF, which is formed from the secretions of follicle cells and substances delivered from the bloodstream, bathes the eggs as they develop within their follicles. For pregnancy to happen, the egg must be capable of being fertilised by a sperm cell, developing into an embryo and implanting it in the womb. FF has evolved to support the egg to achieve this. Using the cow as a model, this study looks at the composition of FF during the final hours before ovulation, when the egg becomes mature and ready for fertilisation. More than 600 different substances were identified, providing new information, that has the potential to improve egg quality.

Keywords: ▸ ovulation ▸ oocyte ▸ maturation ▸ metabolome ▸ cattle

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Introduction

Genetic and environmental factors are major determinants of oocyte developmental potential, measured as the ability of the oocyte to complete meiosis, undergo fertilization and subsequent mitotic cleavage, develop into a blastocyst, establish pregnancy, and generate a healthy offspring (Fair 2010, Lonergan & Fair 2016). Thus, an appropriate biochemical environment is required to support optimal oocyte growth and maturation *in vivo* or *in vitro*. Follicular fluid (FF) is a product of both the transfer of blood plasma constituents across the blood–follicle barrier and of the secretory activity of granulosa and thecal cells, as well as the cumulus–oocyte complex (COC) (Gosden *et al.* 1988). The FF and cumulus cells (CC) support oocyte integrity and competence, by conferring protection against proteolysis, as well as providing the necessary intracellular metabolites for ovulation. These metabolites include hormones, amino acids, lipids, carbohydrates, nucleotides, and other small molecules derived from serum and the metabolic activity of follicular cells (Da Broi *et al.* 2018). Moreover, these constituents are modified during follicle development (Fortune *et al.* 2004), suggesting that FF is adapted to provide an optimal microenvironment which promotes the quality and subsequent developmental competence of the oocyte within (Bender *et al.* 2010, Leroy *et al.* 2011, Matoba *et al.* 2014, Forde *et al.* 2016). The chemical composition of peri-ovulatory FF is particularly important, because this medium bathes the oocyte, serving as an energy source during oocyte meiotic resumption and maturation (Dumesic *et al.* 2015).

Several analytical techniques have been used to evaluate and characterize the biochemical profile of bovine FF with a particular emphasis on the usefulness of the FF metabolomic profile as a non-invasive predictor of bovine oocyte developmental potential (Fair 2014). The fatty acid and amino acid profiles of bovine FF were reported to be predictive of oocyte *in vitro* developmental competence (Matoba *et al.* 2014). The predictive nature of the FF, in terms of association with cow fertility or oocyte competence, was also demonstrated by Moore *et al.* (2017), who identified several fatty acids and amino acids in the FF of cows that correlated with cow fertility. More recently, differential metabolite concentrations in FF samples between inactive vs physiological bovine ovaries at 45–60 days postpartum were detected using ultra-HPLC tandem mass spectrometry ((UHPLC)-MS/MS) technology (Bai *et al.* 2020).

While the bovine FF metabolomic profile has been investigated during the pre-ovulatory (Orsi *et al.* 2005, Forde *et al.* 2016) and peri-ovulatory (Bender *et al.* 2010) period, to our knowledge, this is the first study to compare the global metabolomic FF landscape of pre- and peri-ovulatory follicles in beef cattle. Ovulation is a well-controlled inflammatory process, where immune cells are recruited to the ovulatory follicle from the circulation (Espey 1980, Okuda & Sakumoto 2003, Townson & Liptak 2003). Moreover, immune cell activation, differentiation, and function have specific metabolic requirements to meet their energetic and biosynthetic demands, and successful pregnancy has been attributed to the involvement of a number of metabolic pathways in conjunction with the maternal immune system (Thiele *et al.* 2018). However, knowledge of immunometabolism in respect of ovulation is scant. Therefore, the objectives of the current study were to identify and record alterations of the FF metabolomic profile during the ovulatory window, placing particular emphasis on factors associated with oocyte meiotic maturation and maturation and identifying potential immunomodulatory biomarkers of an optimal peri-ovulatory environment. To achieve this, FF from synchronized cattle was obtained either 24 or 2 h before the estimated time of ovulation and subjected to high throughput untargeted UHPLC-MS/MS.

Our overarching hypothesis was that the broad metabolomic profiles of pre- vs peri-ovulatory FF would differ, particularly in steroid hormone and immune-metabolite composition.

Materials and methods

All experimental procedures involving live animals were approved by the Institutional Animal Research Ethics Committee of University College, Dublin (UCD), and licensed by the Irish Health Products Regulatory Authority in accordance with European Union (EU) Protection of Animals used for Scientific Purposes regulations (2010/63/EU). All animals were housed at UCD Lyons Farm.

Animal synchronization and husbandry

A description of the experimental design, animal synchronization, and follicle dissection and processing has been presented previously (Alrabiah *et al.* 2021). In brief, following confirmation of reproductive tract normality by transrectal ultrasonographical assessment, the oestrous cycles of 16 nulliparous beef (predominantly Limousin and Charolais cross) heifers, with a mean age of 2.0 ± 0.5 years and mean weight of 587.6 ± 98 kg, were synchronized as follows: gonadotropin-releasing hormone (GnRH) analogue (Ovarelin, Ceva Santé Animale) administration

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by i.m. injection, immediately followed by insertion of a progesterone (P4)-releasing intravaginal device (PRID; Ceva Santé Animale). After 7 days, all heifers received a prostaglandin F2α (PGF2α) analogue (Enzaprost; Ceva Santé Animale) intramuscularly followed by PRID removal the next day. A second GnRH injection was administered 36 h post PRID removal (Fig. 1A). Heifers were transported to a local EU-licensed abattoir and ovaries were retrieved immediately post-mortem, corresponding to either 24 or 2 h before the estimated time of ovulation. Previous studies report that the peak of oestrus onset occurs at about 36 h after PRID removal and the LH surge occurs within ~2 h of oestrus onset (van de Leemput *et al.* 1999). Ultrastructural analysis of oocytes recovered from the peri-ovulatory follicle 19 h after the LH peak classifies them as oocytes in the final stage of maturation (Kruip & Dieleman 1985, Hyttel *et al.* 1986), and thus, FF collection at ~24 h before ovulation is the approximate time of the systemic luteinizing hormone (LH) surge which induces oocyte meiotic resumption and maturation and ~2 h before ovulation corresponds with the peri-ovulatory period when the oocyte should be fully mature and surrounded by an expanded CC mass. Only FF from follicles from which such a COC with expanded cumulus was retrieved was used for metabolomic analysis at T2.

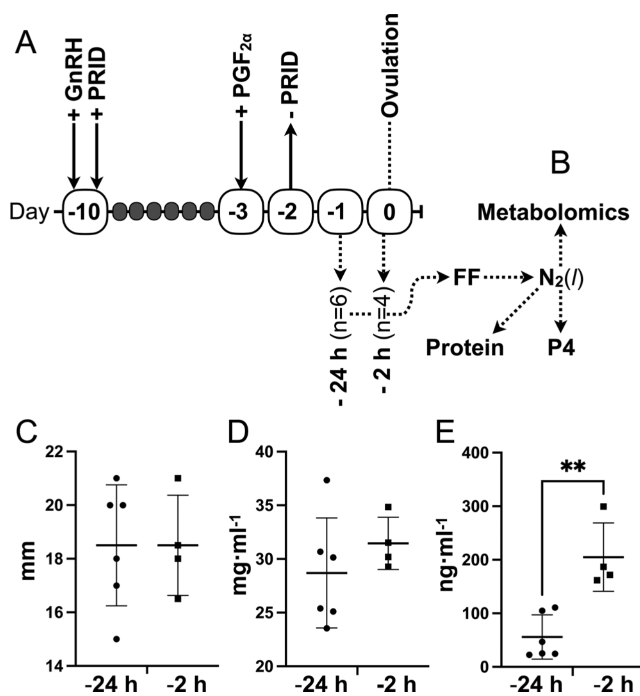

Figure 1 Schematic depiction and validation of experimental design. (A) Ten heifers were synchronized by administration of gonadotropin-releasing hormone (GnRH), progesterone (P4)-releasing intravaginal device (PRID), and prostaglandin (F2α). Follicular fluid (FF) was aspirated 24 (n = 6) and 2 (n = 4) h prior to ovulation. (B) Follicular fluid (FF) was snap-frozen in liquid nitrogen (N_2(l)) until analysis for total protein, P4 content, or high-throughput untargeted metabolomics. (C) Mean diameter (±s.d.) of 24 h (pre (n = 6)) and 2 h (peri (n = 4)) ovulatory follicles. (D) Mean (±s.d.) protein content in 24 h (n = 6) and 2 h (n = 4) ovulatory FF. (E) Mean (±s.d.) FF P4 levels in 24 h (n = 6) vs 2 h (n = 4) ovulatory FF – wherein ** represents $P \leq 0.01$.

Follicular fluid recovery and processing

Ovaries were returned to the laboratory on ice within 1 h of retrieval. Ultimately, ten heifers were included in this study as the dominant follicles of six were ruptured on retrieval. Differentiated (i.e. 24 h before, (n=6) pre-ovulatory and luteinized (i.e. 2 h before, n=4) peri-ovulatory follicles were dissected from the ovaries, diameters were measured, and follicular tissues and fluid were recovered on ice, as previously described (Alrabiah *et al.* 2021). FF was then centrifuged at 100 ***g*** for 5 min at 4°C. The supernatant was distributed into three aliquots and stored at −80°C until analysis. The first aliquot was sent for metabolomic profiling, the second for steroid hormone analysis, and the third for total protein content quantification, as described below (Fig. 1B).

Metabolomic profiling

High-throughput semi-quantitative untargeted metabolomic profiling was performed by Metabolon Inc. (Durham, NC, USA) by ultra-high performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) as described by Simintiras *et al.* (2021*a*).

Briefly, protein in each sample was precipitated with methanol and extracted using the automated MicroLab STAR system (Hamilton Company) under centrifugation at 680 ***g*** for 2 min (Geno/Grinder 2000, Glen Mills), prior to methanol removal using a TurboVap (Zymark) and overnight incubation in nitrogen. Each deproteinated sample was then divided into four aliquots for analysis as follows: 1 and 2, reverse phase (RP) UPLC-MS/MS with positive ion mode electrospray ionization (ESI) analysis; 3, RP UPLC-MS/MS with negative 4 ion mode ESI analysis; and 4, hydrophilic interaction liquid chromatography (HILIC) UPLC-MS/MS with negative ion mode ESI analysis. Samples were then reconstituted in solvents compatible with each analysis, as described below.

Aliquot 1 (RP-UPLC-MS/MS +ESI) was subject to gradient elution in water and methanol with 0.05% perfluoropentanoic acid and 0.1% formic acid (Waters UPLC BEH 1.7 µm C18 2.1 × 100 mm column). Aliquot 2 (also RP-UPLC-MS/MS +ESI) was identically eluted, using the same column, with the addition of acetonitrile

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to the elution buffer. Aliquot 3 (RP-UPLC-MS/MS –ESI) was similarly eluted using a gradient buffer comprising methanol, water, and 6.5 mM ammonium bicarbonate (pH 10.8). Aliquot 4 (HILIC-UPLC-MS/MS –ESI) was eluted using a HILIC (Waters UPLC BEH Amide 1.7 µm 2.1 × 150 mm column) with a water plus acetonitrile plus 10 mM ammonium formate (pH 10.8) gradient. Each of the four aliquots of each sample was subsequently analysed using a Waters Acquity UPLC coupled to a Thermo Scientific Q-Exactive high-resolution MS interfaced with heated electrospray ionization (HES-II) source and Orbitrap mass analyzer operating at 35,000 mass resolution and with a scan range between 70 and 1000 m/z.

Metabolites were quantified against internal and recovery standards, run in parallel. These controls were (1) a pooled aliquot of all experimental samples, serving as a technical replicate control; (2) ultra-pure water as process blanks, also run in between experimental samples; and (3) a cocktail of quality control (QC) metabolites, absent from endogenous compound measurements, were spiked into each sample. The latter internal standard enabled instrument performance monitoring and chromatographic alignment. Metabolite identification was based on retention time/index (RI), mass to charge ratio (m/z) within ±10 ppm, and MS/MS forward and reverse scores between the experimental data vs Metabolon Inc. in-house authentic standards. Where this was not possible, metabolite identification was predicted by comparing metabolite RI, m/z, and chromatographic (MS/MS spectral data) to those of purified standards. Technical (instrument) median relative s.d. was 3% with a total process variability of 6%.

Progesterone and protein quantification

FF P4 was measured by solid-phase RIA (PROG-RIA-CT KIP1458; DiaSource ImmunoAssays S.A., Belgium), according to manufacturer's instructions. Assay range and sensitivity were 0.12–36 ng/mL and 0.05 ng/mL, respectively. FF protein content was quantified by Bradford assay by Metabolon Inc.

Metabolomic data analysis and interpretation

Raw chromatographic data were logarithmically (ln) transformed (scaled). These values were then either divided by the total protein concentration in each individual corresponding sample (protein-normalized) or not. Protein-normalized data are provided as supplementary material. Non-normalized data are discussed herein unless otherwise stated. Missing values were imputed with the minimum observed value for each compound within each group. Pathway enrichment (E) was calculated using the formula: $(k/m)/(n/N)$ whereby k = number of significant ($P \leq 0.05$) metabolites per pathway, m = total number of detected metabolites per pathway, n = number of significant ($P \leq 0.05$) metabolites in the study, and N = total number of detected metabolites in the study, as described by Brown *et al.* (2016).

Statistical analyses

Follicle diameter (Fig. 1C), FF protein (Fig. 1D), and FF P4 composition (Fig. 1E) data were analyzed by unpaired two-tailed *t*-test using Prism 9.0 (GraphPad). Principal component analysis (Fig. 2D) was performed using the open-access Past4 software (Hammer *et al.* 2001). Transformed metabolomic data (Figs. 3 and 4) were statistically contrasted using Welch's two-sample *t*-test with a $P \leq 0.05$ (significant) or $0.05 < P < 0.10$ (trend) cut-off.

Results

Follicle development, progesterone, and protein composition

Mean diameter (±s.d.) of pre- (18.5 ± 1.9 mm) vs peri (18.5 ± 2.3 mm)-ovulatory follicle did not differ (Fig. 1C). Similarly, mean (±s.d.) protein content (28.7 ± 5.1 and 31.5 ± 2.4 mg/mL in pre- and peri -ovulatory FF, respectively) was not different (Fig. 1D). However, mean (±s.d.) FF P4 concentration increased from 24 h (55.7 ± 41.3 ng/mL) to 2 h (204.9 ± 63.8 ng/mL) before ovulation (Fig. 1E) ($P = 0.0091$), confirming the temporal phenotypic divergence of FF collected.

Follicular fluid qualitative metabolomics

A total of 634 metabolites were identified, see full list in Supplementary Table 1 (see section on supplementary materials given at the end of this article), comprising lipids (37.1%), amino acids (30.0%), xenobiotics (11.5%), nucleotides (6.8%), carbohydrates (4.4%), cofactors and vitamins (4.4%), peptides (3.6%), and energy substrates (2.1%), as represented by Fig. 2A. The qualitative metabolomic profiles of pre- (24 h) and peri-ovulatory (2 h) FF were broadly identical, as metabolite presence was 99% (630 of 634) common to both groups. However, between groups, prostaglandins E2 (PGE2) and F2 alpha (PGF2α) were absent from all 24 h pre-ovulatory FF

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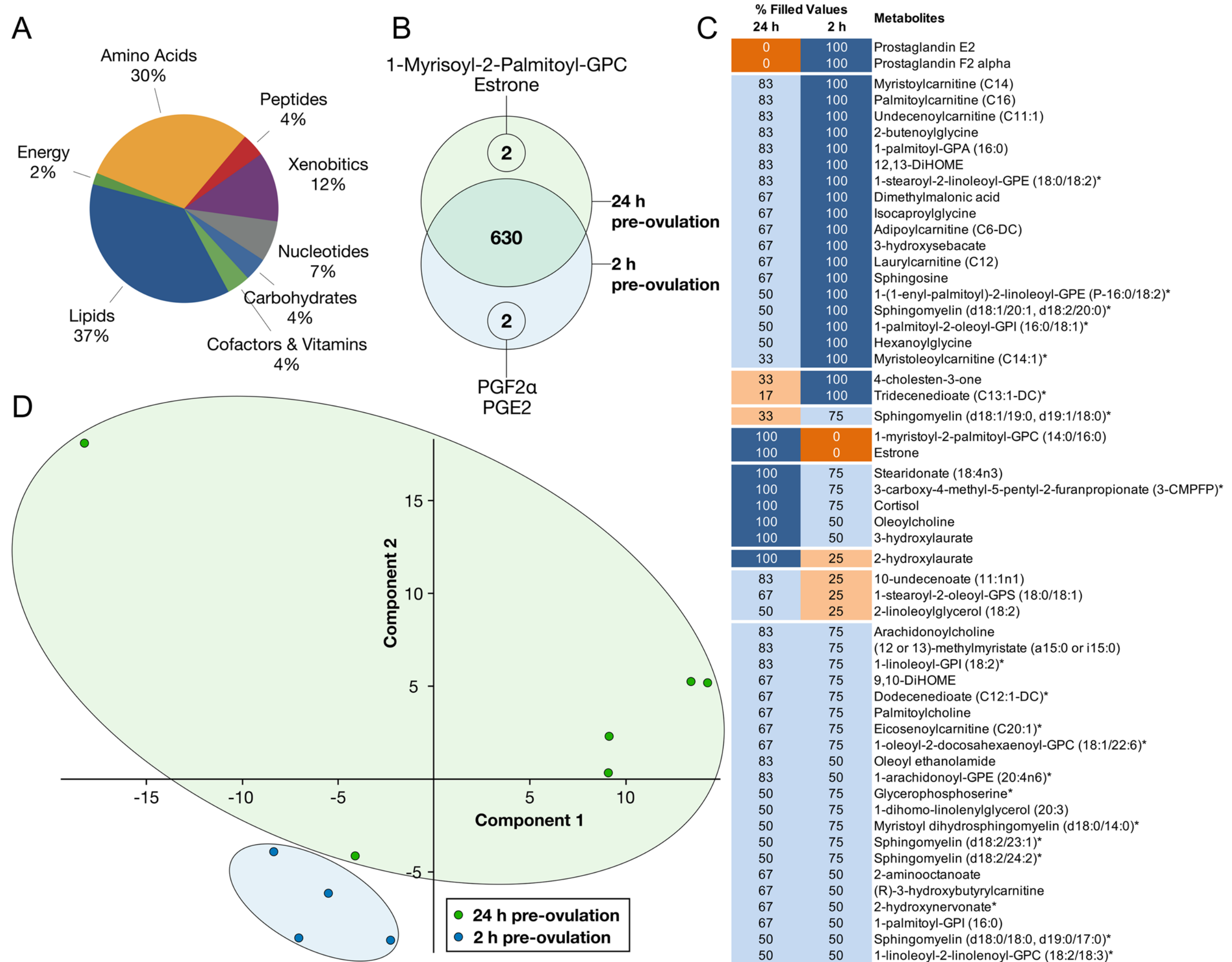

Figure 2 Metabolomic snapshot of pre- and peri-ovulatory follicular fluid (FF). (A) Pie chart of FF metabolite super-pathway distribution. (B) Venn diagram of metabolites unique or common to pre- (green) vs peri- (blue) ovulatory FF. (C) The percentage of samples from each group in which the corresponding metabolite was identified (% filled Values). All other metabolites were identified across all samples. Dark blue cell shading (100%) indicates complete presence, whereas dark orange (0%) highlights complete absence. Light orange indicates presence in 17–25% of samples, whereas light blue shading denotes a percentage fill between 50–83%. (D) Principal component analysis of FF metabolomic profiles 24 h (green; $n = 6$) vs 2 h (blue; $n = 4$) pre-ovulation.

samples, whereas oestrone and 1-myrisoyl-2-palmitoyl-glycerophosphorylcholine were absent from all 2 h peri-ovulatory FF samples (Fig. 2B). Within groups, the presence/absence of 51 metabolites was inconsistent. For example, arachidonoyl-choline was present in 83% of pre- and 75% of peri-ovulatory FF samples. These are listed in Fig. 2C. The original normalized raw area counts for each metabolite are listed in Supplementary Table 2.

Follicular fluid quantitative metabolomics

Despite the near-identical qualitative metabolomic profiles observed, principal component analysis (PCA) revealed distinct separation of the pre- vs peri-ovulatory FF, with greater variation within the 24 h pre-ovulatory group (Fig. 2D). This is attributable to concentration differences of 67 metabolites between the two FF groups (Figs. 3 and 4). More specifically, 21 (33.3%) were reduced ($P \leq 0.05$) by a mean of 9.0-fold, whereas 46 were elevated ($P \leq 0.05$) by a mean of 1.7-fold in peri- vs pre-ovulatory FF, the differences were primarily due to variable lipid (43.3%) and amino acid (28.4%) flux. The most pronounced individual metabolite concentration decreases in the same comparison were hypoxanthine (98.9-fold), xanthine (65.7-fold), 17β-oestradiol (12.4-fold, E2), and inosine (4.55-fold). In contrast, the greatest corresponding increases were retinal (4.9-fold), 1-methyl-5-imidazoleacetate (2.7-fold), and isovalerylcarnitine (2.7-fold).

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A

Lipid Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Long Chain Saturated Fatty Acid	Palmitate (16:0)	1.1094	0.9492	0.0698	1.17
Long Chain Monounsaturated Fatty Acid	Myristoleate (14:1n5)	1.4222	0.7528	0.0151	1.89
Long Chain Polyunsaturated Fatty Acid (n3 and n6)	Stearidonate (18:4n3)	1.3834	0.6040	0.0756	2.29
	Eicosapentaenoate (EPA; 20:5n3)	1.6855	0.5574	0.0035	3.02
	Docosapentaenoate (n3 DPA; 22:5n3)	1.8964	0.6502	0.0132	2.92
	Docosahexaenoate (DHA; 22:6n3)	1.4597	0.5541	0.0011	2.63
	Linoleate (18:2n6)	0.9822	1.3499	0.0819	0.73
	Linolenate [alpha or gamma; (18:3n3 or 6)]	0.8988	1.1461	0.0964	0.78
	Dihomo-linolenate (20:3n3 or n6)	1.3016	0.6590	0.0457	1.98
	Arachidonate (20:4n6)	1.8801	0.6418	0.0116	2.93
	Mead acid (20:3n9)	1.3363	0.6936	0.0268	1.93
Dicarboxylate Fatty Acid	Maleate	1.2761	0.8736	0.0865	1.46
	Octadecanedioate (C18-DC)	1.1462	0.9114	0.0534	1.26
Fatty Acid (inc. Branch-Chain Amino Acid)	Propionylcarnitine (C3)	1.4348	0.8749	0.0955	1.64
Fatty Acid (Acyl Glycine)	Isocaproylglycine	0.7100	1.3308	0.0277	0.53
Fatty Acid Metabolism (Acyl Carnitine; Medium Chain)	Laurylcarnitine (C12)	0.7812	1.1913	0.0379	0.66
Fatty Acid (Monohydroxy)	2-hydroxymyristate	0.7224	1.3376	0.0018	0.54
	2-hydroxypalmitate	0.7979	1.3799	0.0014	0.58
	2-hydroxystearate	0.8451	1.5250	0.0212	0.55
	3-hydroxystearate	1.1873	0.6868	0.0218	1.73
	5-hydroxyhexanoate	1.0404	0.8808	0.0776	1.18
Phosphatidylcholine (PC)	1-myristoyl-2-palmitoyl-GPC (14:0/16:0)	0.9821	0.5217	0.0119	1.88
	1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	0.8527	1.5531	0.0003	0.55
Lysophospholipid	1-palmitoyl-GPA (16:0)	0.8884	2.1020	0.0682	0.42
	1-oleoyl-GPC (18:1)	1.2560	0.6969	0.0020	1.80
	1-linoleoyl-GPC (18:2)	1.1855	0.6218	0.0006	1.91
	1-linolenoyl-GPC (18:3)*	1.3772	0.4849	0.0003	2.84
	1-arachidonoyl-GPC (20:4n6)*	1.2368	0.6190	0.0096	2.00
	1-linoleoyl-GPE (18:2)*	1.4675	0.7224	0.0016	2.03
	1-arachidonoyl-GPE (20:4n6)*	0.9275	0.5152	0.0343	1.80
Glycerolipid	Glycerol	1.0892	0.6139	0.0575	1.77
	Glycerol 3-phosphate	1.6790	0.7047	0.0308	2.38
Sphingomyelin	Behenoyl sphingomyelin (d18:1/22:0)*	0.8834	1.1791	0.0346	0.75
	Tricosanoyl sphingomyelin (d18:1/23:0)*	0.8615	1.1235	0.0601	0.77
	Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)*	0.8589	1.1993	0.0203	0.72
	Sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)	0.8495	1.2293	0.0537	0.69
	Sphingomyelin (d18:1/18:1, d18:2/18:0)	0.9315	1.2200	0.0740	0.76
	Sphingomyelin (d18:2/24:1, d18:1/24:2)*	0.8737	1.1800	0.0297	0.74
Mevalonate	3-hydroxy-3-methylglutarate	0.9169	1.3985	0.0911	0.66
	Mevalonate	1.3710	0.9006	0.0112	1.52
Sterol	7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	0.8430	1.3679	0.0242	0.62
	4-cholesten-3-one	0.7249	1.8183	0.0919	0.40
Corticosteroids	Cortisone	1.2316	0.6694	0.0022	1.84
Estrogenic Steroids	Estrone	1.0203	0.4790	0.0124	2.13
	17β-estradiol	1.5920	0.1287	0.0000	12.37

B

Nucleotide Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Purine [(Hypo)Xanthine/Inosine]	Inosine	3.6267	0.7211	0.0161	5.03
	Hypoxanthine	7.8744	0.0796	0.0032	98.92
	Xanthine	6.2221	0.0947	0.0019	65.70
	Xanthosine	1.7900	0.6619	0.0254	2.70
	Uric acid ribonucleoside*	1.3728	0.8982	0.0798	1.53
Purine [Adenine]	Adenine	1.2027	0.7219	0.0144	1.67
Purine [Guanine]	Guanine	1.3853	0.6373	0.0788	2.17
Pyrimidine [Uracil]	Uracil	2.0726	0.8337	0.0600	2.49
	5-methyluridine (ribothymidine)	1.5275	0.6368	0.0014	2.40
	N-acetyl-beta-alanine	0.7076	1.6841	0.0046	0.42
Pyrimidine [Cytidine]	N4-acetylcytidine	1.5358	0.7741	0.0475	1.98
	2'-deoxycytidine	1.1461	0.8919	0.0642	1.29
	2'-O-methylcytidine	0.9163	1.1308	0.0707	0.81

Figure 3 Quantitative metabolomic analysis of pre- (24 h) and peri-(2 h) ovulatory follicular fluid (FF). Metabolites are grouped by either (A) lipid or (B) nucleotide super-pathway metabolism. Corresponding sub-pathways are also provided. Dark green shading indicates a decrease (metabolite ratio <1.0) between the aforementioned time-points ($P \leq 0.05$), with light green depicting a decreasing trend ($0.05 < P < 0.10$). In contrast, dark red shading indicates an increase (metabolite ratio ≥1.0) between groups ($P \leq 0.05$), with light red depicting an increasing trend ($0.05 < P < 0.10$). Asterisks denote predicted metabolites. RC, relative concentration; RCFC, relative concentration fold-change.

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A Amino Acid Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Glycine, Serine, and Threonine	2-methylserine	0.9186	1.0927	0.0461	0.84
Alanine and Aspartate	Alanine	0.9644	1.1207	0.0708	0.86
	Asparagine	0.9450	1.0821	0.0638	0.87
Glutamate Metabolism	Glutamate	1.1357	0.8117	0.0831	1.40
	Glutamine	1.0175	0.8207	0.0876	1.24
Histidine	1-methyl-5-imidazoleacetate	0.7517	1.8822	0.0181	0.40
	1-ribosyl-imidazoleacetate*	0.6531	1.1529	0.0289	0.57
Lysine	6-oxopiperidine-2-carboxylate	1.4474	0.8655	0.0651	1.67
Tryptophan	Tryptophan	0.9276	1.0320	0.0878	0.90
	Kynurenine	1.1974	0.8228	0.0183	1.46
	Kynurenate	1.2040	0.8915	0.0566	1.35
	Anthranilate	1.1246	0.9008	0.0892	1.25
	Indoleacrylate	0.6600	1.6714	0.0380	0.39
	Indolepropionylglycine	0.5625	1.0672	0.0892	0.53
	7-hydroxyindole sulfate	1.4302	0.6874	0.0832	2.08
Leucine, Isoleucine, and Valine	4-methyl-2-oxopentanoate	0.8720	1.2226	0.0414	0.71
	Isovalerylglycine	0.7929	1.0679	0.0304	0.74
	Isovalerylcarnitine (C5)	0.7923	1.9335	0.0036	0.41
	3-methylcrotonylglycine	0.6848	1.2089	0.0121	0.57
	3-methyl-2-oxovalerate	0.8063	1.2062	0.0270	0.67
	2-methylbutyrylglycine	0.8937	1.1577	0.0773	0.77
	2,3-dimethylsuccinate	1.3811	0.7891	0.0561	1.75
Urea cycle (Arginine and Proline)	3-amino-2-piperidone	0.8941	1.1302	0.0717	0.79
	N-alpha-acetylornithine	0.6788	1.4021	0.0362	0.48
Creatine	N-methylhydantoin	1.1736	0.7328	0.0500	1.60
Glutathione	Cysteinylglycine	0.6454	1.0985	0.0423	0.59
	5-oxoproline	1.1848	0.7804	0.0032	1.52

B Xenobiotic Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Benzoate	Guaiacol sulfate	1.4420	0.6840	0.0468	2.11
	P-cresol sulfate	1.1707	0.7411	0.0623	1.58
	3-(3-hydroxyphenyl)propionate	0.8460	1.0530	0.0851	0.80
Food an Plant Component	Indolin-2-one	1.5935	0.8551	0.0368	1.86
	Mannonate*	0.8384	1.4255	0.0546	0.59
	Thymol sulfate	0.8354	1.7878	0.0369	0.47
Chemical	Dimethyl sulfone	0.8846	1.3030	0.0084	0.68
	6-hydroxyindole sulfate	1.0611	0.8717	0.0850	1.22

C Carbohydrate Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Glycolysis, Gluconeogenesis, and Pyruvate	1,5-anhydroglucitol	1.1905	0.8662	0.0787	1.37
Aminosugar	N-acetylglucosaminylasparagine	1.3139	0.5658	0.0687	2.32

D Cofactor & Vitamin Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Pantothenate and Coenzyme A	Pantoate	1.1839	0.6421	0.0546	1.84
Hemoglobin and Porphyrin	Bilirubin (Z,Z)	0.9448	1.5770	0.0478	0.60
Vitamin A	Retinal	0.6885	3.0961	0.0171	0.22

E Energy Substrate Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Tricarboxylic Adid Cycle	Itaconate	3.7331	0.9170	0.0822	4.07

F Peptide Metabolism Sub-Pathway	Metabolite	RC 24 h	RC 2 h	*t-test* *P*-value	RCFC 24h *vs*. 2h
Dipeptide	Prolylglycine	0.8123	1.3327	0.0383	0.61

Figure 4 Quantitative metabolomic analysis of pre- (24 h) and peri-(2 h) ovulatory follicular fluid (FF) continued. Metabolites are grouped super-pathway metabolism: (A) amino Acid; (B) xenobiotic; (C) carbohydrate; (D) cofactor and vitamin; (E) energy substrate; and (F) peptide metabolism. Corresponding sub-pathways are also provided. Dark green shading indicates a decrease (metabolite ratio <1.0) between the two time-points ($P \leq 0.05$), with light green depicting a decreasing trend ($0.05 < P < 0.10$). In contrast, dark red shading indicates an increase (metabolite ratio ≥1.0) between groups ($P \leq 0.05$), with light red depicting an increasing trend ($0.05 < P < 0.10$). Asterisks denote predicted metabolites. RC, relative concentration; RCFC, relative concentration fold-change.

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Corresponding metabolic pathway enrichment analysis revealed the following pathways as comprising the greatest proportion of temporally dynamic metabolites: estrogenic synthesis (E = 9.2; k = 2, m = 2); advanced glycosylation end-product (E = 9.2; k = 1, m = 1); dipeptide (E = 4.6; k = 1, m = 2); mevalonate (E = 4.6; k = 1, m = 2); and corticosteroid (E = 4.6; k = 1, m = 2) metabolism (Fig. 5). However, as these pathways comprise very few metabolites (m = 1 or 2), the data were re-analysed by percentage difference (PD (i.e. the number of metabolites corresponding to a specific pathway exhibiting significant flux in pre- *vs* peri-ovulatory FF as a percentage of all differences observed)). This revealed leucine, isoleucine, and valine (E = 1.7; PD = 9.5%), food component (E = 0.9; PD = 4.8%), and dicarboxylate fatty acid (E = 0.4; PD = 1.6%) as pathways comprise the most temporally dynamic metabolites (Fig. 5). Sixty-two (62) sub-pathways were unenriched (i.e. E = 0). Further analysis of the global metabolomic data (Supplementary Table 1) shows that FF becomes a less metabolically concentrated environment ($P \leq 0.05$) with time.

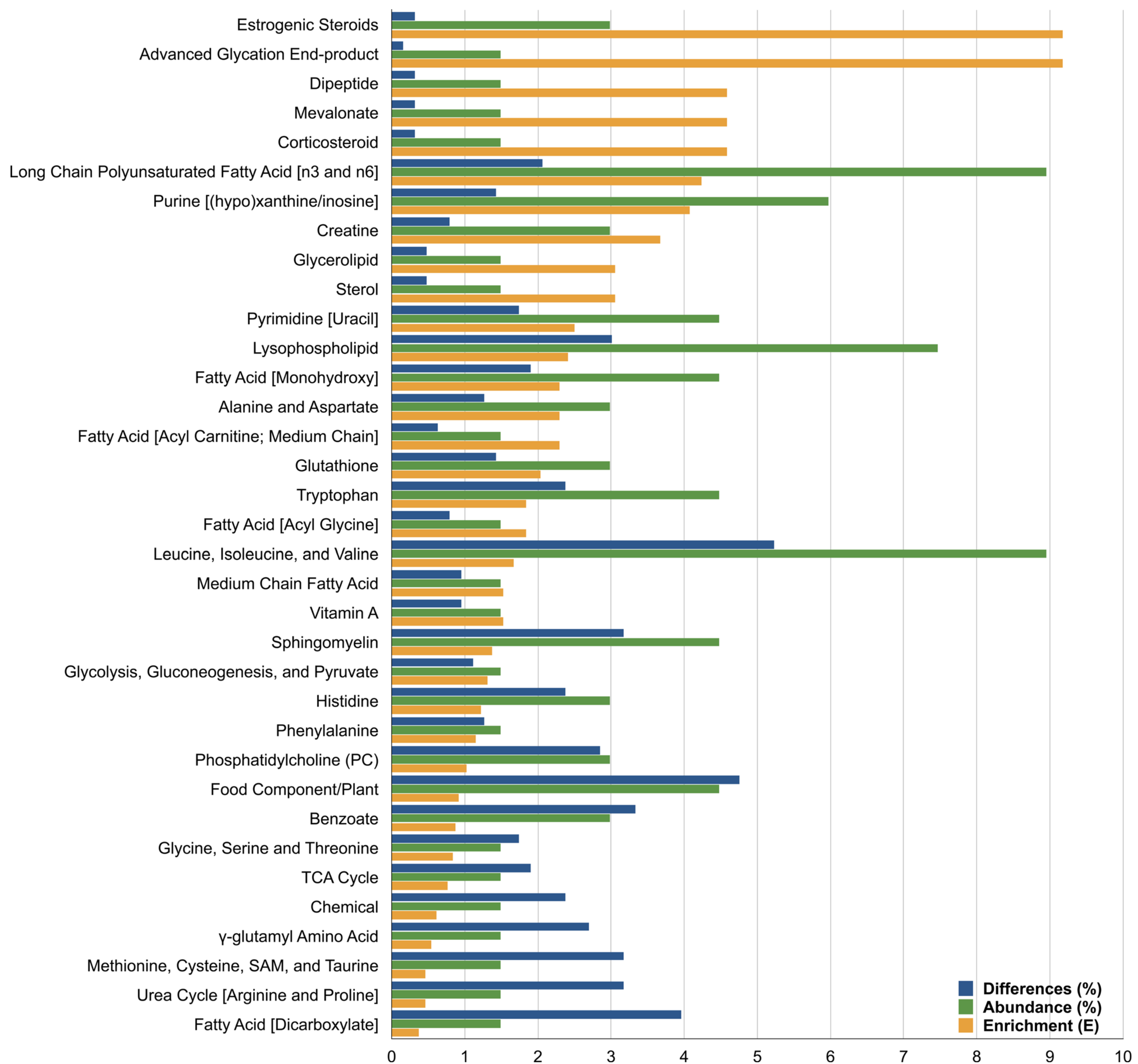

Figure 5 Sub-pathway metabolomic analysis of pre- (24 h) and peri-(2 h) ovulatory follicular fluid (FF). Specifically, pathway representation by percentage differences (i.e. the number of metabolites corresponding to a specific pathway exhibiting significant flux in pre- *vs* peri-ovulatory FF as a percentage of all differences observed in this study); percentage abundance (i.e. the number of metabolites corresponding to a specific pathway as a percentage of all identified metabolites); and enrichment (i.e. measure of intra-pathway metabolite flux relative to inter-pathway metabolite flux).

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Discussion

This study provides a detailed characterization of the metabolic fingerprint of bovine FF at two critical stages of oocyte development around the time of the LH surge-induced resumption of meiotic maturation and just prior to ovulation of the fully mature oocyte. Therefore, dependent experimental variables were inherently (a) local E2 and P4 concentrations and (b) COC maturation. Principal findings include (a) the identification of 634 metabolites in FF, of which 67.1% pertain to lipid and amino acid metabolism; (b) the significant flux of 67 metabolites (just 10.6% of total) over time; (c) FF becomes a less metabolically concentrated environment with time; and (d) a major flux of select metabolites occurs during this critical window. This study advances our understanding of the environment supporting oocyte maturation and ovulation, data which could be exploited to improve oocyte quality both *in vivo* and *in vitro*.

The current study greatly expands on previous work by our group, which used gas chromatography-mass spectrometry to analyse dairy cow preovulatory follicular fluid and identified ~20–30 aqueous metabolites and 23–37 fatty acids ((Bender *et al.* 2010, Forde *et al.* 2016, Moore *et al.* 2017). Here, we reveal FF to be a surprisingly metabolically diverse environment (634 metabolites organised into over 8 super pathways and 97 metabolic activities or sub pathways). Recent studies using the same metabolomic profiling platform have identified 173 metabolites in IVM medium and 369 metabolites in cumulus cells (Uhde *et al.* 2018), between 233 and 317 metabolites in bovine uterine lumen fluid (Simintiras *et al.* 2019, Simintiras *et al.* 2022); between 132 and 280 metabolites in bovine embryo and conceptus conditioned medium (Simintiras *et al.* 2021*b*); and 374 metabolites in human endometrial organoid conditioned medium (Simintiras *et al.* 2021*a*). The lower number of identified metabolites in these studies may be due to sample dilution, which was not required here. As such, our data provide a very high-resolution snapshot of the composition of bovine FF coinciding with final oocyte maturation just prior to ovulation.

Despite this high metabolic diversity, just 67 (10.6 %) metabolites exhibited ($P \leq 0.05$) flux, with a further 42 trending ($0.05 < P < 0.10$) towards exhibiting flux (17.2% in total), within the follicle during the final 22 h period of ovulatory follicle development (Figs. 3 and 4). This modest flux in the FF metabolome is in contrast to the findings *in vitro*, where 25–30% of metabolites exhibited significant fluctuations over a 24 h period (Uhde *et al.* 2018). Thus, despite the complex events of oocyte maturation and follicle luteinization and rupture occurring within the ovulatory follicle, a dynamic equilibrium appears to be maintained during this period by the consumption and secretion activities of the oocyte, cumulus, granulosa, and theca cells, to protect the COC. These metabolites and their associated metabolic and molecular pathways are considered in relation to the competing activities of the COC and the follicle cells during the peri-ovulatory period.

Biomarkers of follicle ovulatory status

The greatest flux magnitudes observed were almost a 100-fold decrease in hypoxanthine (HX) and a 66-fold decline in xanthine levels in less than 22 h. Closely related compounds inosine and xanthosine were also depleted by 5.0- and 2.7-fold, respectively (Fig. 3B). This is reassuring, as HX is a known inhibitor of bovine (Kadam & Koide 1990) as well as murine (Eppig *et al.* 1985, Downs *et al.* 1986) resumption of oocyte meiotic maturation, and starts declining in bovine FF as early as 8 h post the GnRH-induced LH surge (Romero-Arredondo & Seidel 1994). Hypoxanthine has also been identified in porcine (Miyano *et al.* 1995), caprine (Ma *et al.* 2003), and human (Lavy *et al.* 1990) FF. More specifically, granulosa cell inosine-5′-monophosphate (IMP) dehydrogenase (IMPDH), also known as GMP reductase (GMPR), is crucial for maintaining oocyte-follicular synchrony and meiotic arrest via two coordinated pathways. The first revolves around IMP catalysis by IMPDH into xanthosine monophosphate (XMP), eventually further catalyzed by the natriuretic peptide C/natriuretic peptide receptor 2 (NPPC/NPR2) system to cyclic GMP (cGMP) to sustain oocyte meiotic arrest. IMPDH is the rate-limiting step in this cascade (Kiyosu *et al.* 2012, Ni *et al.* 2021). The second mode of IMPDH-regulated meiotic arrest maintenance is the preservation of an intracellular HX pool, which inhibits cyclic nucleotide phosphodiesterases (Downs *et al.* 1989), including adenylyl cyclase, resulting in the accumulation of intracellular cAMP, which too sustains oocyte meiotic arrest (Jones 2004, Pan & Li 2019). The observed steep decline of HX and associated compounds in FF between 24 and 2 h pre-ovulation reflects the culmination of LH-activated signalling cascades within the preovulatory follicle leading to the release of the oocyte from meiotic arrest and resumption of oocyte meiotic maturation. There is an interest in delaying the spontaneous resumption of meiosis *in vitro*, for improving oocyte quality and developmental potential, by recapitulating some of the naturally occurring biochemical and cellular events. The most promising approach is the so-called simulated

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physiological oocyte maturation system of oocyte IVM which incorporates a pre-maturation treatment (Albuz *et al.* 2010, Li *et al.* 2016). This system has improved embryo yield in murine, bovine, porcine, and human IVM COCs and may bridge the efficiency gap between IVM and IVF. However, since the initial reports, few new data have emerged to demonstrate the repeatability of these results, and others have failed to achieve similar outcomes (Guimarães *et al.* 2015). These concerns have been acknowledged (Gilchrist *et al.* 2015). The current data set may enable further refinement of this or similar cAMP-mediated pre-IVM culture systems that have the potential to improve the efficiency of IVM in the future.

The next biggest decline in metabolite concentration was that of E2 (12.37-fold (Fig. 3A)), concomitant with P4 elevation (3.7-fold (Fig. 1A)), hallmarks of the late follicular phase of the bovine oestrous cycle (Dieleman *et al.* 1983). Our data captured the characteristic preovulatory follicle change from an E2-dominated environment at oestrus onset to one that becomes progressively dominated by P4 following the LH surge (Dieleman *et al.* 1983). This window coincides with the resumption of oocyte meiotic maturation and dramatic morphological and metabolic changes to cumulus cells but without a corresponding change in dominant follicle dimensions (reviewed in Lonergan & Fair (2016)), consistent with our observations (Fig. 1C). Further model validation was gleaned from the absence of prostaglandins (PG) PGE2 and PGF2α in pre-ovulatory FF samples and their first detection in peri-ovulatory FF (Fig. 2C).

Polyunsaturated fatty acids (PUFA) are precursors to PG synthesis (Cheng *et al.* 2001), the major one being arachidonic acid (Smyth & Fitzgerald 2003). An almost three-fold depletion of arachidonic acid over time was observed (Fig. 3A). High arachidonic acid levels are associated with bovine ovarian granulosa cell death induction and decreased E2 secretion (Zhang *et al.* 2019). Thus, it is likely that arachidonic acid depletion was due to PGE2 and PGF2α conversion. This corroborates the finding that PG secretion commences late in the periovulatory period, i.e. between 18 and 24 h after GnRH administration (Bridges *et al.* 2006). Moreover, studies on sheep (Wonnacott *et al.* 2010) and cattle (Adamiak *et al.* 2005) support the hypothesis that depleted PUFA is due to their conversion by granulosa cells to PG at ovulation (Algire *et al.* 1992). Intrafollicular PG concentrations increase in the hours preceding ovulation in several species (Armstrong 1981, Sirois & Doré 1997, Sirois *et al.* 2000), while PG synthesis inhibitor administration blocks ovulation in cattle (De Silva & Reeves 1985) and blocks fertilization, embryo development, and implantation in mice (Chakraborty *et al.* 1996). Prostaglandin E2 (PGE2) is the most common PG in animals (Niringiyumukiza *et al.* 2018); a key paracrine mediator of the LH surge, it acts through multiple PGE2 receptors (PTGERs) (Harris *et al.* 2011). Moreover, accumulation of PG, a pro-inflammatory factor (Ricciotti & Fitzgerald 2011) in the peri-ovulatory follicle, supports the concept of ovulation being an inflammatory-like process (Espey 1980), discussed below.

Immunomodulation and metabolism

Polyunsaturated fatty acids are bioactive lipids capable of modulating inflammation and immunity (Michalak *et al.* 2016). Like all fatty acids, they comprise aliphatic hydrocarbon chains with methyl and carboxyl groups at either end. Two main immune metabolically relevant PUFA categories are recognized, those with an unsaturated carbon (double bond) at the third carbon from the methylated end (ω-3 or n-3) and those with a double bond at the sixth carbon (ω-6 or n-6). The ω-3 PUFA include α-linoleic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), whereas ω-6 PUFA include linoleic acid (LA) and arachidonic acid (AA). The ω-3 and ω-6 PUFA are generally considered anti-inflammatory and pro-inflammatory, respectively (Michalak *et al.* 2016). PUFA were jointly the most abundant metabolite class identified and one of the most enriched sub-pathways (Fig. 2). Moreover, of the PUFA we could categorically identify as ω-3 or ω-6, 4 were ω-3 and 2 were ω-6. On the surface, this suggests that the PUFA anti- vs pro-inflammatory balance in FF is tipped towards an anti-inflammatory state. However, the concentrations of all PUFA decreased or trended towards a decrease in FF with time (Fig. 3A) – including ALA. In cattle, FF ALA levels correlate with oocyte competence to form blastocysts (Matoba *et al.* 2014), likely due to scavenging reactive oxygen species (ROS) (Marei *et al.* 2012). Similar results have been reported in pigs (Lee *et al.* 2017). Moreover, reduced pregnancy loss has been reported in cows fed dietary ALA (Ambrose *et al.* 2006). Other antioxidants identified in FF include carotenes, glutathione, urate, and ascorbic acids (Vitamin C), though significant flux in their levels was not observed (Supplementary Table 1).

The metabolite exhibiting the greatest increase in FF over time was retinal (4.9-fold). Retinal is one of three vitamin A (or retinoid) forms, the others being retinol and retinoic acid (RA), of which two forms – trans-retinoic acid and 9-cis retinoid acid – exist. Retinol was also detected in FF but did not exhibit significant flux (Supplementary Table 1). Retinoids act on cells of both the innate and

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adaptive immune systems (Oliveira *et al.* 2018) and are generally considered anti-inflammatory (Huang *et al.* 2011). Numerous alcohol dehydrogenases catalyse the bidirectional conversion of retinol to retinal (Huang *et al.* 2011). Three cytosolic retinaldehyde dehydrogenases (RALDH1, 2, and 3) – also known as ALDH1A1, 2, and 3 irreversibly oxidize retinaldehyde into RA, see reviews by Duester 2008 & Gudas 2022. Within the ovary, it is presumed that granulosa cells uptake retinol and convert it to retinal and RA (Liu *et al.* 2018). However, there is also evidence that CC contain endogenously active retinoid receptors and may also be competent to synthesize RA (Mohan *et al.* 2003). The role of RA as an antioxidant in the bovine ovary has long been hypothesized (Ikeda *et al.* 2005). However, additional roles of RA in female reproduction and more specifically ovarian function were identified. For example, several investigations report RA regulation of steroidogenesis (for review see Damdimopoulou *et al.* (2019). Supplementation of *in vitro* chicken, rat, and murine granulosa cell cultures promoted P4 secretion (Bagavandoss & Midgley 1987, Pawłowska *et al.* 2008, Manna *et al.* 2015). The effect is likely due to RA regulation of steroidogenic enzyme activity; however, evidence of retinoid involvement in FSH-stimulated induction of FSH and LH receptor expression in granulosa cells has been reported in *in vivo* mouse models (Kawai *et al.* 2016, Kawai *et al.* 2018). Interestingly, RA supplementation of oocyte *in vitro* maturation medium within an IVP protocol was shown to enhance blastocyst development rates in cattle (Livingston *et al.* 2004, Lima *et al.* 2006); the effect is likely to be associated with improved oocyte meiotic maturation (Hidalgo *et al.* 2003, Gad *et al.* 2018).

Similarly, tryptophan, an essential amino acid, trended towards an increase in FF at T2. Essential amino acids are involved in immune system regulation (Moffett & Namboodiri 2003, Schröcksnadel *et al.* 2006); Tryptophan metabolism specifically has been implicated in the control of hyperinflammation and long-term immune tolerance induction, as it is a precursor for serotonin and melatonin synthesis (Badawy 2019, Platten *et al.* 2019). Melatonin is a potent antioxidant able to scavenge ROS and reactive nitrogen species (RNS) (Galano *et al.* 2011). Inflammation-related proteolytic enzyme production generates toxic oxygen derivatives (Espey & Lipner 1994). Thus, it could be argued that the increased tryptophan at the time of ovulation may underpin a need to produce melatonin. Indeed, melatonin can reduce oxidative damage in rat oocytes (Tamura *et al.* 2008) and protect bovine CCs from oxidative damage, by promoting CC secretion of self-protective antioxidant proteins, such as CuZn-SOD, Mn-SOD, and glutathione peroxidase (GPx) (Valerino Da Cunha *et al.* 2015). In addition, melatonin reportedly influences bovine oocyte maturation and embryo development by upregulating *ATPase 6*, *BMP-15*, *GDF-9*, *SOD-1*, *GPX-4*, and *BCL-2* mRNA expression and downregulating expression of apoptotic caspase- 3 (Yang *et al.* 2017). Moreover, indoleacrylate, involved in tryptophan metabolism, also elevated in peri-ovulatory FF (Fig. 3A) and promoted anti-inflammatory responses by enhancing *IL10* expression and reducing the expression of *IL6* and *Tnf* in LPS-stimulated murine macrophages (Wlodarska *et al.* 2017).

Several sphingomyelins, specifically, d18:1/22:0 (behenoyl), d17:1/16:0, d18:1/15:0, d16:1/17:0, d18:2/24:1, and d18:1/24:2 were also elevated at the time of ovulation, with sphingomyelins d18:1/16:0 (palmitoyl), 18:1/23:0, d18:1/17:0, d17:1/18:0, d19:1/16:0, d18:1/18:1, d18:2/18:0, d18:2/23:0, d18:1/23:1, and d17:1/24:1 trending ($0.05 < P < 0.10$) towards an increase (Fig. 3A); intriguing observations given that sphingomyelin production is correlated with acute inflammation (Balsinde *et al.* 1997) and dysfunctional ovarian sphingolipid metabolism is associated with polycystic ovarian syndrome (Liu *et al.* 2019).

The FF concentration of lysophospholipid (LysoPCs) metabolites, including 1-oleoyl-GPC (18:1), 1-linoleoyl-GPC (18:2), 1-linolenoyl-GPC (18:3),1-arachidonoyl-GPC (20:4n6), and 1-linoleoyl-GPE (18:2), decreased following the LH surge. Lysophosphatidic acid (LPA) was reported to induce *IL8* and *IL6* expression through LPA receptors and NF-κB-dependent pathways in granulosa-lutein cells recovered from preovulatory follicles of women undergoing IVF (Chen *et al.* 2008). The authors proposed that LysoPCs play a crucial role in CL angiogenesis by increasing endothelial cell permeability (Chen *et al.* 2008). However, higher FF concentrations of LysoPC (18:1), LysoPC (18:2), and LysoPC (18:3) were recently associated with adverse outcomes in women who underwent IVF (Song *et al.* 2019). The published data around the relationship between LysoPCs and oocyte competence are somewhat contradictory; on the one hand, LysoPCs have been highly correlated with apoptosis (Lauber *et al.* 2003), but, LPA supplementation during IVM was shown to improve bovine oocyte maturation, reduce the extent of apoptosis in CCs, and sustain the expression of developmental competence-related factors during oocyte maturation (Boruszewska *et al.* 2015). The authors proposed that LysoPCs may influence the maturation process by providing proper conditions for glucose transport and metabolism by increasing CCs glucose uptake and stimulating lactate production. A regulatory role for lactate during the follicular-luteal

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transition, specifically in attenuating E2 production and promoting luteinization, has been proposed (Baufeld *et al.* 2019, Baufeld & Vanselow 2022), possibly in response to the hypoxic conditions of the ovulatory follicle (Shweiki *et al.* 1992, Levy *et al.* 1995), via hypoxia-inducible factor-independent mechanism of lactate accumulation under hypoxic conditions (Lee *et al.* 2015).

Xenobiotic metabolites

While growing interest in the interaction between the reproductive system and xenobiotics has identified associations between xenobiotics and reproductive dysfunction in livestock species, including infertility, early embryonic loss, decreased oestrus behaviour, and reduced ovulation rate and abortion (Panter & Stegelmeier 2011), the current study is the first to report their presence in bovine preovulatory FF. Moreover, metabolites in this group represent 12% of all identified metabolites and 6.8% of the total differentially abundant metabolites. In livestock species, xenobiotics gain access to the body primarily through feed, drinking water, and veterinary drug administration and indeed the 73 xenobiotics identified in the current study mainly mapped to the sub pathways, food component/plant, drug, benzoate metabolism, and chemical. Feed contaminants may be chemical, such as dioxins, endocrine disruptors, pesticides, fertilizers, and detergents, or biological, ranging from bacterial, fungal, or parasitic pathogens to novel organisms (e.g. genetically engineered feed) (Scialabba 2022). Following ingestion, xenobiotics undergo a broad range of detoxication processes to render them less toxic, more polar, and readily excretable (Patterson *et al.* 2010). The concentration of xenobiotic-derived metabolites included benzoate, 3-(3-hydroxyphenyl) propionate, gluconate, mannonate, thymol sulfate, and dimethyl sulfone, the concentration of which increased in FF ($P < 0.05$) between 24 and 2 h pre-ovulation. The increasing level of xenobiotic metabolites in peri-ovulatory FF may be a consequence of increased blood flow within the preovulatory follicle, which is associated with the LH surge (Acosta *et al.* 2003), and/or the generation of toxic oxygen derivatives during the inflammatory-like process of ovulation (Espey & Lipner 1994), which must be detoxified. Several animal-based studies have shown that chemical mixtures can affect folliculogenesis and steroidogenesis *in vivo* (for review, see Mourikes and Flaws (Mourikes & Flaws 2021)). Most relevant to the current study is working in sheep, exposing pregnant ewes to sewage sludge, recognized source of environmental contaminants, resulted in increased cell death in ovarian follicles, acceleration of follicle development, and altered candidate protein expression in ovarian tissue in their female offspring (Fowler *et al.* 2008). There is some evidence to suggest that these metabolites possess anti-inflammatory properties; for example, gluconate was observed to inhibit tumour growth in mice by blocking citrate transport into cancer cells (Mycielska *et al.* 2019), while thymol, circulating as thymol sulphate (Nagoor Meeran *et al.* 2017), reduces inflammation in a rat ulcerative colitis model, by suppressing PTGS2 protein expression, as well as IL6 and IL1 concentrations (Tahmasebi *et al.* 2019). Additionally, in several rodent models, thymol promotes wound healing by inhibiting leucocyte influx to the site of injury, subsequently preventing oedema (Riella *et al.* 2012). Similarly, dimethyl sulfone is a potent anti-inflammatory agent with anti-oxidant properties, although whether the mechanism is direct or indirect is unknown (Butawan *et al.* 2017).

Summary

This study demonstrates that FF metabolites that are differentially regulated around the time of ovulation may have essential roles in the final stages of oocyte maturation and the ovulatory inflammatory cascade, where increased metabolites, mainly related to inflammatory/immune responses, modulate inflammation and contribute to cellular homeostasis. In addition, the identification of xenobiotic metabolites in bovine preovulatory FF is interesting, as it highlights the exposure of the oocyte within a developing follicle to environmental contaminants and raises questions about their actions. In conclusion, the dynamic pre-ovulatory FF content should be considered in the context of immunomodulation and as an important milieu regulating the balance between oxidants and antioxidants.

This high-resolution analysis of the metabolomic dynamics of preovulatory bovine follicular fluid supports the hypothesis of the ovulatory process as an inflammatory/immune cascade and further describes the environment for final oocyte maturation, implicating additional metabolic pathways as being important including responses to external contaminants.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-22-0090.

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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