Lay Abstract

The ovaries are part of the female reproductive system, 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. Follicular fluid, 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 in the womb. Follicular fluid has evolved to support the egg to achieve this. Using the cow as a model, this study looks at the composition of follicular fluid 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.
Biochemical alterations in the follicular fluid of bovine peri-ovulatory follicles and 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-high-performance liquid chromatography 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 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 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.

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 (Lonergan and Fair, 2016, Fair, 2010). 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 d postpartum were detected using ultra-high-performance liquid chromatography 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 and Sakumoto, 2003, Townson and 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 metabolomics 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 h 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- versus 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, 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 estrous 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 by intramuscular injection, immediately followed by insertion of a progesterone (P4)-releasing intravaginal device (PRID; Ceva Santé Animale). After seven 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 (Figure 1A). Heifers were transported to a local EU-licensed abattoir and ovaries were retrieved immediately post-mortem, corresponding to either 24 h 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 (Kruipl et al., 1983; Hyttel P et al., 1987), 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 were used for metabolomic analysis at T2.

**Follicular fluid recovery and processing**

Ovaries were returned to the laboratory on ice within 1 h of retrieval. Ultimately, 10 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 recovered on ice, as previously described (Alrabiah et al., 2021). FF was then centrifuged at 100 x 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 (Figure 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 in Simintiras et al. (2021a).

Briefly, protein in each sample was precipitated with methanol and extracted using the automated MicroLab STAR system (Hamilton Company) under centrifugation at 680 x 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 & 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 for 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 x 100 mm column). Aliquot 2 (also RP-UPLC-MS/MS +ESI), was identically eluted, using the same column, with the addition of acetonitrile 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 x 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 were subsequently analyzed 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 70-1000 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 versus 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 standard deviation was 3% with a total process variability of 6%.

**Progesterone and protein quantification**

Follicular fluid P4 was measured by solid-phase radioimmunoassay (PROG-RIA-CT KIP1458; DiaSource ImmunoAssays S.A.), according to manufacturer instructions. Assay range and sensitivity were 0.12 to 36 ng·ml⁻¹ and 0.05 ng·ml⁻¹, respectively. Follicular fluid 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: \( E = \frac{k}{m} \cdot \frac{n}{N} \cdot \frac{1}{1} \)

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 (**Figure 1C**), FF protein (**Figure 1D**), and FF P4 composition (**Figure 1E**) data were analyzed by unpaired two-tailed t-test using Prism 9.0 (GraphPad, San Diego, CA, USA). Principal component analysis (**Figure 2D**) was performed using the open-access Past4 software (Hammer et al., 2001). Transformed metabolomic data (**Figures 3 and 4**) were statistically contrasted using a 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 \( [\pm \text{ standard deviation (S.D.)}] \) of pre \((18.5 \pm 1.9 \text{ mm})\) vs. peri \((18.5 \pm 2.3 \text{ mm})\) -ovulatory follicle did not differ (**Figure 1C**). Similarly, mean \( [\pm \text{ S.D.}] \) protein content \((28.7 \pm 5.1 \text{ and } 31.5 \pm 2.4 \text{ mg·ml}^{-1}\) in pre and peri -ovulatory FF, respectively) was not different (**Figure 1D**). However, mean \( [\pm \text{ S.D.}] \)
FF P4 concentration increased from 24 h (55.7 ± 41.3 ng·ml⁻¹) to 2 h (204.9 ± 63.8 ng·ml⁻¹) before ovulation (Figure 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, 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 Figure 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 samples, whereas estrone and 1-myrisoyl-2-palmitoyl-glycerophosphorylcholine were absent from all 2 h peri-ovulatory FF samples (Figure 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 Figure 2C. The original normalized raw area counts for each metabolite is 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 (Figure 2D). This is attributable to concentration differences of 67 metabolites between the two FF groups (Figures 3 and 4). More specifically, 21 (33.3%) were reduced \( (P\leq0.05) \) by a mean of 9.0-fold, whereas 46 were elevated \( (P\leq0.05) \) by a mean of 1.7-fold in peri vs. pre-ovulatory FF, the differences 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).

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=4.6; \( k=1; m=2 \)); mevalonate (E=4.6; \( k=1; m=2 \)); and corticosteroid (E=4.6; \( k=1; m=2 \)) metabolism (Figure 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 comprising the most temporally dynamic metabolites (Figure 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 \( (P\leq0.05) \) with time.

**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 using gas chromatography mass spectrometry to analyse dairy cow preovulatory follicular fluid, 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) and between 233-317 metabolites in bovine uterine lumen fluid (Simintiras et al., 2019, Simintiras et al., 2022); between 132-280 metabolites in bovine embryo and conceptus conditioned medium (Simintiras et al., 2021b); and 374 metabolites in human endometrial organoid conditioned medium (Simintiras et al., 2021a). 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 \( P\leq0.05 \) 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 (Figures 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, 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 (Figure 3B). This is reassuring, as HX is a known inhibitor of bovine (Koide and Kadam, 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 and Seidel Jr, 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 guanosine monophosphate (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
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 cyclic adenosine monophosphate cAMP, which too sustains oocyte meiotic arrest (Jones, 2004, Pan and 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 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 has 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 (Figure 3A)], concomitant with P4 elevation [3.7-fold (Figure 1A)], hallmarks of the late follicular phase of the bovine estrous 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 and Fair (2016)), consistent with our observations (Figure 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 (Figure 2C).

Polyunsaturated fatty acids (PUFA) are precursors to PG synthesis (Cheng et al., 2001), the major one being arachidonic acid (Smyth and FitzGerald, 2003). An almost 3-fold depletion of arachidonic acid over time was observed (Figure 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 18-24 h after GnRH administration (Bridges et al., 2006). Moreover, work in sheep (Wonnacott et al., 2010), and cattle (Adamiak et al., 2004), 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 (Sirois and Doré, 1997, Armstrong, 1981, Sirois et al., 2000), while PG synthesis inhibitor administration blocks ovulation in cattle (De Silva and 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 and 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), eicosapentanoic 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 (Figure 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 toward a decrease in FF with time (Figure 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 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) and (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 for 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 and Midgley, 1987, Pawlowska 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 (Lima et al., 2006, Livingston et al., 2004); the effect likely to be associated with improved oocyte meiotic maturation (Hidalgo et al., 2003, Gad et al., 2018).

Similarly, tryptophan, an essential amino acid, trended toward an increase in FF at T2. Essential amino acids are involved in immune system regulation (Moffett and 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 (Platten et al., 2019, Badawy, 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 and 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) (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 (Table 2A), promotes 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 (Figure 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 lysosphospholipid (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. Lysoosphatidic 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) was 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 is 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 via providing the 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 transition, specifically in attenuating E2 production and promoting luteinization has been proposed (Baufeld and Vanselow, 2022, Baufeld et al., 2019), possibly in response to the hypoxic conditions of the ovulatory follicle (Levy et al., 1995, Shweiki et al., 1992), 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, reduced ovulation rate and abortion (Panter and
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, mannionate, thymol sulfate and dimethyl sulfone, the concentration of which increased in FF (P<0.05) between 24h to 2h 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 and 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 and Flaws, 2021)). Most relevant to the current study is work 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 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 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.
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Contribution to the Field Statement
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.
Figure Legends

**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) hours 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 [± standard deviation (S.D.)] of 24h [pre (n=6)] and 2h [peri (n=4)] ovulatory follicles. (D) Mean (± S.D.) protein content in 24h (n=6) vs. 2h (n=4) ovulatory FF. (E) Mean (± S.D.) FF P4 levels in 24h (n=6) vs. 2h (n=4) ovulatory FF – wherein ** represents \( P \leq 0.01 \).

**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 in 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.

**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 \( \geq 1.0 \)) between groups \( P \leq 0.05 \), with light red depicting an increasing trend \( 0.05 < P < 0.10 \). Asterisks denote predicted metabolites. Abbreviations: Relative concentration (RC); and Relative concentration fold-change (RCFC).

**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 \( \geq 1.0 \)) between groups \( P \leq 0.05 \), with light red depicting an increasing trend \( 0.05 < P < 0.10 \). Asterisks denote predicted metabolites. Abbreviations: Relative concentration (RC); and Relative concentration fold-change (RCFC).

**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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Table 1. 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<0.05), with light green depicting a decreasing trend (0.05<P<0.10). In contrast, dark red shading indicates an increase (metabolite ratio >1.2) between groups (P<0.05), with light red depicting an increasing trend (0.05<P<0.10). Asterisks denote predicted metabolites. Abbreviations: Relative concentration (RC), and Relative concentration fold-change (RCFC).

Table 2. 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) Nucleotide, (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 aforementioned time-points (P<0.05), with light green depicting a decreasing trend (0.05<P<0.10). In contrast, dark red shading indicates an increase (metabolite ratio >1.2) between groups (P<0.05), with light red depicting an increasing trend (0.05<P<0.10). Asterisks denote predicted metabolites. Abbreviations: Relative concentration (RC), and Relative concentration fold-change (RCFC).

Figure 1. Schematic depiction of experimental design. (A) Ten heifers were synchronized by administration of gonadotropin-releasing hormone (GnRH), progesterone releasing intrauterine device (PRED), and prostaglandin (PGs). Follicular fluid (FF) was aspirated 24 (n=4) and 2 (n=4) hours prior to ovulation. (B) FF was snap-frozen in liquid nitrogen (L.N.) until analysis for total protein content or by high-throughput untargeted metabolomics.

Figure 2. Metabolomic snapshot of pre- and peri-ovulatory follicular fluid (FF). (A) Principal component analysis of FF metabolomic profiles 24 h (green; n=6) vs. 2 h (blue; n=4) pre-ovulation. (B) Pie chart of FF metabolite super-pathway distribution. (C) Venn diagram of metabolites unique or common to pre- (green) vs. peri- (blue) ovulatory FF. (D) The percentage of samples from each group in which the corresponding metabolite was identified (% Filled Values). All other metabolites were identified in 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 50-85%.

Figure 3. Sub-pathway metabolomic analysis of pre- (24 h) and peri- (2 h) ovulatory follicular fluid (FF). Specifically, Pathway representation by abundance i.e. the number of metabolites corresponding to a specific pathway as a percentage of all identified metabolites and 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.
Biochemical composition of follicular fluid from the bovine pre- and peri-ovulatory follicle

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Figure 1. Schematic depiction of experimental design. (A) Ten heifers were synchronized by administration of gonadotropin-releasing hormone (GnRH), progesterone releasing intravaginal device (PRID), and prostaglandin (PGE2). Follicular fluid (FF) was aspirated 24 (n = 6) and 2 (n = 4) hours prior to ovulation. (B) FF was snap-frozen in liquid nitrogen (LN2) until analysis for total protein content or by high-throughput untargeted metabolomics.

Figure 2. Metabolite snapshot of pre- and peri-ovulatory follicular fluid (FF). (A) Principal component analysis of FF metabolomic profiles 24 h (green: n=6) vs. 2 h (blue: n=4) pre-ovulation. (B) Pie chart of FF metabolite super-pathway distribution. (C) Venn diagram of metabolites unique to pre- (green) or peri- (blue) ovulatory FF. (D) The percentage of samples from each group in which the corresponding metabolite was identified (% Filled Values). All other metabolites were identified in almost all samples. Dark blue cell shading (100%) indicates complete presence whereas dark orange (0%) indicates complete absence. Light orange indicates presence in 17-25% of samples whereas light blue shading denotes a percentage fit between 50-85%.

Figure 3. Sub-pathway metabolite analysis of pre- (24 h) and peri- (2 h) ovulatory follicular fluid (FF). Specifically, Pathway representation by abundance (i.e. number of metabolites corresponding to a specific pathway as a percentage of all identified metabolites) and 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).

Table 1. Quantitative metabolite 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 (PI0.05), 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 (PI0.05), with light red depicting an increasing trend (0.05<P<0.10). Asterisks denote predicted metabolites. Abbreviations: Relative concentration (RC), and Relative concentration fold-change (RCFC).

Table 2. Quantitative metabolite analysis of pre- (24 h) and peri- (2 h) ovulatory follicular fluid (FF). Metabolites are grouped super-pathway metabolism: (A) Amino Acid; (B) Nucleotide; (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 aforementioned time-points (PI0.05), 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 (PI0.05), with light red depicting an increasing trend (0.05<P<0.10). Asterisks denote predicted metabolites. Abbreviations: Relative concentration (RC), and Relative concentration fold-change (RCFC).
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