



HAL
open science

Differences in the metabolomic signatures of porcine follicular fluid collected from environments associated with good and poor oocyte quality

Michael Bertoldo, Lydie Nadal-Desbarats, Nadine Gérard, Alexis Dubois, Patricia K. Holyoake, Christopher Gerald Grupen

► **To cite this version:**

Michael Bertoldo, Lydie Nadal-Desbarats, Nadine Gérard, Alexis Dubois, Patricia K. Holyoake, et al.. Differences in the metabolomic signatures of porcine follicular fluid collected from environments associated with good and poor oocyte quality. *Reproduction*, 2013, 146 (3), pp.221-231. 10.1530/REP-13-0142 . hal-01129742

HAL Id: hal-01129742

<https://hal.science/hal-01129742>

Submitted on 29 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Differences in the metabolomic signatures of porcine follicular fluid collected from environments associated with good and poor oocyte quality

Michael J Bertoldo^{1,2}, Lydie Nadal-Desbarats^{3,4}, Nadine Gérard¹, Alexis Dubois³, Patricia K Holyoake^{2,5} and Christopher G Grupen²

¹INRA, UMR85 Physiologie de la Reproduction et des Comportements, Équipe Interactions Cellulaires et Fertilité, 37380 Nouzilly, France, ²Faculty of Veterinary Science, The University of Sydney, Camden, New South Wales 2570, Australia, ³INSERM U930, Équipe Neurogénétique et Neurométabolomique, Université François-Rabelais, 37044 Tours, France, ⁴INSERM U930, PPF Analyse des Systèmes Biologiques, Université François-Rabelais 37044 Tours, France and ⁵Pig Health and Research Unit, Department of Primary Industries, Epsom, Victoria 3551, Australia

Correspondence should be addressed to C G Grupen; Email: christopher.grupen@sydney.edu.au

M J Bertoldo and L Nadal-Desbarats contributed equally to this work

Abstract

The microenvironment of the developing follicle is critical to the acquisition of oocyte developmental competence, which is influenced by several factors including follicle size and season. The aim of this study was to characterise the metabolomic signatures of porcine follicular fluid (FF) collected from good and poor follicular environments, using high-resolution proton nuclear magnetic resonance (¹H-NMR) spectroscopy. Sow ovaries were collected at slaughter, 4 days after weaning, in summer and winter. The contents of small (3–4 mm) and large (5–8 mm) diameter follicles were aspirated and pooled separately for each ovary pair. Groups classified as summer-small ($n=8$), summer-large ($n=15$), winter-small ($n=9$) and winter-large ($n=15$) were analysed by ¹H-NMR spectroscopy. The concentrations of 11 metabolites differed due to follicle size alone ($P<0.05$), including glucose, lactate, hypoxanthine and five amino acids. The concentrations of all these metabolites, except for glucose, were lower in large FF compared with small FF. Significant interaction effects of follicle size and season were found for the concentrations of glutamate, glycine, *N*-acetyl groups and uridine. Succinate was the only metabolite that differed in concentration due to season alone ($P<0.05$). The FF levels of progesterone, androstenedione and oestradiol were correlated with the concentrations of most of the metabolites examined. The results indicate that there is a distinct shift in follicular glucose metabolism as follicles increase in diameter and suggest that follicular cells may be more vulnerable to oxidative stress during the summer months. Our findings demonstrate the power of ¹H-NMR spectroscopy to expand our understanding of the dynamic and complex microenvironment of the developing follicle.

Reproduction (2013) **146** 221–231

Introduction

During the latter stages of folliculogenesis, the cumulus–oocyte complex (COC) is bathed in follicular fluid (FF), which provides an important and specialised microenvironment for *in vivo* oocyte maturation. It is well known that FF consists of exudates from serum and factors produced by the oocyte and somatic compartment. Characterisation of FF can provide useful information about the growth and differentiation of the follicle (Edwards 1974). Various studies performed in mammals clearly demonstrate that FF contains components essential to oocyte maturation and fertilisation and molecules related to follicular cell proliferation and differentiation (Gérard *et al.* 2002, Pinero-Sagredo

et al. 2010). Owing to the close physical relationship between the COC and the FF, analysis of this fluid is of extreme interest for elucidating the determinants of oocyte quality.

Investigation of FF has increased over the last decade but its composition is still relatively unknown. Previous studies have considered the concentration of various hormones (Takahashi *et al.* 2008, Bertoldo *et al.* 2011a), growth factors (Wang *et al.* 2006, Wu *et al.* 2007), reactive oxygen species (Oyawoye *et al.* 2003, Das *et al.* 2006) and other biomarkers (Wallace *et al.* 2012, Yang *et al.* 2012) and have attempted to correlate the biochemistry of FF with oocyte developmental competence and embryo development with varying success. Nonetheless, targeted analysis of certain amino acids and

steroids has revealed interesting correlations with oocyte developmental competence and IVF outcomes (D'Aniello *et al.* 2007, Boxmeer *et al.* 2009, Bertoldo *et al.* 2011a).

The modern domestic sow (*Sus domesticus*) generally breeds throughout the year and is capable of producing two litters annually. However, a reduction in fertility is observed during the late summer and early autumn months in many countries around the world (Love *et al.* 1993, Peltoniemi *et al.* 1999, Bertoldo *et al.* 2009). This phenomenon has been referred to as seasonal infertility and its primary manifestation is increased early pregnancy loss (reviewed by Bertoldo *et al.* (2012)). The mechanisms that underpin this pregnancy loss are highly complex and remain poorly understood. Recent observations have implicated follicle development and ovarian progesterone in the reduction of sow fertility (Bertoldo *et al.* 2011a), and there is clear evidence that oocyte developmental competence is reduced during the seasonal infertility period (Bertoldo *et al.* 2010). Oocytes recovered from large follicles (5–8 mm diameter) in summer had a significantly reduced capacity to form blastocysts compared with those recovered in winter, whereas oocytes recovered from small follicles (3–4 mm diameter) had a limited capacity to form blastocysts regardless of season (Bertoldo *et al.* 2010). Therefore, the seasonal and follicle size differences observed in the sow make it an ideal comparative model to reveal changes in FF composition that support the acquisition of oocyte developmental competence.

Metabolomics is the non-targeted identification and quantification of all the metabolites present in a biological sample, with the aim of measuring the response of an organism to environmental stimuli or modification. In contrast to transcriptomic or proteomic analysis, which observes effects on gene and protein expression, respectively, metabolomics has the advantage of understanding the biological processes being completed as it is capable of providing biological endpoint markers that occur as a result of environmental change or altered gene function (Lindon *et al.* 2004, Baskind *et al.* 2011). By analysing different FF samples, the metabolic profiles obtained can act as fingerprints or signatures for certain physiological states (Lindon *et al.* 2004). High-resolution proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy is a unique tool for studying the composition of biofluids as it provides qualitative and quantitative data of all the metabolites present in a sample (Revelli *et al.* 2009). Using the previously observed seasonal and follicle size effects to provide models of good and poor follicular environments, the aim of this study was to characterise the metabolomic signatures of porcine FF samples collected from small and large follicles in winter and summer.

Materials and methods

Sample collection

FF samples were collected from the ovaries of adult Large White/Landrace cross-bred sows as described by Bertoldo *et al.* (2011a). The sows were sourced from commercial piggeries located in Victoria and South Australia and selected for culling following removal of piglets at weaning (mean parity of 6.0). Only sows culled for reasons unrelated to infertility (referred to as 'non-reproductive' culls), such as lameness, were included in the study. The piglets were weaned 21 days after birth, and the sows were killed at a commercial abattoir 4 days later. This ensured that ovaries were collected during the follicular phase of the oestrous cycle, as a weaning to oestrus interval of 5–7 days is typically exhibited in domestic sows. The ovaries were excised from the reproductive tracts (each ovary pair was kept together in a small sealable plastic bag) and transported to the laboratory in a thermos flask containing saline (0.9% w/v NaCl; Baxter Healthcare, Deerfield, IL, USA) supplemented with 100 U/ml penicillin, 10 mg/ml streptomycin and 25 mg/ml amphotericin (Invitrogen Australia) and maintained at 34–38 °C. Upon arrival, ovaries were washed thoroughly and immersed in saline at room temperature. Ovaries with corpora haemorrhagica or corpora lutea present were excluded from the study. Follicles were measured individually with calipers and aspirated within 2 h of ovary collection using a 21-gauge needle through which constant suction (1.5 l/min) was applied. For each ovary pair, the aspirated material from small follicles (3–4 mm diameter) was pooled in one tube and that from large follicles (5–8 mm diameter) was pooled in another tube. Follicles <3 or >8 mm in diameter or that were observed to have viscous FF (indicating that the LH surge had already occurred) were excluded from the study. Cellular material (COCs and granulosa cells) was removed from the samples by centrifuging them at 3000 g for 20 min and transferring the supernatant (FF) to new tubes. Samples were collected on at least three separate occasions in each season. The volume of FF obtained from each sow ranged from 50 to 1000 μl for small follicles and 400 to 1000 μl for large follicles, but only samples that contained sufficient FF (500 μl) were analysed (summer-small ($n=8$), summer-large ($n=15$), winter-small ($n=9$) and winter-large ($n=15$)). All samples were stored at -20 °C within 1 h of follicle aspiration.

Sample preparation

For $^1\text{H-NMR}$ spectroscopy, the FF samples were thawed at room temperature and then centrifuged at 3000 g for 5 min. Samples were prepared by mixing 500 μl FF, 100 μl deuterium oxide (D_2O) solution and 100 μl phosphate buffer to obtain a pH value of 7.4 ± 0.5 . The samples were then transferred to 5 mm NMR tubes (CortecNet, Paris, France) for $^1\text{H-NMR}$ analysis.

$^1\text{H-NMR}$ spectroscopy

The $^1\text{H-NMR}$ spectroscopy was performed on a Bruker DRX-500 spectrometer (Bruker SADIS, Wissembourg, France), operating at 11.7 T, with a broadband inverse probe head

equipped with a Z gradient coil. NMR measurements were performed at 298 K. Conventional ¹H-NMR spectra were recorded with 90° pulse ($p_1=10\ \mu\text{s}$, $pl=0\ \text{dB}$) using a pulse-and-acquire sequence with residual water pre-saturation (single-frequency irradiation during the relaxation delay). ¹H spectra were collected with 128 transients (and eight dummy scans) in 32 K data points with a spectral width of 7500 Hz and a recycling time of 15 s. Carr-Purcell-Meiboom-Gill (CPMG) spin echo spectra were carried out with 80 ms total echo times and 32 K data points. This spin echo sequence avoided broad short T_2 resonance. Sample shimming was performed automatically on the water signal. Spectra were processed using WinNMR version 3.5 Software (Bruker Daltonik, Karlsruhe, Germany). Prior to Fourier transformation, the free induction decay (FID) signals were zero-filled to 64 K data points, which provided sufficient data points for each resonance and a line-broadening factor of 0.3 Hz was applied. All spectra were corrected for phase distortion and the baseline was manually corrected for each spectrum. To quantify this, the electronic reference to *in vivo* concentrations (ERETIC) signal (Barantin *et al.* 1997) was used. It was generated using one of the frequency channels of the spectrometer and was sent to the carbon channel of the probe during the acquisition of the NMR signal (Akoka *et al.* 1999). The ERETIC signal consists of an exponentially decaying function that mimics the behaviour of a FID signal. The NMR parameters of this ERETIC FID were under control. The ERETIC peak is provided as a quantitative reference signal that has the same area in all spectra (Barantin *et al.* 1997). Spectral ¹H assignments were made based on the literature values of chemical shifts in various media and biofluids (Nicholson *et al.* 1995, Maillot *et al.* 1998). The ¹H-NMR spectra were referenced to the lactate resonance at 1.33 p.p.m. and automatically reduced to ASCII files using AMIX Software package (Analysis of MIXture, version 3.1.5, Bruker Biospin, Karlsruhe, Germany). The regions from 4.20 to 6.4 p.p.m. were removed from each spectrum to eliminate baseline effects of imperfect water saturation. Spectral intensities were scaled to the total intensity and reduced to equidistant integrated regions of 0.001 p.p.m. (buckets) over the chemical shift range of 0.7–9.5 p.p.m. Before the multivariate analysis, the NMR spectral data set was pre-processed using peak alignment algorithm icoshift (<http://www.models.life.ku.dk>; Savorani *et al.* 2010) to minimize spectral peak shift due to residual pH differences within samples. The corresponding realigned bucket tables were then exported to the SIMCA-P⁺ Software (version 12.0, Umetrics, Umea, Sweden) for analysis.

Data analyses

A supervised method, partial least-squares discriminant analysis (PLS-DA), was performed using SIMCA-P⁺ Software (Eriksson *et al.* 2008). Data were scaled using unit Pareto (Par) scaling prior to PLS-DA. PLS-DA is a multivariate projection method that can be used to classify samples according to their common spectral characteristics. PLS-DA takes advantage of class information to attempt to maximize the separation between groups of observations. A plot of the first two principal components (scores plot) provided the most effective 2D

representation of the information contained in the data set. The overall quality of the models was judged by cumulative R^2 and the predictive ability by cumulative Q^2 extracted according to the internal cross-validation default method of SIMCA-P⁺ Software.

An orthogonal PLS-DA (OPLS-DA) as a supervised model of classes, was also performed using SIMCA-P⁺ Software (Cloarec *et al.* 2005, Eriksson *et al.* 2008, Sadeghi-Bazargani *et al.* 2011). Data were scaled using unit Par scaling prior to OPLS-DA. OPLS-DA is a prediction and regression method that finds information in the X data (variables) that is related to known information, the Y data (classes). OPLS-DA takes advantage of class information to attempt to maximize the separation between groups of observations. The overall quality of the models was judged by cumulative R^2 and the predictive ability by cumulative Q^2 . R^2 is defined as the proportion of variance in the data explained by the model and indicates goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability. In addition, to identify putative biomarkers from the data, the contribution plot was used. The contribution plot provides information on the variables that influence any observed clustering of samples (negative values refer to variables associated with one group and positive values refer to variables associated with the second group).

Statistical analyses

A linear mixed model was fitted to the data using the statistical software package GenStat 14th edition (VSN International, Ltd., Hemel Hempstead, UK). For each metabolite, the effects of 'follicle size' and 'season' were assessed for significance ($P<0.05$), as was the interaction effect. The term 'pig', which specified individual animals, was included as a random term. Residual plots were examined to confirm model assumptions. When an interaction effect was detected, approximate least significant differences (LSDs) were used to compare pairs of means.

Correlations of metabolite concentrations with steroid concentrations were initially assessed by including progesterone, androstenedione and oestradiol (E_2) in a separate linear mixed model series. As the steroid concentrations were strongly correlated with each other, the two steroids that are found to be least significantly correlated with the metabolite were then sequentially removed from the model. The correlation coefficient (r) was then calculated for the metabolite and each steroid.

Results

FF ¹H-NMR spectroscopic profiles

Representative ¹H-NMR spectra of FF samples are shown in Fig. 1. The FF samples contained low-molecular-weight metabolites including amino acids (alanine, glycine, phenylalanine and tyrosine), creatine-creatinine, organic acids (lactate, acetate, pyruvate and formate), carbohydrates (glucose), trimethylamine groups, *N*-acetyl groups and some lipid groups.

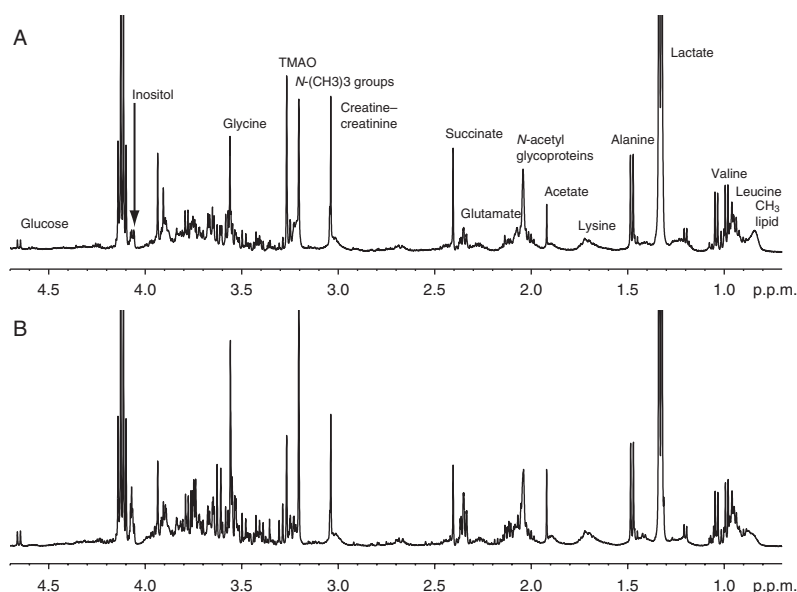


Figure 1 Representative $^1\text{H-NMR}$ spectra (500 MHz) of porcine follicular fluid samples collected from large follicles in winter (A) and small follicles in summer (B).

Multivariate analysis of the $^1\text{H-NMR}$ spectral data

The scores scatter plot resulting from the PLS-DA of the $^1\text{H-NMR}$ spectral data is shown in Fig. 2A ($R^2Y=0.47$ and $Q^2=0.29$). The plot shows a slight follicle size effect (open vs closed symbols) and a slight season effect (squares vs circles) on the metabolic profile of FF. However, the goodness of prediction of the model ($Q^2=0.29$) was quite low. Figure 2B shows that the OPLS-DA of the $^1\text{H-NMR}$ spectral data achieved a clearer separation between the small FF and large FF groups ($R^2Y=0.685$ and $Q^2=0.50$). Figure 2C shows that the OPLS-DA of the $^1\text{H-NMR}$ spectral data also achieved a more obvious separation between the summer FF and winter FF groups ($R^2Y=0.69$ and $Q^2=0.52$). The corresponding contribution plots (not shown) were used to identify the metabolites responsible for the discriminations. The areas of the spectral peaks assigned to these metabolites were then used to quantitate their concentrations in each FF sample.

Effects of follicle size and season on the concentrations of metabolites in FF

Follicle size was found to affect the concentrations of 11 metabolites independent of season (Fig. 3). Interestingly, the concentrations of all but one of these metabolites, glucose, were significantly greater in FF from small follicles (3–4 mm diameter; small FF) than those in FF from large follicles (5–8 mm diameter; large FF). These ten metabolites included acetate, lactate, hypoxanthine, inositol, trimethylamine and five amino acids (alanine, leucine, lysine, methionine and phenylalanine). Glucose was the only metabolite affected by follicle size alone that had an increased concentration in large FF compared with

small FF ($P<0.05$). The corresponding percentage differences in metabolite concentrations between large FF and small FF, for those metabolites significantly affected by follicle size alone, are shown in Fig. 4. The concentration of hypoxanthine in large FF was 44% lower than that in small FF, whereas the concentration of glucose in large FF was 16% greater than that in small FF.

The only metabolite whose concentration was significantly affected by season, independent of follicle size, was succinate. The concentration of succinate in FF collected in winter (winter FF, 0.253 ± 0.015 mM) was significantly greater than that in FF collected in summer (summer FF, 0.218 ± 0.007 mM), representing a 17% difference. The winter FF and summer FF concentrations of three other metabolites, glucose (0.890 ± 0.048 and 0.785 ± 0.044 mM respectively; $P=0.095$), methionine (0.136 ± 0.008 and 0.114 ± 0.008 mM respectively; $P=0.077$) and lipid methyl (0.876 ± 0.041 and 0.955 ± 0.026 mM respectively; $P=0.093$), tended to differ.

Significant interaction effects between follicle size and season were found for the concentrations of four metabolites (Fig. 5). Glutamate concentrations were greater in small FF compared with large FF, and the difference was greater in summer ($P<0.05$). The concentrations of glycine and uridine in small FF collected in summer were greater than those in small FF collected in winter and were also greater than those in large FF collected in either season ($P<0.05$). Likewise, the concentration of *N*-acetyl groups in small FF collected in summer was greater than that in small FF collected in winter ($P<0.05$), but the concentrations in large FF collected in either season were intermediate.

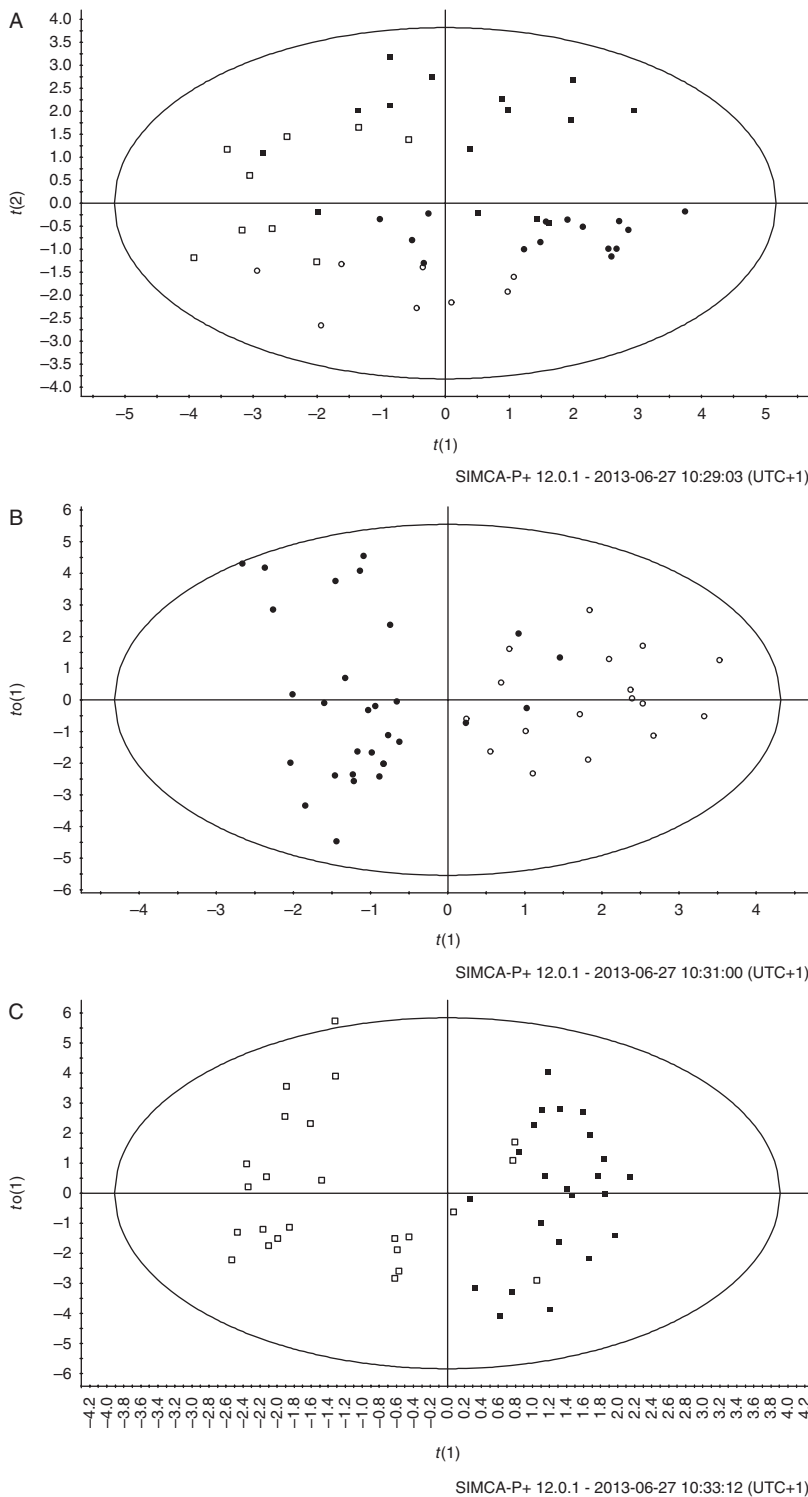


Figure 2 (A) PLS-DA scores scatter plot obtained from ¹H-NMR spectral data of porcine follicular fluid (FF) samples of the winter-small ($n=9$; open squares), winter-large ($n=15$; closed squares), summer-small ($n=8$; open circles) and summer-large ($n=15$; closed circles) groups. The R^2Y and Q^2 values are 0.47 and 0.29 respectively, showing a slight follicle size effect on axis 1 (open vs closed symbols) and a slight season effect on axis 2 (squares vs circles) on the metabolic profiles. (B) OPLS-DA scores scatter plot obtained from ¹H-NMR spectral data of porcine FF samples of the small ($n=17$; open circles) and large ($n=30$; closed circles) follicle size groups. The two populations are well separated with R^2Y and Q^2 values of 0.685 and 0.50 respectively, showing a clear effect of follicle size on the metabolic profiles. (C) OPLS-DA scores scatter plot obtained from ¹H-NMR spectral data of porcine FF samples of the summer ($n=23$; closed squares) and winter ($n=24$; open circles) groups. The two populations are well separated with R^2Y and Q^2 values of 0.69 and 0.52 respectively, showing a clear effect of season on the metabolic profiles.

Relationships between the concentrations of metabolites and steroids in FF

A previous analysis of the samples assessed in this study showed a significant interaction effect of follicle size and season on the FF concentration of progesterone and that FF concentrations of androstenedione and E₂ were

affected by follicle size but not by season (Bertoldo *et al.* 2011a). Further analysis of the metabolite concentrations revealed numerous correlations with FF steroid concentrations (Table 1). The concentrations of ten metabolites, acetate, alanine, glutamate, hypoxanthine, lactate, leucine, lysine, methionine,

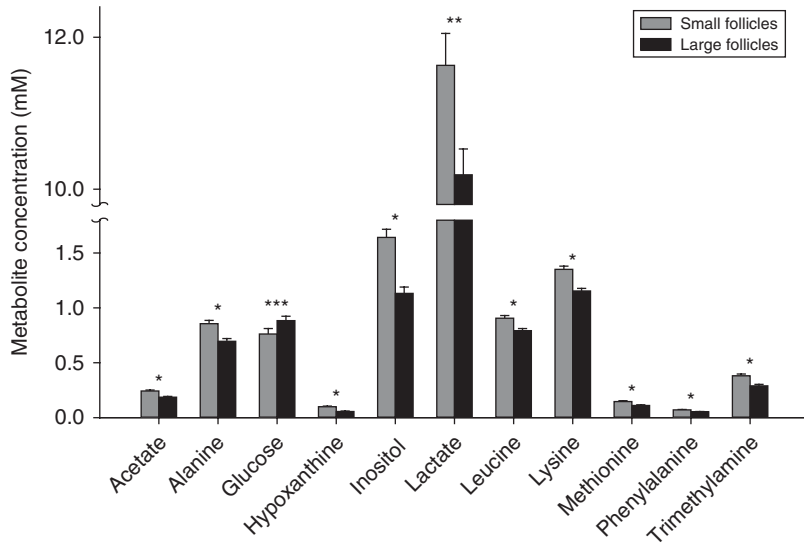


Figure 3 Porcine follicular fluid metabolites that differed significantly in concentration between small (grey bars) and large (black bars) follicles. Asterisks indicate the *P* value determined for the differing concentration values (means ± S.E.M.) for each metabolite (**P* < 0.001, ***P* < 0.01 and ****P* < 0.05).

phenylalanine and succinate, were most strongly correlated with the concentration of E₂, and all were negatively correlated. The concentrations of four metabolites, glycine, inositol, trimethylamine and uridine, were most strongly correlated with the concentration of progesterone, and all were negatively correlated. The concentration of only one metabolite, trimethylamine oxide, was correlated with the concentration of androstenedione. In contrast with the other correlations, the concentrations of trimethylamine and androstenedione were positively correlated.

Discussion

Using ¹H-NMR spectroscopy, this study demonstrated that the metabolomic signatures of porcine FF from small and large antral follicles differ markedly and are

influenced by the season. As the developmental potential of oocytes recovered from large follicles has been shown to be greater than that of oocytes recovered from small follicles in numerous species (Crozet *et al.* 1995, Marchal *et al.* 2002, Iwata *et al.* 2004, Kauffold *et al.* 2005, Lequarre *et al.* 2005, Bagg *et al.* 2007, Grupen *et al.* 2007), the differences found may provide insight into the cellular processes involved during the acquisition of oocyte developmental competence. Similarly, the observed differences may provide clues to the possible cause of reduced oocyte quality in sows during the period of seasonal infertility (Bertoldo *et al.* 2010, 2011b). Importantly, the seasonally induced differences in the FF metabolomic signatures differed considerably from those attributed to follicle size, suggesting that disparate mechanisms are responsible for assuring that good oocyte quality is acquired.

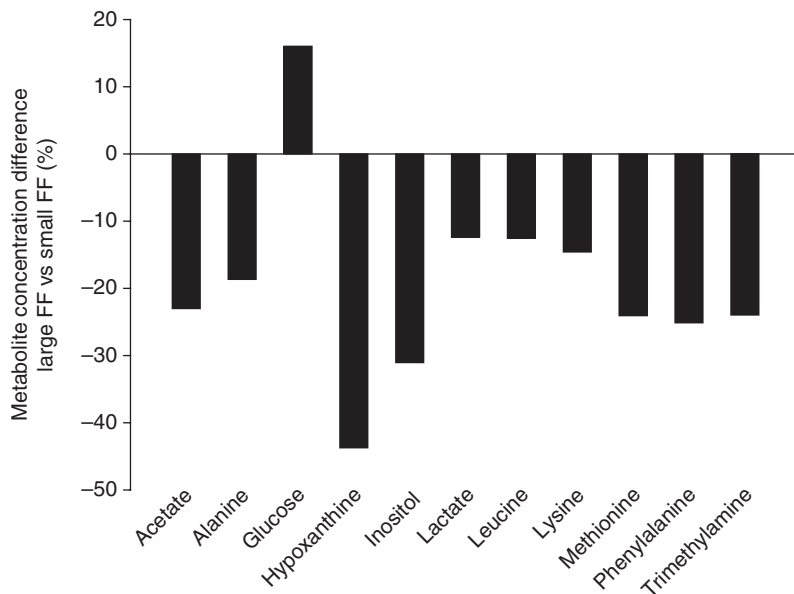


Figure 4 Porcine follicular fluid (FF) metabolites that were found to differ significantly in concentration between small and large follicles as shown in Fig. 3. The value shown for each metabolite is the percentage that the mean concentration in FF from large follicles (5–8 mm) differed from the mean concentration in FF from small follicles (3–4 mm).

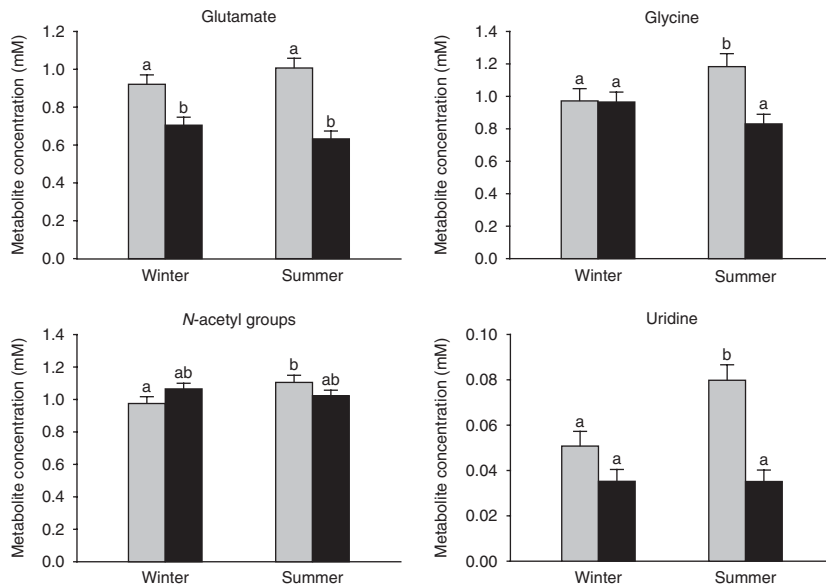


Figure 5 Concentrations (mean ± S.E.M.) of metabolites in follicular fluid samples collected from small (grey bars) and large (black bars) follicles in winter and summer, where an interaction effect of season and follicle size was detected using a linear mixed model. Approximate least significant differences were used to compare pairs of means. Bars with different letters differ significantly ($P < 0.05$).

Our analysis revealed an effect of follicle size, independent of season, on the concentrations of 11 metabolites. Only glucose levels were greater in large follicles compared with small follicles, with the levels of acetate, lactate, hypoxanthine, inositol, trimethylamine and the amino acids alanine, leucine, lysine, methionine and phenylalanine being lower. The finding that glucose concentration increased as follicular diameter increased is consistent with analyses of sheep (Ying *et al.* 2011), cattle (Leroy *et al.* 2004) and buffalo (Nandi *et al.* 2008) FF, but contrary to findings in camels (Ali *et al.* 2008) and horses (Collins *et al.* 1997, Gérard *et al.* 2002). Furthermore, Leroy *et al.* (2004) also observed a concomitant reduction in lactate levels as follicle size increased. The opposing change in the levels of glucose and lactate may be due to a reduction in glucose

catabolism and lactate secretion by granulosa cells as follicular development progresses. However, given the importance of glucose during oocyte maturation, this seems unlikely. Glucose is metabolised by COCs via the glycolytic pathway to provide pyruvate for energy generation (Sutton-McDowall *et al.* 2010). Glucose is also utilized by the pentose phosphate pathway to regulate nuclear maturation and redox state and by the hexosamine biosynthesis pathway to provide substrates required for processes such as cumulus expansion and cell signalling (Sutton-McDowall *et al.* 2010). An alternative explanation is that as follicle size increases, there are relatively fewer granulosa cells consuming glucose and secreting lactate into the relatively greater volume of FF. While it can be argued that post-mortem turnover of glucose to lactate by anaerobic glycolysis

Table 1 Correlations between metabolite and steroid concentrations in porcine follicular fluid.

| Metabolite | Effect ^a | Progesterone ^b | Androstenedione ^b | Oestradiol ^b |
|----------------------|---------------------|----------------------------|------------------------------|----------------------------|
| Acetate | F | -0.3988 (0.009) | -0.3922 (0.010) | -0.4779 (0.001) |
| Alanine | F | -0.3148 (0.042) | -0.4735 (0.002) | -0.5456 (<0.001) |
| Glucose | F | 0.1450 (0.360) | 0.1551 (0.327) | 0.0985 (0.535) |
| Hypoxanthine | F | -0.5210 (<0.001) | -0.5123 (<0.001) | -0.5624 (<0.001) |
| Inositol | F | -0.5243 (<0.001) | -0.2997 (0.054) | -0.3181 (0.040) |
| Lactate | F | -0.1979 (0.209) | -0.3993 (0.009) | -0.4739 (0.002) |
| Leucine | F | -0.2379 (0.129) | -0.4545 (0.003) | -0.5080 (<0.001) |
| Lysine | F | -0.3930 (0.010) | -0.5006 (<0.001) | -0.5304 (<0.001) |
| Methionine | F | -0.2383 (0.129) | -0.3347 (0.030) | -0.4498 (0.003) |
| Phenylalanine | F | -0.2496 (0.111) | -0.5668 (<0.001) | -0.5956 (<0.001) |
| Trimethylamine | F | -0.4136 (0.007) | -0.1187 (0.454) | -0.1102 (0.487) |
| Glutamate | F×S | -0.4040 (0.008) | -0.5086 (<0.001) | -0.5745 (<0.001) |
| Glycine | F×S | -0.3590 (0.020) | -0.1894 (0.230) | -0.3132 (0.043) |
| N-acetyl groups | F×S | -0.0240 (0.880) | -0.0063 (0.968) | 0.0224 (0.888) |
| Uridine | F×S | -0.4480 (0.003) | -0.3639 (0.018) | -0.3656 (0.017) |
| Succinate | S | -0.1842 (0.243) | -0.2074 (0.188) | -0.3127 (0.044) |
| Trimethylamine oxide | - | -0.0635 (0.690) | 0.3449 (0.025) | 0.1554 (0.326) |

^aThe observed effect on metabolite concentration was due to follicle size alone (F), season alone (S) or an interaction effect (F×S). ^bValues shown are the correlation coefficients (*r*) describing the metabolite–steroid relationships, with the corresponding *P* values in parentheses. For each metabolite, the value for the strongest significant metabolite–steroid correlation is shown in bold.

(Leroy *et al.* 2004) may have contributed to the observed changes in glucose and lactate concentrations, any follicular cell death that may have occurred prior to sampling does not explain the seasonal and interaction effects. Furthermore, as already mentioned, the changes in glucose and lactate levels were consistent with those reported by Leroy *et al.* (2004) in cattle FF, which was sampled following transportation of ovaries on ice (4 °C).

When the interaction effects of follicle size and season were also taken into consideration, the increase in glucose concentration in large FF corresponded to a decrease in the concentrations of numerous amino acids, including alanine, glutamate, glycine, leucine, lysine, methionine and phenylalanine. Hong & Lee (2007) reported a very similar effect of follicle size on the amino acid concentrations in porcine FF, even though the follicle size categories differed slightly to those described here. The glucogenic amino acids can be degraded to pyruvate or an intermediate of the tricarboxylic acid (TCA) cycle for the production of glucose under conditions of low glucose, and the ketogenic amino acids can be converted to acetoacetate and acetyl-CoA when energy sources are low. Acetate, another metabolite that decreased in concentration as follicle size increased, can also be converted to acetyl-CoA, which may in turn be utilized by the TCA cycle. A previous study in mares showed that the FF concentrations of alanine and acetate also decreased during follicular growth and preovulatory follicular maturation respectively (Gérard *et al.* 2002). Taken together, the results indicate that the TCA cycle, a key energy-generating metabolic pathway, is employed by granulosa cells to a greater extent in large follicles than in small follicles and represents an increase in the energy demands of the growing follicle.

The importance of the TCA cycle during the acquisition of oocyte quality is further implicated by the fact that succinate, the only metabolite found to differ in concentration between seasons independently of follicle size, was at lower levels in summer FF compared with winter FF. Succinate is an intermediate of the TCA cycle and donates electrons to the electron transport chain when oxidized to fumarate. Lower levels of succinate in summer FF may therefore result in follicular cells having a reduced capacity to generate energy via oxidative phosphorylation.

Large FF also had lower concentrations of trimethylamine, inositol and hypoxanthine compared with small FF. Gérard *et al.* (2002) reported a similar change in the FF level of trimethylamine between the late dominant and preovulatory stages in mares and proposed that the change in trimethylamine concentration was linked to polyamine metabolism (Gérard *et al.* 2002). Inositol is a precursor of the inositol phospholipids, which regulate many cellular processes in response to hormonal or other types of stimuli. The results of a study in women undergoing IVF treatment indicated that the FF level of inositol was positively associated with oocyte

developmental potential (Chiu *et al.* 2002). Therefore, the effect of follicle size on inositol concentration in this study seems counterintuitive, although clearly the conditions of FF sampling were very different to those of the IVF study, in which FF was collected well after the LH surge. In this study, the FF samples were collected from individual sows slaughtered for non-reproductive reasons (e.g. lameness) 4 days after weaning, when the vast majority of sows (about 90%) (Langendijk *et al.* 2000) experience the onset of oestrus. Therefore, the FF metabolomic profiles reported here are representative of the microenvironments that oocytes are exposed to during the follicular phase of the cycle, just prior to the LH surge.

The concentration of hypoxanthine in large FF was 44% lower than that in small FF, the largest relative difference in concentration of all the metabolite comparisons. A previous NMR analysis of porcine FF failed to detect the presence of hypoxanthine (Gosden *et al.* 1990), but the concentrations reported here are still less than a tenth of that determined previously using u.v. absorption spectra and ion-exchange and HPLC (Downs *et al.* 1985). Hypoxanthine is a non-specific phosphodiesterase inhibitor that prevents oocyte meiotic resumption by maintaining elevated intra-oocyte levels of cAMP (Downs *et al.* 1985). A lower concentration of hypoxanthine in large FF therefore seems logical, as this would allow the resumption of oocyte meiosis to occur more readily in response to the impending LH surge.

In small FF, the concentrations of uridine and *N*-acetyl groups were greater in summer than in winter. The increased concentration of uridine in small FF during summer indicates that there was an increased level of circulating uridine or a decreased uptake of uridine by granulosa cells. Uridine is the primary circulating pyrimidine in humans (Wurtman *et al.* 2000). Following cellular uptake, uridine is converted to UTP, which can then be utilized for the synthesis of RNA, phosphocholine (Cansev *et al.* 2008) or glycogen via glycogenesis. The concomitant increase in the concentration of *N*-acetyl groups, which are associated with glycoproteins, glycolipids and important antioxidant constituents, may be indicative of a follicular environment that was more sensitive to oxidative stress and/or increased inflammation and cell damage (Kolwijck *et al.* 2009, Sun *et al.* 2012).

All the FF samples analysed in this study were the remaining FF after separate, smaller volumes of each sample had been removed to assay for the concentrations of progesterone, androstenedione and E₂ in a previous study (Bertoldo *et al.* 2011a). As follicle size and season were found to exert effects on the concentrations of steroids in these FF samples (Bertoldo *et al.* 2011a), the differences in metabolite concentrations would be expected to correlate with the steroid changes previously observed. In the majority of cases (10 of 15 metabolites), the metabolite concentrations in

FF were most strongly correlated with the concentration of E₂. The associations were negative, indicating that as E₂ production increased in the developing follicle, concentrations of these metabolites fell. The concentrations of four metabolites, including two of the metabolites found to be greater in small FF during the summer months, were most strongly correlated with the concentration of progesterone. Again, the associations were negative. Reduced production of progesterone by follicular cells both *in vivo* (Grupen *et al.* 2003, Bagg *et al.* 2007, Bertoldo *et al.* 2010) and *in vitro* (Grupen & Armstrong 2010) has been associated with reduced oocyte quality in pigs. Whether changes in steroid production altered the metabolism of follicular cells, or whether the metabolic environment influenced follicular steroidogenic activity, requires further investigation.

The complexity of the follicular environment has made the analysis of FF extremely challenging. Numerous methods have been employed to characterise components of FF in efforts to increase our understanding of oocyte maturation and identify predictors of oocyte quality. To date, there are relatively few reports of the use of NMR spectroscopy to analyse FF samples (reviewed by Baskind *et al.* (2011)), and to our knowledge, there is only one other report of this method being used to determine the composition of porcine FF (Gosden *et al.* 1990). Recent reports of the use of this technology to identify potential biomarkers of oocyte quality (Pinero-Sagredo *et al.* 2010, McRae *et al.* 2012, Wallace *et al.* 2012) and polycystic ovarian syndrome (Atiomo & Daykin 2012) in FF collected from women undergoing fertility treatments highlight its potential. Our findings demonstrate the effectiveness of ¹H-NMR spectroscopy in detecting metabolite differences in intact, untreated FF collected from individual animals and extend the knowledge gained from ¹H-NMR studies in other species. It should be noted that the small FF and large FF samples collected from each animal were not derived from individual follicles but consisted of fluid aspirated from all the small (3–4 mm diameter) and large (5–8 mm diameter) antral follicles respectively in the ovary pair. Follicular dynamics in the pig obviously differ from that in monovular species, in which only the dominant follicle ovulates. Nevertheless, given the variation in oocyte quality, one would assume that minor differences existed between individual follicles of the same size category. Despite this, a large number of metabolite differences were found between groups.

In conclusion, the current study used a model that represented good and poor follicular environments to reveal differences in the metabolomic signatures of FF that may be associated with the acquisition of oocyte developmental competence. Follicle size was found to affect the concentrations of numerous metabolites that can be linked to the TCA cycle, indicating that there is a distinct energy-generating shift during the final stages

of follicle development. The resumption of meiosis in oocytes of large follicles would also be facilitated by the observed decrease in the concentration of hypoxanthine. The reduction in oocyte quality during the period of seasonal infertility appears to involve metabolite changes in FF that are very different from those associated with follicle size. We propose that the effect of season on oocyte quality is in part due to a follicular environment that is less able to support cells exposed to oxidative stress. Finally, for nearly all the metabolites shown to differ in concentration in this study, strong correlations with steroid levels were found. While further studies are needed to clarify the effects of the observed metabolite changes on oocyte quality, these findings clearly demonstrate the power of ¹H-NMR spectroscopy to expand our understanding of the dynamic and complex microenvironment of the developing follicle.

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.

Funding

This work was supported by the Australian Pork Cooperative Research Centre and the Australian Government Science and Innovation Awards for Young People in Agriculture, Fisheries and Forestry sponsored by Australian Pork Limited.

Acknowledgements

The authors thank Leslie Ritter and Drs Xavier Druart and Claire Kershaw-Young for their technical assistance.

References

- Akoka S, Barantin L & Trierweiler M 1999 Concentration measurement by proton NMR using the ERETIC method. *Analytical Chemistry* **71** 2554–2557. (doi:10.1021/ac981422i)
- Ali S, Ahmad N, Akhtar N, Zia ur R & Noakes DE 2008 Metabolite contents of blood serum and fluid from small and large sized follicles in dromedary camels during the peak and the low breeding seasons. *Animal Reproduction Science* **108** 446–456. (doi:10.1016/j.anireprosci.2007.10.001)
- Atiomo W & Daykin CA 2012 Metabolomic biomarkers in women with polycystic ovary syndrome: a pilot study. *Molecular Human Reproduction* **18** 546–553. (doi:10.1093/molehr/gas029)
- Bagg MA, Nottle MB, Armstrong DT & Grupen CG 2007 Relationship between follicle size and oocyte developmental competence in prepubertal and adult pigs. *Reproduction, Fertility, and Development* **19** 797–803. (doi:10.1071/RD07018)
- Barantin L, LePape A & Akoka S 1997 A new method for absolute quantitation of MRS metabolites. *Magnetic Resonance in Medicine* **38** 179–182. (doi:10.1002/mrm.1910380203)
- Baskind NE, Mcrae C, Sharma V & Fisher J 2011 Understanding subfertility at a molecular level in the female through the application of nuclear magnetic resonance (NMR) spectroscopy. *Human Reproduction Update* **17** 228–241. (doi:10.1093/humupd/dmq039)

- Bertoldo M, Grupen CG, Thomson PC, Evans G & Holyoake PK 2009 Identification of sow-specific risk factors for late pregnancy loss during the seasonal infertility period in pigs. *Theriogenology* **72** 393–400. (doi:10.1016/j.theriogenology.2009.03.008)
- Bertoldo M, Holyoake PK, Evans G & Grupen CG 2010 Oocyte developmental competence is reduced in sows during the seasonal infertility period. *Reproduction, Fertility, and Development* **22** 1222–1229. (doi:10.1071/RD10093)
- Bertoldo M, Holyoake PK, Evans G & Grupen CG 2011a Follicular progesterone levels decrease during the period of seasonal infertility in sows. *Reproduction in Domestic Animals* **46** 489–494. (doi:10.1111/j.1439-0531.2010.01695.x)
- Bertoldo MJ, Holyoake PK, Evans G & Grupen CG 2011b Seasonal effects on oocyte developmental competence in sows experiencing pregnancy loss. *Animal Reproduction Science* **124** 104–111. (doi:10.1016/j.anireprosci.2011.02.012)
- Bertoldo MJ, Holyoake PK, Evans G & Grupen CG 2012 Seasonal variation in the ovarian function of sows. *Reproduction, Fertility, and Development* **24** 822–834. (doi:10.1071/RD11249)
- Boxmeer JC, Macklon NS, Lindemans J, Beckers NG, Eijkemans MJ, Laven JS, Steegers EA & Steegers-Theunissen RP 2009 IVF outcomes are associated with biomarkers of the homocysteine pathway in mono-follicular fluid. *Human Reproduction* **24** 1059–1066. (doi:10.1093/humrep/dep009)
- Cansev M, Wurtman RJ, Sakamoto T & Ulus IH 2008 Oral administration of circulating precursors for membrane phosphatides can promote the synthesis of new brain synapses. *Alzheimer's & Dementia* **4** S153–S168. (doi:10.1016/j.jalz.2007.10.005)
- Chiu TT, Rogers MS, Law EL, Britton-Jones CM, Cheung LP & Haines CJ 2002 Follicular fluid and serum concentrations of myo-inositol in patients undergoing IVF: relationship with oocyte quality. *Human Reproduction* **17** 1591–1596. (doi:10.1093/humrep/17.6.1591)
- Cloarec O, Dumas ME, Trygg J, Craig A, Barton RH, Lindon JC, Nicholson JK & Holmes E 2005 Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in ¹H-NMR spectroscopic metabolomic studies. *Analytical Chemistry* **77** 517–526. (doi:10.1021/ac048803i)
- Collins A, Palmer E, Bezdard J, Burke J, Duchamp G & Buckley T 1997 A comparison of the biochemical composition of equine follicular fluid and serum at four different stages of the follicular cycle. *Equine Veterinary Journal Supplement* **29** 12–16. (doi:10.1111/j.2042-3306.1997.tb05092.x)
- Crozet N, Ahmed-Ali M & Dubos MP 1995 Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. *Journal of Reproduction and Fertility* **103** 293–298. (doi:10.1530/jrf.0.1030293)
- D'Aniello G, Grieco N, Di Filippo MA, Cappiello F, Topo E, D'Aniello E & Ronsini S 2007 Reproductive implication of D-aspartic acid in human pre-ovulatory follicular fluid. *Human Reproduction* **22** 3178–3183. (doi:10.1093/humrep/dem328)
- Das S, Chattopadhyay R, Ghosh S, Goswami SK, Chakravarty BN & Chaudhury K 2006 Reactive oxygen species level in follicular fluid – embryo quality marker in IVF? *Human Reproduction* **21** 2403–2407. (doi:10.1093/humrep/del156)
- Downs SM, Coleman DL, Ward-Bailey PF & Eppig JJ 1985 Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. *PNAS* **82** 454–458. (doi:10.1073/pnas.82.2.454)
- Edwards RG 1974 Follicular fluid. *Journal of Reproduction and Fertility* **37** 189–219. (doi:10.1530/jrf.0.0370189)
- Eriksson L, Trygg J & Wold S 2008 CV-ANOVA for significance testing of PLS and OPLS (R) models. *Journal of Chemometrics* **22** 594–600. (doi:10.1002/cem.1187)
- Gérard N, Loiseau S, Duchamp G & Seguin F 2002 Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (¹H-NMR). *Reproduction* **124** 241–248. (doi:10.1530/rep.0.1240241)
- Gosden RG, Sadler IH, Reed D & Hunter RHF 1990 Characterization of ovarian follicular fluids of sheep, pigs and cows using proton nuclear-magnetic-resonance spectroscopy. *Experientia* **46** 1012–1015. (doi:10.1007/BF01940658)
- Grupen CG & Armstrong DT 2010 Relationship between cumulus cell apoptosis, progesterone production and porcine oocyte developmental competence: temporal effects of follicular fluid during IVM. *Reproduction, Fertility, and Development* **22** 1100–1109. (doi:10.1071/RD09307)
- Grupen CG, McIlfatrick SM, Ashman RJ, Boquest AC, Armstrong DT & Nottle MB 2003 Relationship between donor animal age, follicular fluid steroid content and oocyte developmental competence in the pig. *Reproduction, Fertility, and Development* **15** 81–87. (doi:10.1071/RD02086)
- Grupen CG, Gilchrist RB, Nayudu PL, Barry MF, Schulz SJ, Ritter LJ & Armstrong DT 2007 Effects of ovarian stimulation, with and without human chorionic gonadotrophin, on oocyte meiotic and developmental competence in the marmoset monkey (*Callithrix jacchus*). *Theriogenology* **68** 861–872. (doi:10.1016/j.theriogenology.2007.07.009)
- Hong J & Lee E 2007 Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development. *Theriogenology* **68** 728–735. (doi:10.1016/j.theriogenology.2007.06.002)
- Iwata H, Hashimoto S, Ohota M, Kimura K, Shibano K & Miyake M 2004 Effects of follicle size and electrolytes and glucose in maturation medium on nuclear maturation and developmental competence of bovine oocytes. *Reproduction* **127** 159–164. (doi:10.1530/rep.1.00084)
- Kauffold J, Amer HA, Bergfeld U, Weber W & Sobiraj A 2005 The *in vitro* developmental competence of oocytes from juvenile calves is related to follicular diameter. *Journal of Reproduction and Development* **51** 325–332. (doi:10.1262/jrd.17002)
- Kolwijck E, Engelke UF, van der Graaf M, Heerschap A, Blom HJ, Hadfoune M, Buurman WA, Massuger LF & Wevers RA 2009 N-acetyl resonances in *in vivo* and *in vitro* NMR spectroscopy of cystic ovarian tumors. *NMR in Biomedicine* **22** 1093–1099. (doi:10.1002/nbm.1417)
- Langendijk P, van den Brand H, Soede NM & Kemp B 2000 Effect of boar contact on follicular development and on estrus expression after weaning in primiparous sows. *Theriogenology* **54** 1295–1303. (doi:10.1016/S0093-691X(00)00436-2)
- Lequarre AS, Vigneron C, Ribaucour F, Holm P, Donnay I, Dalbies-Tran R, Callesen H & Mermillod P 2005 Influence of antral follicle size on oocyte characteristics and embryo development in the bovine. *Theriogenology* **63** 841–859. (doi:10.1016/j.theriogenology.2004.05.015)
- Leroy JL, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PE & de Kruif A 2004 Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. *Animal Reproduction Science* **80** 201–211. (doi:10.1016/S0378-4320(03)00173-8)
- Lindon JC, Holmes E, Bollard ME, Stanley EG & Nicholson JK 2004 Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* **9** 1–31. (doi:10.1080/13547500410001668379)
- Love RJ, Evans G & Klupiec C 1993 Seasonal effects on fertility in gilts and sows. *Journal of Reproduction and Fertility. Supplement* **48** 191–206.
- Maillet S, Vion-Dury J, Confort-Gouny S, Nicoli F, Lutz NW, Viout P & Cozzone PJ 1998 Experimental protocol for clinical analysis of cerebrospinal fluid by high resolution proton magnetic resonance spectroscopy. *Brain Research Protocols* **3** 123–134. (doi:10.1016/S1385-299X(98)00033-6)
- Marchal R, Vigneron C, Perreau C, Bali-Papp A & Mermillod P 2002 Effect of follicular size on meiotic and developmental competence of porcine oocytes. *Theriogenology* **57** 1523–1532. (doi:10.1016/S0093-691X(02)00655-6)
- McRae C, Baskind NE, Orsi NM, Sharma V & Fisher J 2012 Metabolic profiling of follicular fluid and plasma from natural cycle *in vitro* fertilization patients – a pilot study. *Fertility and Sterility* **98** 1449–1457. (doi:10.1016/j.fertnstert.2012.07.1131)
- Nandi S, Girish Kumar V, Manjunatha BM, Ramesh HS & Gupta PS 2008 Follicular fluid concentrations of glucose, lactate and pyruvate in buffalo and sheep, and their effects on cultured oocytes, granulosa and cumulus cells. *Theriogenology* **69** 186–196. (doi:10.1016/j.theriogenology.2007.08.036)
- Nicholson JK, Foxall PJD, Spraul M, Farrant RD & Lindon JC 1995 750-MHz ¹H and ¹H-¹³C NMR spectroscopy of human blood-plasma. *Analytical Chemistry* **67** 793–811. (doi:10.1021/ac00101a004)

- Oyawoye O, Abdel Gadir A, Garner A, Constantinovici N, Perrett C & Hardiman P 2003 Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. *Human Reproduction* **18** 2270–2274. (doi:10.1093/humrep/deg450)
- Peltoniemi OA, Heinonen M, Leppavuori A & Love RJ 1999 Seasonal effects on reproduction in the domestic sow in Finland – a herd record study. *Acta Veterinaria Scandinavica* **40** 133–144.
- Pinero-Sagredo E, Nunes S, de los Santos MJ, Celda B & Esteve V 2010 NMR metabolic profile of human follicular fluid. *NMR in Biomedicine* **23** 485–495. (doi:10.1002/nbm.1488)
- Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M & Rinaudo P 2009 Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reproductive Biology and Endocrinology* **7** 40. (doi:10.1186/1477-7827-7-40)
- Sadeghi-Bazargani H, Bangdiwala SI, Mohammad K, Maghsoudi H & Mohammadi R 2011 Compared application of the new OPLS-DA statistical model versus partial least squares regression to manage large numbers of variables in an injury case–control study. *Scientific Research and Essays* **6** 4369–4377. (doi:10.5897/SRE10.1147)
- Savorani F, Kristensen M, Larsen FH, Astrup A & Engelsen SB 2010 High throughput prediction of chylomicron triglycerides in human plasma by nuclear magnetic resonance and chemometrics. *Nutrition and Metabolism* **7** 43. (doi:10.1186/1743-7075-7-43)
- Sun L, Hu W, Liu Q, Hao Q, Sun B, Zhang Q, Mao S, Qiao J & Yan X 2012 Metabonomics reveals plasma metabolic changes and inflammatory marker in polycystic ovary syndrome patients. *Journal of Proteome Research* **11** 2937–2946. (doi:10.1021/pr3000317)
- Sutton-McDowall ML, Gilchrist RB & Thompson JG 2010 The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* **139** 685–695. (doi:10.1530/REP-09-0345)
- Takahashi C, Fujito A, Kazuka M, Sugiyama R, Ito H & Isaka K 2008 Anti-Müllerian hormone substance from follicular fluid is positively associated with success in oocyte fertilization during *in vitro* fertilization. *Fertility and Sterility* **89** 586–591. (doi:10.1016/j.fertnstert.2007.03.080)
- Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M & Brennan L 2012 An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. *Fertility and Sterility* **97** 1078–1084 (e1–8). (doi:10.1016/j.fertnstert.2012.01.122)
- Wang TH, Chang CL, Wu HM, Chiu YM, Chen CK & Wang HS 2006 Insulin-like growth factor-II (IGF-II), IGF-binding protein-3 (IGFBP-3), and IGFBP-4 in follicular fluid are associated with oocyte maturation and embryo development. *Fertility and Sterility* **86** 1392–1401. (doi:10.1016/j.fertnstert.2006.03.064)
- Wu YT, Tang L, Cai J, Lu XE, Xu J, Zhu XM, Luo Q & Huang HF 2007 High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Human Reproduction* **22** 1526–1531. (doi:10.1093/humrep/dem029)
- Wurtman RJ, Regan M, Ulus I & Yu L 2000 Effect of oral CDP-choline on plasma choline and uridine levels in humans. *Biochemical Pharmacology* **60** 989–992. (doi:10.1016/S0006-2952(00)00436-6)
- Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ & Robker RL 2012 Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus–oocyte complexes. *Fertility and Sterility* **97** 1438–1443. (doi:10.1016/j.fertnstert.2012.02.034)
- Ying S, Wang Z, Wang C, Nie H, He D, Jia R, Wu Y, Wan Y, Zhou Z, Yan Y *et al.* 2011 Effect of different levels of short-term feed intake on folliculogenesis and follicular fluid and plasma concentrations of lactate dehydrogenase, glucose, and hormones in Hu sheep during the luteal phase. *Reproduction* **142** 699–710. (doi:10.1530/REP-11-0229)

Received 7 April 2013

First decision 13 May 2013

Accepted 24 June 2013