

Fluctuations in bovine ovarian follicular fluid composition throughout the oestrous cycle

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Abstract

Bovine oocyte maturation *in vitro* frequently results in abnormal cytoplasmic maturation and failure to acquire developmental competence. This is, in part, likely to be due to the non-physiological nutritional milieu to which oocytes are exposed. Improvements in oocyte developmental potential may be achieved by modelling nutrient profiles on those of preovulatory follicular fluid (FF). However, little is known about fluctuations in FF nutrient levels according to follicle dominance and oestrous cyclicity. This study therefore characterised the carbohydrate and amino acid profile of FF according to these parameters, and compared preovulatory FF composition with that of maturation medium. Carbohydrate concentrations ($n = 121$) were determined enzymatically whilst amino acid profiles ($n = 40$) were determined by reverse-phase HPLC. Pyruvate and glucose concentrations were unaffected by follicle dominance, whereas Stage III–IV lactate profiles were higher in non-dominant FF ($P < 0.01$). While most dominant FF amino acid concentrations were affected by oestrous stage, only glutamate, alanine, leucine and lysine levels fluctuated in non-dominant FF. Glucose and lactate concentrations were significantly negatively correlated, whereas most amino acids were significantly positively correlated with each other. Maturation medium had higher pyruvate and lower lactate concentrations than preovulatory FF ($P < 0.001$), whereas glucose level was similar. All amino acid levels (except histidine, taurine, alanine and tryptophan) differed significantly between maturation medium and preovulatory FF. These data indicated that FF composition varies throughout the oestrous cycle. Preovulatory FF nutrient profile differed from that of maturation medium, perhaps accounting for the poor developmental competence of *in vitro* matured oocytes. These data may contribute to the formulation of a nutritionally more physiological maturation medium.

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Introduction

Routine maturation of bovine oocytes *in vitro* underpins commercial embryo production and emerging biotechnologies such as cloning and transgenics (Kubota *et al.* 1998, Chen *et al.* 2002, Galli *et al.* 2003). However, current *in vitro* production protocols are inefficient at producing viable embryos (Lonergan *et al.* 2001), partly due to abnormal oocyte cytoplasmic maturation *in vitro* (Krisher & Bavister 1998). Optimisation of *in vitro* maturation (IVM) protocols is vital not only for generating viable embryos but also to support the development of subsequent offspring into normal adults (Eppig & O'Brien 1998).

Glucose, lactate, pyruvate and amino acids are prerequisites for the maturation and fertilisation of bovine

oocytes *in vitro*. The oxidative metabolism of pyruvate and amino acids is the major energy-generating pathway in bovine oocytes (Rieger & Loskutoff 1994). Furthermore, pyruvate and glucose support high levels of maturation, and amino acids aid pronuclear formation in fertilised oocytes (Donahue & Stern 1968, Lim *et al.* 1999). Nutrient concentrations are non-physiological in mammalian IVM systems (Nakazawa *et al.* 1997); improvements in oocyte energy metabolism and developmental potential may be achieved by modelling nutrient profiles on those of preovulatory follicular fluid (FF) (Krisher & Bavister 1998, Smitz & Cortvrindt 1999).

FF has a variety of oocyte-related functions: maintenance of meiotic arrest (McNatty 1978), protection against proteolysis, extrusion during ovulation (Espey & Lipner

1994), enhancement of spermatozoa attraction, motility and acrosome reaction (Dell'Aquila *et al.* 1997, Rodriguez *et al.* 2001, Wang *et al.* 2001), and buffering against adverse haematic influences (Gosden *et al.* 1988). The components of FF are derived from plasma in early antral follicles (Gosden *et al.* 1988). In particular, antral lactate and pyruvate concentrations appear to result from granulosa cell glycolytic metabolism (Donahue & Stern 1968, Gull *et al.* 1999).

Although undiluted FF is largely ineffective at supporting bovine oocyte maturation *in vitro*, it has been used to supplement media with varying degrees of success in equine, bovine and porcine systems, where oocyte maturation rates are affected by oestrous stage and follicle size (Gerritse *et al.* 1988, Sirard & First 1988, Ayoub & Hunter 1993, Choi *et al.* 1998, Takagi *et al.* 1998, Coy *et al.* 1999, Aguilar *et al.* 2001, Bøgh *et al.* 2002, Avery *et al.* 2003). This strategy exposes oocyte-cumulus complexes (OCCs) to a more 'physiological' – albeit undefined – hormonal and nutritional milieu. By contrast, undefined/defined mammalian oocyte maturation systems – despite endocrine profiles conducive to cytoplasmic and nuclear maturation – contain a nutrient profile optimised for the culture of somatic cells rather than the combination of oocyte and cumulus cells (Zuelke & Brackett 1993, Carolan *et al.* 1996, Eppig *et al.* 1996, Lebedeva *et al.* 1998, Smitz & Cortvrindt 1999, Watson *et al.* 2000, Nagai 2001).

Some progress has been made towards the characterisation of FF in a variety of mammalian species. Glucose, pyruvate, lactate, histidine, phenylalanine and asparagine concentrations have been determined in human FF (Leese & Lenton 1990, Jimena *et al.* 1993, Gull *et al.* 1999). Similarly, Guérin *et al.* (1995) determined amino acid profiles in FF from a variety of domestic species, albeit without consideration for follicle size or oestrous stage. Gérard *et al.* (2002) also quantified alanine, glutamate and glutamine profiles in equine FF.

The aim of the present study was to determine the carbohydrate and amino acid profile of bovine FF according to follicle dominance and oestrous stage. The data were compared with the nutrient composition of bovine plasma to help elucidate the role of granulosa cells in FF formation. Finally, dominant preovulatory FF composition (representing the *in vivo* environment) was compared with that of maturation medium (the *in vitro* environment). These data may contribute to the formulation of a nutritionally more 'physiological' bovine oocyte maturation medium.

Materials and Methods

Collection of FF and plasma

Entire bovine reproductive tracts at different stages of the oestrous cycle were collected on ice and processed on site at a local abattoir within a few minutes of death to

minimise post mortem changes in nutrient concentrations. Cycle stage (I–IV) was assessed on the basis of ovarian luteal morphology, according to the criteria defined by Ireland *et al.* (1980), on both ovaries from the same female. Briefly, Stage I (days 1–4) was defined as the interval between ovulation and the time when the epithelium grows over the rupture point, thus forming the apex of a new corpus luteum (CL). Stage II (days 5–10) covered the complete formation of a new CL with vasculature at its periphery; when bisected, the apex was red/brown while the remainder was orange/yellow. Stage III (days 11–17) was characterised by an absence of red/brown colouration, leaving the CL wholly orange/yellow, with visible apical vasculature later in this stage. During Stage IV (days 18–20), the ovary contained at least one large follicle and a regressed CL with no surface vasculature. Tracts with ovaries not clearly matching these criteria were discarded. Individual follicles were graded on the basis of dominance (assessed as whether they were the largest follicle or not, since steroid profiles were not determined) and FF volume. Mean (range) follicle diameter for Stages I–IV were 7.49 ± 0.489 , 10.48 ± 1.17 , 9.12 ± 0.26 and 11.52 ± 6.07 (6.76–9.63, 9.63–30.63, 7.65–11.38 and 4.50–33.25) mm for dominant follicles, and 5.30 ± 0.30 , 7.18 ± 0.31 , 6.46 ± 0.26 and 7.86 ± 0.68 (4.38–6.02, 7.00–10.50, 2.95–5.15 and 7.03–11.25) mm for non-dominant follicles respectively. Fluid was collected in 2 or 5 ml sterile syringes (Becton Dickinson, Plymouth, Devon, UK) fitted with 19 gauge needles (Becton Dickinson). Cell debris was removed by centrifugation at 900 *g* for 5 min using a microcentrifuge (Micro Centaur; MSE Scientific, Loughborough, Leics, UK). The supernatant was diluted with HPLC grade water (Fisher Scientific, Loughborough, Leics, UK) for amino acid, glucose and lactate analyses, whilst undiluted fluid was used for determining pyruvate concentration.

Carotid blood was collected in 10 ml EDTA tubes (NHS Logistics, Alfreton, Derbyshire, UK) from heifers immediately after slaughter. Blood samples were centrifuged at 250 *g* for 10 min, and the supernatant plasma was processed as described above. All samples were processed and frozen on dry ice within 10–15 min of slaughter and stored at -80°C until analysis.

Composition of bovine maturation medium

The bovine oocyte maturation medium used was based on bicarbonate-buffered TCM-199 supplemented with fetal calf serum (Sigma, Poole, Dorset, UK), as used routinely in our laboratories (original protocol from C Galli & G Lazzari; Laboratorio di Tecnologie della Riproduzione (LTR-CIZ), Via Porcellasco 7/f, 26100 Cremona, Italy) (Gopichandran & Leese 2003, Orsi & Leese 2004a,b). Details of its supplements are given in Table 1. Four independent batches of maturation medium (using the same batch of serum) were tested, and samples were processed as described above.

Table 1 Supplements for bovine maturation medium (^aFerring Pharmaceuticals, Berks, Slough, UK).

Component	Concentration
Oestradiol-17 β	2 μ g/ml
Apo-transferrin	25 μ g/ml
β -Mercaptoethanol	7 nl/ml (97 μ mol/l)
Epidermal growth factor	0.47 μ g/ml
Fibroblast growth factor	10.9 ng/ml
FSH/LH ^a	0.025 IU/ml of each
Fetal bovine serum	10% (v/v)

Measurement of carbohydrate concentrations

Glucose, pyruvate and lactate concentrations in FF ($n = 121$), plasma ($n = 7$) and maturation medium ($n = 4$) were measured enzymatically on a COBAS MIRA auto-analyser (Roche Instruments, Lewes, East Sussex, UK), as previously described (Leese & Lenton 1990).

Measurement of amino acid profiles

Amino acid concentrations were measured by reverse-phase HPLC, as previously described (Partridge & Leese 1996). Sample profiles ($n = 10$ FFs for each oestrous stage; $n = 7$ for plasma; $n = 4$ for maturation medium) were compared with 10 μ M amino acid standards. All were analysed on a Kontron 500 Series automated HPLC system fitted with a Jasco F920 fluorescence detector and 4.5 \times 250 mm Hypersil ODS-16 column (Jones Chromatography, Mid Glamorgan, Pontypridd, UK). Tetrahydrofuran concentration was modified over the 7–12% range according to the elution profile. All samples were analysed at two different dilutions to allow for the elevated concentrations of glycine, glutamine and alanine. This method did not allow the detection of proline or cysteine.

Measurement of osmolality

Osmolality was measured using pooled FF, independent of oestrous stage. Samples (100 μ l) were measured in triplicate on an osmometer (Camlab, Over, Cambs, UK).

Data presentation and statistical analysis

Data are presented as means \pm S.E.M. and tested for normality using Anderson–Darling tests. Significant differences in FF nutrient profile across the oestrous cycle and comparisons with plasma were tested by one-way ANOVA, followed by Fisher's LSD test post hoc, or by Kruskal–Wallis test followed by Mann–Whitney U tests post hoc. Differences between dominant and non-dominant follicle parameters were compared by Student's *t*-test or Mann–Whitney U test, as were the differences between the nutrient composition of preovulatory FF and IVM medium.

Results

Carbohydrate concentrations of FF and plasma

There were no significant differences in pyruvate concentrations according to oestrous cycle stage or between plasma and FF in dominant follicles (Table 2). By contrast, FF glucose was slightly, but significantly, lower than that of plasma in all instances (except Stage II), while the opposite trend was observed for lactate, particularly Stage I follicles ($P < 0.05$).

Non-dominant FF pyruvate levels were similar across oestrous cycle stages and in plasma, while glucose concentration was significantly lower than that of plasma in all cases (except Stage II) ($P < 0.05$) (Table 3). Lactate concentration was higher in FF than plasma, particularly in Stage I follicles where levels were significantly higher than at other stages ($P < 0.05$).

There was no significant difference in pyruvate and glucose concentrations on the basis of follicle dominance throughout the oestrous cycle (Table 3). However, Stage III and IV lactate profiles were higher in non-dominant follicles compared with their stage-matched dominant counterparts ($P < 0.01$). Independent of dominance, glucose and lactate levels were lower and higher respectively in Stage I follicles.

Amino acid profiles of FF and plasma

Most amino acid concentrations were affected by oestrous stage, although this was largely attributable to differences between dominant Stage I FF and the other stages, where amino acid concentrations were consistently

Table 2 Carbohydrate concentrations of dominant bovine ovarian follicles throughout the oestrous cycle and compared with plasma (comparisons are confined to within columns).

	Pyruvate (mmol/l)	Glucose (mmol/l)	Lactate (mmol/l)
Stage I	0.01 \pm 0.01	4.71 \pm 0.31 ^a	8.16 \pm 1.60 ^a
Stage II	0.04 \pm 0.01	5.08 \pm 0.20 ^{ab}	5.01 \pm 0.39 ^b
Stage III	0.05 \pm 0.01	4.99 \pm 0.09 ^a	5.22 \pm 0.26 ^b
Stage IV	0.03 \pm 0.01	4.84 \pm 0.14 ^a	5.09 \pm 0.37 ^b
Plasma	0.01 \pm 0.01	5.57 \pm 0.14 ^b	3.94 \pm 0.35 ^c

Means without common superscripts differ ($P < 0.05$).

Table 3 Pyruvate, glucose and lactate concentrations of non-dominant bovine ovarian follicles throughout the oestrous cycle compared with plasma.

	Pyruvate (mmol/l)	Glucose (mmol/l)	Lactate (mmol/l)
Stage I	0.00 \pm 0.00	4.25 \pm 0.35 ^a	10.16 \pm 1.60 ^a
Stage II	0.02 \pm 0.01	5.05 \pm 0.24 ^{ab}	7.39 \pm 0.72 ^b
Stage III	0.03 \pm 0.01	4.49 \pm 0.12 ^a	8.51 \pm 2.01 ^{b,**}
Stage IV	0.03 \pm 0.01	4.54 \pm 0.26 ^a	5.65 \pm 0.11 ^{b,**}
Plasma	0.01 \pm 0.01	5.57 \pm 0.14 ^b	3.94 \pm 0.35 ^c

Means without common superscripts differ ($P < 0.05$). ** $P < 0.01$ in lactate profile in stage-matched dominant versus non-dominant FF.

lower (Table 4). Amino acids affected by oestrous stage included: glutamate, asparagine, histidine, glutamine, threonine, arginine, taurine, alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, leucine and lysine. The Stage I FF amino acid profile was very similar to that of plasma, particularly as regards aspartate, glutamate, serine, glycine, threonine, arginine, taurine, phenylalanine and isoleucine. By contrast, Stages II–IV profiles were significantly lower than plasma for aspartate, glutamate, asparagine (Stage III only), serine, taurine (except Stage II), alanine (Stage II only), methionine (except Stage II), valine (except Stage II), leucine (Stage III only) and lysine (except Stage III). Overall, plasma levels were markedly higher

than those of FF for glutamate, serine and glutamine and, to a lesser extent, for asparagine, threonine, taurine, tyrosine, methionine, phenylalanine and lysine. By contrast, FF valine concentration was significantly higher than in plasma.

Alterations in amino acid profile with respect to oestrous stage and plasma were less evident for non-dominant follicles (Table 5). Only glutamate, alanine, leucine and lysine were affected by oestrous stage. Compared with plasma, FF had lower aspartate, glutamate (except Stage I) and serine (except Stage I), while glutamine (except Stages I and II), alanine (only Stage I) and lysine (except Stage II) levels were higher.

Table 4 Bovine FF amino acid profile of dominant follicles at different stages of oestrus compared with plasma.

Amino acid	Stage I (mmol/l)	Stage II (mmol/l)	Stage III (mmol/l)	Stage IV (mmol/l)	Plasma (mmol/l)
ASP	0.03 ± 0.01 ^{ab}	0.02 ± 0.00 ^b	0.01 ± 0.00 ^b	0.012 ± 0.002 ^b	0.055 ± 0.014 ^a
GLU	0.18 ± 0.01 ^a	0.07 ± 0.03 ^b	0.05 ± 0.01 ^b	0.06 ± 0.01 ^b	0.20 ± 0.02 ^a
ASN	0.06 ± 0.01 ^a	0.03 ± 0.00 ^{bc}	0.02 ± 0.00 ^c	0.03 ± 0.00 ^{bc}	0.04 ± 0.01 ^b
SER	0.14 ± 0.00 ^{ab}	0.07 ± 0.01 ^b	0.05 ± 0.01 ^b	0.06 ± 0.01 ^b	0.21 ± 0.07 ^a
HIS	0.15 ± 0.00 ^a	0.09 ± 0.02 ^b	0.06 ± 0.00 ^b	0.08 ± 0.01 ^b	0.06 ± 0.02 ^b
GLN	0.88 ± 0.12 ^a	0.44 ± 0.04 ^b	0.34 ± 0.03 ^b	0.35 ± 0.04 ^b	0.32 ± 0.03 ^b
GLY	0.87 ± 0.18	0.55 ± 0.05	0.42 ± 0.05	0.49 ± 0.05	0.51 ± 0.10
THR	0.37 ± 0.03 ^a	0.26 ± 0.02 ^{abc}	0.21 ± 0.03 ^{bc}	0.22 ± 0.02 ^c	0.31 ± 0.05 ^{ac}
ARG	0.22 ± 0.01 ^a	0.21 ± 0.01 ^{ab}	0.13 ± 0.01 ^b	0.14 ± 0.01 ^{ab}	0.18 ± 0.04 ^{ab}
TAU	0.14 ± 0.02 ^a	0.09 ± 0.01 ^{ab}	0.08 ± 0.01 ^b	0.08 ± 0.01 ^b	0.12 ± 0.02 ^a
ALA	0.68 ± 0.04 ^a	0.54 ± 0.06 ^b	0.38 ± 0.03 ^c	0.46 ± 0.02 ^{bc}	0.41 ± 0.04 ^c
TYR	0.13 ± 0.01 ^a	0.09 ± 0.01 ^b	0.06 ± 0.01 ^b	0.08 ± 0.01 ^b	0.10 ± 0.01 ^b
TRP	0.12 ± 0.01 ^a	0.06 ± 0.00 ^b	0.05 ± 0.01 ^b	0.06 ± 0.01 ^b	0.04 ± 0.01 ^b
MET	0.06 ± 0.00 ^a	0.05 ± 0.00 ^{bc}	0.03 ± 0.01 ^b	0.04 ± 0.01 ^b	0.04 ± 0.01 ^c
VAL	0.53 ± 0.03 ^a	0.36 ± 0.01 ^{bc}	0.30 ± 0.03 ^b	0.35 ± 0.03 ^b	0.04 ± 0.02 ^c
PHE	0.13 ± 0.01 ^a	0.10 ± 0.01 ^{bc}	0.08 ± 0.01 ^b	0.08 ± 0.01 ^b	0.11 ± 0.01 ^{ac}
ILE	0.20 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.17 ± 0.11
LEU	0.33 ± 0.03 ^a	0.24 ± 0.01 ^{bc}	0.19 ± 0.02 ^b	0.21 ± 0.02 ^{bc}	0.26 ± 0.01 ^c
LYS	0.21 ± 0.01 ^a	0.20 ± 0.03 ^a	0.11 ± 0.01 ^b	0.17 ± 0.02 ^a	0.10 ± 0.01 ^b

Means without common superscripts differ ($P < 0.05$).

Table 5 Bovine FF amino acid profiles of non-dominant follicles at different stages of oestrus compared with plasma.

Amino acid	Stage I (mmol/l)	Stage II (mmol/l)	Stage III (mmol/l)	Stage IV (mmol/l)	Plasma (mmol/l)
ASP	0.03 ± 0.01 ^a	0.01 ± 0.00 ^a	0.02 ± 0.00 ^{a,*}	0.02 ± 0.00 ^a	0.06 ± 0.01 ^b
GLU	0.20 ± 0.04 ^{ac}	0.09 ± 0.02 ^b	0.13 ± 0.03 ^{ab,*}	0.13 ± 0.03 ^{ab}	0.20 ± 0.02 ^c
ASN	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01
SER	0.09 ± 0.02 ^{ab}	0.05 ± 0.01 ^a	0.07 ± 0.01 ^a	0.07 ± 0.01 ^a	0.21 ± 0.07 ^b
HIS	0.10 ± 0.01	0.07 ± 0.01	0.08 ± 0.01 [*]	0.09 ± 0.12	0.06 ± 0.02
GLN	0.51 ± 0.09 ^{ab}	0.34 ± 0.09 ^{ab}	0.53 ± 0.10 ^a	0.54 ± 0.04 ^{a,*}	0.32 ± 0.03 ^b
GLY	0.86 ± 0.15	0.38 ± 0.07	0.56 ± 0.05	0.63 ± 0.07	0.51 ± 0.10
THR	0.26 ± 0.05	0.21 ± 0.03	0.26 ± 0.04	0.29 ± 0.02 [*]	0.31 ± 0.05
ARG	0.21 ± 0.01	0.15 ± 0.02	0.18 ± 0.01 [*]	0.19 ± 0.01 [*]	0.18 ± 0.04
TAU	0.15 ± 0.04	0.07 ± 0.01	0.13 ± 0.03	0.13 ± 0.02	0.12 ± 0.02
ALA	0.81 ± 0.10 ^a	0.48 ± 0.05 ^b	0.55 ± 0.08 ^b	0.51 ± 0.02 ^b	0.41 ± 0.04 ^b
TYR	0.11 ± 0.01	0.07 ± 0.02	0.09 ± 0.01 [*]	0.11 ± 0.01 [*]	0.10 ± 0.01
TRP	0.08 ± 0.02	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.04 ± 0.01
MET	0.05 ± 0.00	0.03 ± 0.01	0.05 ± 0.01 [*]	0.05 ± 0.00 [*]	0.04 ± 0.01
VAL	0.42 ± 0.01	0.27 ± 0.06	0.37 ± 0.04	0.41 ± 0.01	0.43 ± 0.02
PHE	0.13 ± 0.01	0.08 ± 0.01	0.11 ± 0.01 [*]	0.10 ± 0.01 [*]	0.11 ± 0.01
ILE	0.18 ± 0.01	0.15 ± 0.03	0.16 ± 0.03	0.20 ± 0.01 [*]	0.17 ± 0.01
LEU	0.32 ± 0.03 ^{ac}	0.20 ± 0.04 ^b	0.27 ± 0.02 ^{ac}	0.30 ± 0.01 ^{c,**}	0.26 ± 0.01 ^{ac}
LYS	0.22 ± 0.03 ^{ac}	0.13 ± 0.04 ^{ab}	0.16 ± 0.02 ^{ac,*}	0.22 ± 0.03 ^c	0.10 ± 0.01 ^b

Different superscripts indicate significant differences in amino acid concentration between groups ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$ statistically significant differences in amino acid profile between stage-matched dominant versus non-dominant FF.

There were no significant differences in amino acid profile between dominant and non-dominant FF for the early oestrous stages, although asparagine and methionine profiles were noticeably lower in Stage I non-dominant FF, while taurine concentration was reduced in their Stage II counterparts (all $P = 0.052$) (Table 5). By contrast, non-dominant Stage III FF had significantly higher concentrations of aspartate, glutamate, histidine, arginine, tyrosine, methionine, phenylalanine and lysine. Similarly, Stage IV non-dominant FF was characterised by significantly higher levels of glutamine, threonine, arginine tyrosine, methionine, phenylalanine, isoleucine and leucine.

Correlations between FF volume, carbohydrate and amino acid profiles

Glucose concentration was not significantly correlated with FF volume (although a trend was observed), while pyruvate and lactate concentrations were related to FF volume positively and negatively respectively (Table 6). Pyruvate concentration was not correlated with that of the other carbohydrates, whereas glucose and lactate concentrations were significantly negatively correlated.

Almost all amino acids were significantly positively correlated with each other (Table 7). Correlations were weakest between amino acids and FF volume. Aspartate, glutamate, alanine, tyrosine, methionine, phenylalanine and leucine were all significantly negatively correlated with FF volume and arginine weakly so.

There was no significant correlation between pyruvate profile and that of any of the amino acids (Table 8). However, there was a trend for a negative correlation between glucose and amino acid profiles, which was significant for arginine, tyrosine, methionine, phenylalanine, isoleucine, leucine and lysine ($P < 0.05$). In contrast, there was a trend for a positive correlation between lactate and amino acid profiles, which was significant for aspartate, glutamate, alanine, tyrosine and phenylalanine ($P < 0.05$), and approached significance for arginine.

Comparison of FF with maturation medium

There was a significantly higher pyruvate concentration in the medium used for *in vitro* maturation compared with Stage IV FF, whereas the reverse was true for lactate ($P < 0.001$) (Table 9). Glucose concentration was similar in maturation medium and Stage IV FF.

Table 6 Bovine FF volume and carbohydrate correlation coefficients; r and P values.

	Volume	Pyruvate	Glucose
Pyruvate	0.346; 0.022	–	–
Glucose	0.271; 0.075	–0.001; 0.997	–
Lactate	–0.326; 0.031	0.139; 0.367	–0.309; 0.041

Bold indicates significance ($P < 0.05$).

All amino acid concentrations (Fig. 1) differed significantly between maturation medium and preovulatory FF, except those of histidine, taurine, alanine and tryptophan. Apart from the elevated concentrations of aspartate, glutamate and serine in maturation medium, the overall amino acid ratios were similar in both groups, with FF concentrations being consistently lower.

The osmolality of FF (292.4 ± 2.1 mOsm/kg) was not significantly different from that of maturation medium (284.5 ± 4.9 mOsm/kg) and plasma (299.0 ± 5.0 mOsm/kg).

Discussion

Carbohydrate profiles

We have measured the pyruvate, glucose, lactate and amino acid composition of bovine FF throughout the oestrous cycle. FF pyruvate concentration was largely unaffected by oestrous stage and follicle dominance. Apart from Stage IV dominant FF, it increased moderately in line with oestrous stage, possibly as a result of increased production by granulosa/cumulus cell activity (Leese & Barton 1985). By contrast, glucose concentration was relatively uniform throughout the oestrous cycle and independent of follicle dominance, with lowest values reported for Stage I. These follicles were, on average, smaller than their later stage counterparts and, in this respect, Leese & Lenton (1990) also found that concentrations of glucose were affected by follicle volume in the human. However, the present study reports significantly higher lactate concentrations in Stage I follicles, independent of dominance, a trend which agrees with a previous report (Leroy *et al.* 2004). The glucose and lactate levels may be accounted for by (i) the elevated metabolic activity of small follicles (Hammon *et al.* 2000a) and/or (ii) the possible atretic fate of a significant number of the antral follicles sampled (Erickson 1966, Choudary *et al.* 1968, Kruij & Dieleman 1982, Wise 1987). The trend for lactate concentration to be higher in non-dominant follicles (significant for later oestrous stages) supports the second explanation.

The plasma glucose concentrations reported are at the upper end of the physiological range for cattle (40–100 mg/dl; Iowa State University Clinical Pathology Laboratory) and the FF concentrations are higher than those quoted in a recent study (Leroy *et al.* 2004). We therefore repeated these analyses (and those of fresh follicles obtained from a different slaughterhouse for comparison) using undiluted samples with an alternative assay (Glucose PAP; Audit Diagnostics, Doughcloyne, Cork, Ireland) on a different autoanalyser (COBAS MIRA S Plus; Roche Instruments, UK). Our measurements were reproducible; these discrepancies may be a reflection of stress associated with progress along the abattoir line and/or, possibly, to post mortem changes in the study of Leroy *et al.* (2004). Plasma lactate levels were also mildly elevated, whereas

Table 7 Correlation coefficients between bovine FF volume and amino acid concentrations: *r* and *P* values.

	Volume	ASP	GLU	ASN	SER	HIS	GLN	GLY	THR	ARG	TAU	ALA	TYR	TRP	MET	VAL	PHE	ILE	LEU
ASP	-0.372 0.013	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
GLU	-0.397 0.008	0.703 0.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
ASN	-0.218 0.156	0.296 0.051	0.331 0.028	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
SER	-0.165 0.283	0.589 0.000	0.413 0.005	0.555 0.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
HIS	-0.204 0.185	0.432 0.003	0.346 0.021	0.727 0.000	0.533 0.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—
GLN	-0.256 0.094	0.277 0.069	0.281 0.065	0.812 0.000	0.587 0.000	0.677 0.000	—	—	—	—	—	—	—	—	—	—	—	—	—
GLY	-0.230 0.139	0.554 0.000	0.522 0.000	0.527 0.000	0.708 0.000	0.651 0.000	0.570 0.000	—	—	—	—	—	—	—	—	—	—	—	—
THR	-0.108 0.486	0.296 0.051	0.300 0.048	0.716 0.000	0.529 0.000	0.597 0.000	0.688 0.000	0.495 0.001	—	—	—	—	—	—	—	—	—	—	—
ARG	-0.288 0.058	0.551 0.000	0.471 0.001	0.714 0.000	0.630 0.000	0.645 0.000	0.726 0.000	0.696 0.000	0.681 0.000	—	—	—	—	—	—	—	—	—	—
TAU	-0.169 0.272	0.332 0.028	0.440 0.003	0.436 0.003	0.423 0.004	0.349 0.020	0.604 0.000	0.340 0.025	0.581 0.000	0.435 0.003	—	—	—	—	—	—	—	—	—
ALA	-0.297 0.050	0.508 0.000	0.683 0.000	0.566 0.003	0.459 0.002	0.586 0.000	0.475 0.001	0.566 0.000	0.401 0.007	0.630 0.000	0.534 0.000	—	—	—	—	—	—	—	—
TYR	-0.353 0.019	0.566 0.000	0.500 0.001	0.776 0.000	0.582 0.000	0.750 0.000	0.669 0.000	0.665 0.000	0.651 0.000	0.809 0.000	0.401 0.007	0.646 0.000	—	—	—	—	—	—	—
TRP	-0.233 0.128	0.242 0.113	0.372 0.013	0.719 0.000	0.591 0.000	0.658 0.000	0.725 0.000	0.535 0.000	0.623 0.000	0.487 0.001	0.607 0.000	0.581 0.000	0.626 0.000	—	—	—	—	—	—
MET	-0.338 0.025	0.428 0.001	0.379 0.011	0.785 0.000	0.572 0.000	0.652 0.000	0.749 0.000	0.580 0.000	0.636 0.000	0.855 0.000	0.511 0.000	0.612 0.000	0.901 0.000	0.656 0.000	—	—	—	—	—
VAL	-0.239 0.119	0.295 0.052	0.360 0.016	0.754 0.000	0.588 0.000	0.679 0.000	0.698 0.000	0.595 0.000	0.848 0.000	0.677 0.000	0.510 0.000	0.515 0.000	0.741 0.000	0.767 0.000	0.722 0.000	—	—	—	—
PHE	-0.461 0.002	0.536 0.000	0.559 0.000	0.647 0.000	0.528 0.000	0.681 0.000	0.630 0.000	0.677 0.000	0.504 0.000	0.765 0.000	0.405 0.006	0.708 0.000	0.888 0.000	0.633 0.000	0.864 0.000	0.716 0.000	—	—	—
ILE	-0.196 0.203	0.375 0.012	0.320 0.034	0.663 0.000	0.529 0.000	0.540 0.000	0.559 0.000	0.490 0.000	0.496 0.001	0.676 0.000	0.376 0.012	0.492 0.001	0.742 0.000	0.524 0.000	0.716 0.000	0.651 0.000	0.661 0.000	—	—
LEU	-0.337 0.025	0.480 0.001	0.446 0.002	0.586 0.000	0.632 0.000	0.553 0.000	0.588 0.000	0.691 0.000	0.704 0.000	0.746 0.000	0.485 0.001	0.544 0.000	0.789 0.000	0.628 0.000	0.771 0.000	0.842 0.000	0.814 0.000	0.745 0.000	—
LYS	-0.213 0.165	0.410 0.006	0.254 0.097	0.618 0.001	0.382 0.010	0.695 0.001	0.455 0.002	0.580 0.001	0.541 0.001	0.729 0.001	0.282 0.064	0.498 0.001	0.849 0.001	0.407 0.006	0.776 0.001	0.615 0.001	0.727 0.001	0.699 0.001	0.705 0.001

Bold indicates ($P < 0.05$).

pyruvate levels were comparable with published physiological values (<http://dairynet.traill.uiuc.edu>). FF lactate profiles were lower overall than those of Leroy *et al.* (2004), possibly as a result of post mortem glycolysis-induced changes in composition in the latter study. The present carbohydrate profiles compared favourably with the values reported in the human by Leese & Lenton (1990) (0.45, 3.29 and 6.12 mmol/l pyruvate, glucose and lactate respectively), perhaps due to the similar follicle size in both species. Antral follicles with different granulosa cell area:antral volume ratios, such as those of the mouse, have a different carbohydrate profile (Harris *et al.* 2003), likely due to granulosa/theca cell glycolytic metabolism (Boland *et al.* 1993, 1994).

Whereas pyruvate and lactate were correlated with FF volume positively and negatively respectively, glucose was not. These findings differed from those reported for human FF, possibly due to differences in theca/granulosa cell metabolism or oestrous stage at the time of sampling (Leese & Lenton 1990). Nonetheless, glucose and lactate

concentrations were significantly negatively correlated, as reported by Leese & Lenton (1990), implying the participation of follicular glycolysis superimposition upon the flux of these carbohydrates from plasma.

Amino acid profiles

To the best of our knowledge, there is only one earlier study on the amino acid composition of bovine FF, which found qualitatively similar profiles to those given here (Guérin *et al.* 1995). Quantitative discrepancies may be accounted for by differences in analytical technique, smaller sample size and the absence of follicular segregation according to oestrous stage in the work of Guérin *et al.* (1995). Amino acid profiles were very homogeneous, independent of oestrous stage or follicle status, except for taurine, arginine and leucine, suggesting that follicle size has minor effects on amino acid levels. Although certain amino acids (glycine, alanine, proline, serine, tyrosine, glutamate and lysine) are taken up by

Table 8 Correlation coefficients between bovine FF carbohydrate (Pyr, pyruvate; Glc, glucose; Lac, lactate) and amino acid concentrations; *r* and *P* values.

	ASP	GLU	ASN	SER	HIS	GLN	GLY	THR	THR	ARG	TAU	ALA	TYR	TRP	MET	VAL	PHE	ILE	LEU	LYS
Pyr	-0.161	-0.107	0.129	-0.057	0.076	0.115	0.174	0.036	0.036	0.152	-0.003	0.246	0.152	0.033	0.163	0.038	0.171	-0.038	0.008	0.216
Glc	0.298	0.490	0.405	0.713	0.623	0.457	0.264	0.814	0.325	0.984	0.107	0.107	0.324	0.831	0.289	0.808	0.267	0.805	0.958	0.158
Lac	-0.522	-0.269	-0.243	-0.248	-0.209	-0.134	-0.234	-0.100	-0.324	-0.129	-0.129	-0.191	-0.464	-0.063	-0.417	-0.118	-0.438	-0.300	-0.307	-0.439
	0.000	0.077	0.111	0.105	0.173	0.386	0.130	0.517	0.032	0.404	0.404	0.214	0.002	0.685	0.005	0.444	0.003	0.048	0.043	0.003
	0.503	0.589	0.087	0.051	0.132	-0.008	0.250	-0.091	0.295	0.008	0.610	0.000	0.342	0.006	0.258	0.022	0.447	0.150	0.178	0.271
	0.001	0.000	0.573	0.741	0.392	0.957	0.105	0.559	0.052	0.957	0.000	0.000	0.023	0.970	0.091	0.890	0.002	0.330	0.247	0.075

Bold indicates (*P* < 0.05).**Table 9** Pyruvate, glucose and lactate concentrations in Stage IV dominant bovine ovarian follicles and maturation medium.

	Pyruvate (mmol/l)	Glucose (mmol/l)	Lactate (mmol/l)
Stage IV	0.03 ± 0.01	4.84 ± 0.14	5.09 ± 0.37
Maturation	1.01 ± 0.10***	5.41 ± 0.76	2.23 ± 0.45***

*** *P* < 0.001.

granulosa cells and transferred to the oocyte (Colonna & Mangia 1983), only serine and glutamate followed such a pattern consistently throughout the oestrous cycle. This suggests that passage of amino acids into the antrum may satisfy the requirements of developing granulosa cells. Interestingly, FF amino acid concentrations were lower than those found in the oviduct, despite having qualitatively similar profiles. Furthermore, they were comparable with plasma amino acid levels (Guérin *et al.* 1995, present authors, unpublished observations).

Interestingly, amino acid concentration was negatively correlated with follicular volume, significantly so in the case of aspartate, glutamate, alanine, tyrosine, methionine, phenylalanine and leucine (slightly so for arginine). This may have reflected selective mural granulosa utilisation/production, although the profile of these amino acids followed no clear pattern according to whether they are essential or non-essential. With a few very marginal exceptions, all amino acid concentrations were very significantly positively correlated with each other, suggesting that the regulatory influences that affect their profile influence them in a similar manner overall (i.e. arguing against differential metabolism).

Pyruvate and amino acid concentrations were not correlated. Although this suggests that amino acid metabolism (and, speculatively, protein synthesis) is not correlated with pyruvate production by cumulus cells, it is likely that the impact of cumulus cells to FF composition is minimal compared with mural granulosa cells, due to the large size of bovine follicles. By contrast, glucose concentration was negatively correlated with arginine, tyrosine, methionine, phenylalanine, isoleucine, leucine and lysine concentrations. Except arginine (which is nevertheless readily consumed by proliferating cells (Sakagami *et al.* 1998)) and tyrosine, all of these are essential amino acids and thus substrates for follicular development, like glucose. Lactate profile, on the other hand, was positively correlated with aspartate, glutamate, alanine, tyrosine and phenylalanine. Except phenylalanine, these are non-essential amino acids and can be readily produced by cells, like lactate (Salway 1996).

Comparison of preovulatory FF composition with IVM medium

Glucose concentrations were similar in FF and IVM medium, since both reflect plasma concentrations, whereas pyruvate and lactate levels differed markedly.

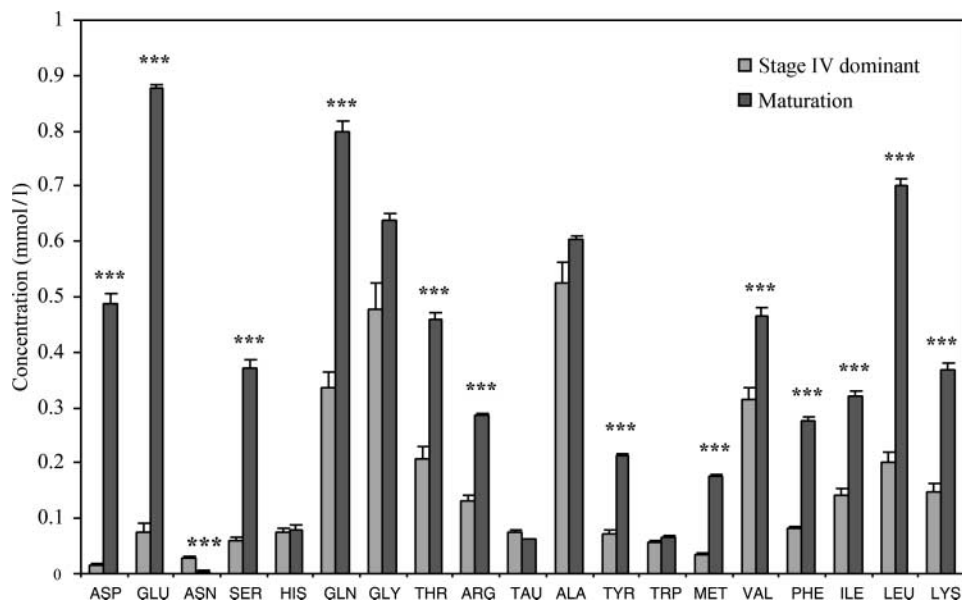


Figure 1 Amino acid profile of Stage IV dominant bovine ovarian follicles and maturation medium. *** $P < 0.001$.

The difference in pyruvate concentration is accounted for by base medium supplementation with 1 mmol/l pyruvate. Although pyruvate confers protection against oxidative stress (Salahudeen *et al.* 1991, O'Fallon & Wright 1995, Morales *et al.* 1999, Orsi & Leese 2001), these levels are excessive to support the nutritional requirements of the oocyte. The differences in FF and maturation medium lactate levels are accounted for by the absence of this compound in maturation medium, except for that derived from foetal calf serum. FF lactate, by contrast, is likely derived from granulosa/theca cell glycolytic metabolism (Hillier *et al.* 1985, Boland *et al.* 1993). The large disparities in pyruvate:lactate ratio between FF and IVM medium will almost certainly have a biological impact by influencing oocyte metabolism, REDOX potential (Harvey *et al.* 2002) and meiotic regulation through alterations in pH (Downs & Mastropolo 1997).

The higher amino acid levels in maturation medium are derived from three sources: TCM-199, additional glutamine and serum. These may be beneficial by regulating metabolism (as shown for early mouse embryos (Lane & Gardner 1998)) and protein synthesis (Kuran *et al.* 2002), and by conferring protection against osmotic shock (Lane 2001) and oxidative stress (Lindenbaum 1973). However, they also result in ammonium production as a result of spontaneous degradation and catabolism (Lane & Gardner 1994, 1995, Orsi & Leese 2004a), although this is unlikely to accumulate to toxic levels over the 24-h maturation period, since bovine OCCs are relatively tolerant to ammonium (Hammon *et al.* 2000a,b). The ratio of different amino acids may also be important for the oocyte. By analogy, in mammalian embryos, the free amino acid ratio – whether consumed as free molecules (Jung *et al.* 1998) or as proteins to be hydrolysed (Orsi & Leese 2004b) – impacts on metabolism, gene expression,

developmental potential and quality (Jung *et al.* 1998, Liu *et al.* 2002). A similar rationale may also apply to cattle oocytes during maturation. In particular, as the protein profiles of FF at different stages of oestrus and plasma differ (Spitzer *et al.* 1996), so will the resultant amino acid mixture obtained by hydrolysis.

The comparable osmolalities of IVM medium and FF suggest that OCCs are unlikely to be subjected to osmotic shock in the present IVM system. However, the individual concentration of different electrolytes may nonetheless play a significant role in supporting oocyte viability.

In conclusion, cycle stage and follicle status markedly impact on follicular carbohydrate and – to a lesser extent – amino acid concentrations, supporting the proposition that FF composition varies throughout the oestrous cycle (Wise 1987, Gosden *et al.* 1988, Spitzer *et al.* 1996). Pre-ovulatory FF nutrient profile differs markedly from that of maturation medium, which may account for the poor developmental competence of IVM oocytes induced by a suboptimal IVM medium. We hope that our data will lead to the formulation of a more physiological medium for the *in vitro* maturation of bovine oocytes.

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