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Revised

A study relating the composition of follicular fluid and blood plasma from individual Holstein dairy cows to the *in vitro* developmental competence of pooled abattoir-derived oocytes.

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ABSTRACT

The fertility of high performance (high milk yield) dairy breeds such as the Holstein within the Australian dairy herd has been on the decline for the past two decades. The 12-month calving interval for pasture-based farming practises results in oocyte maturation coinciding with peak lactation, periods of negative energy balance and energy partitioning for lactation, causing energy deficiency in some organ systems, including the reproductive system. Oocyte developmental competence (the ability to undergo successful fertilisation, embryo development and establishment of pregnancy) is intrinsically linked with the composition of follicular fluid (FF). The aim of this study was to determine if there was a relationship between the fat and carbohydrate levels in plasma and FF and the ability to support in vitro oocyte maturation (IVM). Plasma and FF were collected *in vivo* from eight Holstein cows between 52-151 days post-partum. Plasma glucose trended ($P = 0.072$) higher and triglyceride levels were significantly higher than in FF ($P < 0.05$) but there were no relationships between FF and plasma composition. Glucose FF concentration was negatively related to follicular lactate and NEFA levels and days post-partum. Conversely, FF triglyceride concentrations were positively related to FF NEFA levels and negatively related to milk fat and protein composition. Abattoir-derived cumulus oocyte complexes (COCs) were cultured in either 50% FF (FF-IVM) or 50% plasma (plasma-IVM), with on time embryo development then assessed. While there were no differences between animals, blastocyst rates following FF-IVM were negatively related to plasma glucose and days post-partum and positively related to body condition score (BCS) and plasma NEFA levels. In comparison to previous studies, total NEFA levels in FF were not related to animal parameters and did not influence oocyte developmental competence in vitro. Results from this study suggest that days post-partum and BCS influence carbohydrate metabolism within the follicular environment and this may be attributed to the pasture-based feed system applied in the Australian dairy industry.

KEYWORDS

Dairy cattle; cumulus oocyte complex; follicular fluid; plasma; developmental competence

1. INTRODUCTION

Worldwide, the Holstein is the predominant dairy cattle breed, due to its superior milk yield capabilities, with high performance cows averaging 10000-20000 litres per cow per year. However, the fertility of these high performance Holstein dairy cattle has declined as milk yields have increased [1,2] and subfertility is one of the key priorities of dairy industries worldwide [3-7]. Specifically, in the Australian dairy industry, the number of cows not pregnant (dry or in calf) by the completion of the mating season has more than doubled from 9% in 2000 to 20% in 2009 [8].

The Australian dairy industry is largely pasture based [9], where seasonal grass growth is matched with periods of the peak lactation period from 0-70 days post-partum. Hence, for producers to time calving to coincide with peak pasture production, a seasonal 12-month calving interval is required, where cows need to successfully conceive by 60-80 days post-partum. The peak lactation period corresponds with loss of body condition and negative energy balance (NEB), as 80% of glucose is partitioned for lactation [10,11], compromising function of other organs such as the reproductive system [6,12]. In addition, adipose tissue fat stores are mobilised, increasing circulating levels of specific fats such as non-esterified fatty acids (NEFAs), with the severity of NEB positively correlated to NEFA levels [12]. As the final stages of oocyte growth and development occurs 90 days prior to ovulation in the cow [13], oocyte developmental competence (the ability for the oocyte to successfully undergo fertilisation and early embryo development) may be compromised, leading to early pregnancy loss; also known as “phantom cow” syndrome, where cows have not returned to oestrus by the second service but are not pregnant with a viable fetus [14]. The detrimental impact of lactation on fertility is best demonstrated by higher rates of early pregnancy loss in cows compared to heifers, with

70-90% embryo survival rates seen in non-lactating heifers vs. 45-63% in cows on Day 7-8 post-insemination [6,15-17].

Within antral and pre-ovulatory ovarian follicles, the cumulus oocyte complex (COC) is surrounded by follicular fluid (FF) that contains proteins, cytokines, growth factors, steroids and metabolites and has similar composition to filtered venous plasma [18]. FF supplies the COC with necessary signals and metabolites for nourishment, which is critical for developmental competence; hence FF composition is innately related to the health of the COC [18,19].

The follicular environment also reflects the maternal metabolic condition. For example, increasing lipid content is seen in the FF from women with increasing body mass index [20]. Furthermore, the ability of mouse COCs to complete maturation was impaired following culture in 50% human FF with high lipid content, compared to COCs cultured in the presence of FF with low lipid content [21]. Given that lipids, in particular NEFA levels, are elevated in FF of high performance dairy cattle during peak lactation [22], the developmental competence of abattoir derived cattle COCs cultured in *in vivo* collected FF and plasma from lactating Australian Holstein cows may be a bioassay for fertility. The aim of this study was to determine if animal parameters, such as milk yield, milk composition, body condition scores and days post-partum correlated with FF and plasma composition from cows at various times post-partum and then relate that to the developmental competence of abattoir-derived COCs cultured in 50% FF (FF-IVM) or plasma (plasma-IVM).

2. MATERIAL AND METHODS

Unless specified, all chemicals and materials were purchased from Sigma Aldrich (St Louis MO).

2.1. Follicular fluid and plasma collection

The follicular fluid (FF) and plasma were collected from eight animals from three different herds in Northern Victoria, coinciding with commercial oocyte retrieval for *in vitro* embryo production. The donor cows were treated with 1 ml of Gonabreed (100 mg/ml gonadorelin acetate; Parnell Technologies Pty Ltd, Alexandra NSW Australia) by intramuscular injection. Seven days later, the location, size and number of follicles greater than 5 mm were recorded using a PIE240 and PIE OPU pick probe ultrasound (Pie Medical, Holland). Oocytes and FF were aspirated from the dominant follicle using an 18 G needle at an 11 ml/minute flow rate using a vacuum pump (IVF Ultra Quiet; COOK Medical, IN USA). Blood contamination in FF samples was minimised by the samples being collected by an experienced operator and single needle placement into the dominant follicle. Using this method, only one sample had visual blood contamination and this sample was disregarded. The follicular aspirates without any visible blood contamination were allowed to settle for 5 minutes and then the top half of the fluid was drawn off. The drawn off fluid was then split between two cryovials, sealed and then frozen and stored in a -28°C freezer. Coinciding with the ovarian aspiration, blood samples were collected from the tail vein in EDTA/heparin coated tubes. The samples were centrifuged at 700 rpm for 15 minutes. The plasma layer was drawn off and the sample was split between two cryovials, sealed and frozen and stored in a -28°C freezer. Samples were transported in dry ice from Kyabram (Victoria) to The University of Adelaide.

Details of days post-partum (dpp), milk parameters, body condition score (BCS) and feeding are presented in **Table 1**. All herds were fed a mixture of bail and pasture and had similar levels of protein (14-16%) and energy (8-10 MJ). Samples from herd 1 were collected in the afternoon, post-milking and herds 2 and 3 collection occurred in the morning, post-milking.

Glucose, lactate, triglyceride and non-esterified fatty acid (NEFA) concentrations were measured in the FF and plasma samples using a COBAS Integra400 chemical analyser (Roche, Basel, Switzerland), based on colorimetric and enzymatic assays. Inter and intra assay variances were less than 5%.

2.2. Oocyte maturation (50% FF or plasma) and embryo production

Cattle ovaries were transported from a local abattoir in warm saline (30-35°C). Follicles were aspirated using an 18-gauge needle and a 10 ml syringe. Intact cumulus oocyte complexes (COCs) with greater than four intact, unexpanded cell layers and non-granulated ooplasm were selected in undiluted FF, washed once in undiluted IVM media, and then transferred into corresponding IVM drops. The base IVM media was VitroMat (IVF Vet Solutions, Adelaide, Australia), which contains 2.3 mM glucose, 0.4 mM pyruvate, non-essential and essential amino acids and no NEFAs or triglycerides. VitroMat was supplemented with 4 mg/ml fatty acid free (FAF) BSA (MP Biomedicals, Solon, OH USA) and 0.1 IU/ml FSH (Puregon, Organon, Oss, Netherlands). For IVM, 50% FF or plasma was added to VitroMat + 0.1 IU/ml FSH. A pooled FF sample from abattoir-derived ovaries served as the control. Groups of 10 COCs were cultured in 100 μl drops of pre-equilibrated IVM media (50% FF or 50% plasma), overlaid with paraffin oil (Merck, Darmstadt, Germany) and were cultured at 38.5°C in 6% CO_2 in humidified air for 23 h.

At the completion of IVM (day 0, D0), COCs were washed once in VitroWash (IVF Vet Solutions) + 4 mg/ml FAF BSA, once in IVF medium (VitroFert, IVF Vet Solutions + 4 mg/ml FAF BSA + 10 IU/ml heparin) and then transferred into 500 μl IVF wells overlaid with paraffin oil. Two thawed straws of sperm from a single sire of proven fertility were prepared using a discontinuous Percoll gradient (45%:90%; GE Healthcare, Uppsala, Sweden) and sperm was added to the IVF wells at a final concentration of 1 x 10^6 sperm/ml. COCs were co-incubated with sperm for 23h at 38.5°C in 6% CO_2 in humidified air.

Presumptive zygotes were mechanically denuded of their cumulus vestment by repeated pipetting in VitroWash + 4 mg/ml FAF BSA, washed once in VitroCleave (IVF Vet Solutions) + 4 mg/ml FAF BSA and groups of 5 embryos were transferred into 20 μl of pre-equilibrated VitroCleave + 4 mg/ml FAF BSA and cultured at 38.5°C in 6% CO_2, 7% O_2 in nitrogen balance. On D5, embryos were washed once in VitroBlast (IVF Vet Solutions) + 4 mg/ml FAF BSA and groups of 5 embryos were transferred into 20 μl of pre-equilibrated VitroBlast + 4 mg/ml FAF BSA and cultured at 38.5°C in 6% CO_2, 7% O_2 in nitrogen balance. On-time embryo development (blastocyst stage) was assessed on D8.

Three replicate experiments were performed for FF and plasma samples from all animals, with 30-40 COCs used per replicate.

2.3. Statistical Analyses

Statistical differences between animals and herds were determined using a general linear model and Bonferroni post-hoc test. Cleavage and blastocyst rates were arcsine transformed prior to statistical analyses. Relationships between animal parameters, embryo development following FF-IVM and plasma-IVM, FF and plasma composition were determined using regression analyses and a negative value for the slope indicated a negative relationship between measurements. All statistic tests were performed using SPSS version 20 statistical software and P-values less than 0.05 were considered statistically significant and P-values less than 0.1 were considered as trending toward differences.

3. RESULTS

Plasma and FF samples were collected from eight cows representing three different herds. The average days post-partum was 73.3 ± 11.8 days, and ranged from 52 days to 151 days (**Table 1**). There were no differences in milk fat and protein and body condition score (BCS) between the three herds. Milk yield trended to be lower in Herd 2 compared to the other herds (**Table 1**, P = 0.085).

3.1. FF and Plasma Composition

Glucose, lactate, triglyceride and non-esterified fatty acid (NEFA) levels were measured in FF and plasma samples. There were no significant differences between FF and plasma concentrations of lactate and NEFAs (**Figure 1**). However, glucose was trending to be 1.2-fold (P = 0.07) higher and and triglyceride levels were 2.6-fold higher (P < 0.001) in plasma compared to FF (**Figure 1**).

Analyses of the differences in FF and plasma composition between herds demonstrated that the concentration of NEFAs in FF from Herd 3 was significantly higher than Herds 1 and 2 (**Table 2**; P < 0.001). There was also a trend for differences in glucose concentrations in FF between Herds 2 and 3 (3.56 ± 0.07 mM vs. 2.55 ± 0.31 mM; P = 0.083). There were no significant differences between any of the other measured parameters between herds.

The relationships between FF and plasma composition and animal parameters were determined (**Table 3**). Follicular glucose levels were negatively correlated with lactate levels in FF (r^2 = 0.78, P = 0.004) and days post-partum (r^2 = 0.711, P = 0.008) and there was a trend for a negative correlation between glucose levels and NEFA in FF (r^2 = 0.48, P = 0.057).

Lactate levels in FF were positively correlated to days post-partum ($r^2 = 0.921$, $P < 0.001$). However, there was no relationship between plasma and FF glucose and lactate levels.

There was a trend for a positive relationship between triglyceride and NEFA levels in FF ($r^2 = 0.48$, $P = 0.057$). Plasma lactate levels were negatively related either significantly or as a trend, respectively to FF triglycerides ($r^2 = 0.58$, $P = 0.028$) and FF NEFA ($r^2 = 0.44$, $P = 0.074$). Triglyceride levels in FF tended to be negatively related with milk composition such as the proportion of protein and fat (protein: $r^2 = 0.305$, $P = 0.09$ and fat: $r^2 = 0.417$, $P = 0.084$).

Despite plasma triglycerides levels being significantly higher than FF triglyceride levels (**Figure 1**, $P < 0.001$), there were no correlations between plasma triglycerides and NEFAs and any of the other animal parameters measured (**Table 3**).

3.2. 50% FF and Plasma IVM Cultures

Abattoir-derived cattle COCs were cultured in either 50% FF (FF-IVM) or 50% plasma (plasma-IVM) to determine if the composition of either would influence oocyte developmental competence, by assessing on time embryo development to the blastocyst stage following in vitro fertilisation. On Day 8, there were no significant differences in cleavage, total blastocysts, expanded and hatched blastocyst rates between animals, following FF-IVM and plasma-IVM (**Figure 2**).

When embryo development was compared between the different herds, there were no differences in development following FF-IVM. However, total blastocyst development was significantly lower following plasma-IVM from Herd 1 compared to Herd 3 (main effect; $P < 0.05$).

Regression analyses revealed negative relationships between glucose levels in plasma and the blastocyst development outcomes of COCs cultured in FF-IVM (**Table 4**: total blastocyst r^2 = 0.15, P = 0.033; expanded blastocyst r^2 = 0.25, P = 0.004 and hatched blastocyst r^2 = 0.25, P = 0.004), while plasma NEFA levels were positively related to expanded blastocyst development (r^2 = 0.15, P = 0.029). Lactate in FF was the only follicular parameter measured that affected embryo development (negative relationship; r^2 = 0.13, P = 0.045) and there were no relationships between follicular glucose, triglyceride and NEFA levels.

Cleavage (r^2 = 0.1, P = 0.085), expanded (r^2 = 0.12, P = 0.058) and hatched blastocyst developmental rates (r^2 = 0.13, P = 0.042) of COCs exposed to FF-IVM were trending or significantly and negatively related to days post-partum (**Table 4**). Cleavage and hatched blastocyst rates tended to be (r^2 = 0.097, P = 0.089) or were significantly reduced (r^2 = 0.15, P = 0.034) respectively following FF-IVM in FF samples from cows with high milk protein. Body condition score (BCS) was the only parameter to be positively related with the developmental competence of oocytes matured in FF-IVM, in regards to total blastocyst development (r^2 = 0.13, P = 0.045) and hatched blastocyst development rates (r^2 = 0.11, P = 0.074).

There were few relationships observed for plasma-IVM and subsequent embryo development. These were restricted to weak trends between follicular triglyceride or NEFA levels and total blastocyst development (**Table 4**; r^2 = 0.096, P = 0.095 and r^2 = 0.11, P =0.077) and NEFA levels and expanded blastocyst development (r^2 = 0.11, P = 0.071). As with the FF-IVM cultures, the levels of glucose, lactate, triglycerides and NEFAs in plasma did not relate to embryo development outcomes following IVM in 50% plasma.

4. DISCUSSION

Using biomarkers in plasma to predict fertility would be beneficial to milk producers and many groups have analysed the composition of blood to identify compounds that are related to oocyte developmental competence and fertility. Factors such as metabolites (carbohydrates and fats), hormones/steroids and growth factors have been analysed and despite many potential markers assessed, no clear candidate for fertility has emerged [23-25].

In contrast, few studies have focused on the changing composition of the follicular environment during peak lactation. The follicular environment of high performance dairy cattle during peak lactation is high in NEFA, and FF levels differed from serum levels from 16 days post-partum, decreasing by 44 days post-partum [22]. This corresponds with elevations in plasma NEFA and β-hydroxybutyrate concentrations between 0-21 days post-partum and levels stabilising by 42 days post-partum [24]. While these collection periods were taken during the early period of peak lactation, coinciding with decreases in BCS, the samples in the current study were collected towards the end of the peak lactation period (mean = 73.3 ± 11.8 days post-partum), around the time of servicing and artificial insemination. By this time, animals may be recovering from NEB and loss of BCS, resulting in a recovery in blood and FF fat levels [24]. While we saw similar levels of total NEFAs in FF and plasma as Leroy and colleagues [22], we did not investigate changes in the concentrations of individual NEFAs. Supplementing IVM cultures with elevated levels of NEFAs found in FF during lactation, namely palmitic acid (C16:0), steric acid (C18:0) and oleic acid (C18:1), compromises oocyte developmental competence [26,27], hence alterations in specific NEFAs is likely to contribute to compromised fertility, rather than total NEFA concentrations.

A summary of our findings is detailed in **Table 5**. There were correlations between glucose and lactate concentrations in FF and days post-partum. Glucose concentrations were negatively correlated with both lactate levels and days post-partum, while there was a significantly positive correlation between lactate and days post-partum. Interestingly, increased days post-partum was associated with declining embryo development outcomes such as cleavage, expanded blastocyst and hatched blastocyst rates of COCs matured in FF-IVM. Glucose is one of the major energy sources of the COC, with a large proportion metabolised via glycolysis [28]. As the major end point of glycolysis is lactate, the results from the current study suggest that with increasing days post-partum, more glucose is converted to lactate. However, serial collections from the same animal would be needed to confirm this and rule out daily fluctuations in lactate levels. Regardless of the source, increasing lactate production could have a detrimental effect on oocyte developmental competence, as is the case for *in vitro* embryo cultures [29].

While there were no relationships between plasma and FF glucose and lactate concentrations (indeed plasma glucose levels trended to be higher than FF levels; P = 0.072), there was a highly significant relationship between glucose plasma levels and blastocyst development rates in COCs cultured in FF-IVM. Hence, carbohydrate levels in plasma and FF change during different stages of the lactation cycle and, either directly or indirectly affect the composition of the follicular environment and oocyte developmental competence.

Surprisingly, there were few correlations between FF fat levels and animal parameters and no correlations between plasma levels and animal measurements. Within the follicular microenvironment, triglyceride and NEFA levels were positively correlated and increasing milk fat and protein content was related to increasing triglycerides in FF, most likely due to increased fat mobilisation for increased lactation. In the current study, there were no correlations between plasma concentrations of glucose, lactate, triglycerides and NEFA and animal parameters such as days post-partum, body condition score and milk composition.

Measurement of follicular fluid levels of carbohydrates has been reported, varying according to the source of materials (abattoir, post-mortem ovaries vs *in vivo* collection), breed and handling of samples post-collection, revealing the sensitivity of carbohydrate measurements to the follicular fluid environment. We have previously reported that the follicular levels of glucose and lactate ranged from 1.4-2.3 mM glucose and 3-6.4 mM lactate [30], when collected from abattoir-derived ovaries of predominantly beef cattle, vs. 1.7-1.9 mM glucose (Holstein, abattoir [31] and 2-3.8 mM glucose and 5.6-14.4 mM lactate (Holstein, abattoir [32]). High levels of lactate suggest post-mortem metabolism, hence accounting for some of the variability between these studies.

While the current study suggests a role for an imbalance in plasma carbohydrate levels in influencing oocyte developmental competence, a larger study including more animals from different herds and a broader range of days post-partum is required. Furthermore, oocyte developmental competence is positively related to follicular progesterone and negatively related to oestradiol levels when samples were collected from hyperstimulated, non-lactating Holstein-Fresian cows [33], so steroid levels should also be examined. In addition, amino acid turnover by cattle COCs is related to on-time embryo development [34], suggesting FF amino acid concentration should also be examined in a larger study.

5. Conclusion

Sub-fertility of high performance Holstein cows during later stages of peak lactation is associated with plasma carbohydrate levels, rather than alterations in the levels of fats such and triglycerides and total NEFAs. This is supported by the fact that changes in post-partum energy balance are related to dominant follicle function and IGF-I levels [35]. The Australian dairy industry relies on a pasture-based management system, and differs in comparison to North American and European management systems, which employ more intensive feeding practices and supplements. This alone may impact the composition of the follicular environment, leading to potentially different metabolic mechanisms leading to poor oocyte competence during peak lactation.

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FIGURE CAPTIONS

Figure 1. Glucose, lactate, triglyceride (TG) and non-esterified fatty acid (NEFA) concentrations in FF and plasma collected from eight dairy cows at difference stages of the lactation cycle. Data is presented as means + SEM. * P = 0.072 and ** P < 0.001.

Figure 2. Cleavage and blastocyst development on day 8 following IVM in 50% FF (A, C, E, G) or 50% plasma (B, D, F, G) collected from 8 cows. Bars with similar colours/patterns were in the same herd and data is presented as means + SEM. Initials as in Table 1. FF= control FF (pooled from abattoir ovaries)

Table 1. Characteristics of cows from which follicular fluid and plasma samples were collected.

Animal	Post Partum (days)	Milk Fat (%)	Milk Protein (%)	BCS (1-5 scale)	Daily Milk Yield (L)	Feeding in (kg)
Skychief Farwina (SF)	88	3.9	3.1	4	56.1	9.5
Shottle Betsyann (SB)	61	4.1	3	4	45.2	9.5
Outside Mary (OM)	52	4.5	3.2	4	33	4
Orange 3078	60	3.9	3.4	3	34	4
245	59	4.8	3.6	3	40	4
Leduc Valerie (LV)	151	3.85	3.33	4	39.3	6
Fab Myrtle (FM)	55	3.2	2.7	4	52	6
Roy Ding (RD)	60	4	3	4	43.5	6
Herd 1	74.5 ± 13.5	4 ± 0.1	3.1 ± 0.05	4 ± 0	50.7 ± 5.5	9.5
Herd 2	57 ± 2.5	4.4 ± 0.26	3.4 ± 0.12	3.3 ± 0	35.7 ± 2.2	4
Herd 3	88.7 ± 31.2	3.7 ± 0.25	3 ± 0.18	4 ± 0	44.9 ± 3.7	6
Overall	73.3 ± 11.8	4.0 ± 0.17	3.2 ± 0.1	3.8 ± 0.2	42.9 ± 2.9	6.1 ± 0.2

BCS= body condition score. Herd and overall values are means ± SEM.

Table 2. The composition of follicular fluid (FF) and plasma collected from dairy cattle originating from three herds

	Glucose (mM)		Lactate (mM)		Triglycerides (mM)		NEFA (mM)	
	FF	**Plasma**	**FF**	**Plasma**	**FF**	**Plasma**	**FF**	**Plasma**
Control FF (pooled)	0.73		16.68		0.44		0.76	
Skychief Farwina	2.72	3.86	0.82	0.92	0.02	0.12	0.08	0.1
Shottle Betysann	3.47	3.53	0.69	0.47	0.06	0.18	0.08	0.07
Outside Mary	3.51	2.79	0.17	0.55	0.04	0.06	0.1	0.61
Orange 3078	3.7	3.58	0.35	0.98	0.04	0.12	0.09	0.14
245	3.46	4.01	0.34	0.67	0.04	0.16	0.1	0.62
Leduc Valerie	1.93	3.97	2.51	0.58	0.06	0.11	0.51	0.12
Fab Myrtle	2.87	3.27	0.74	0.34	0.1	0.18	0.5	0.59
Roy Ding	2.85	3.43	0.56	0.32	0.06	0.12	0.58	0.31
Herd 1	3.1 ± 0.38	3.7 ± 0.17	0.76 ± 0.07	0.7 ± 0.23	0.04 ± 0.02	0.15 ± 0.03	0.08 ± 0	0.09 ± 0.02
Herd 2	3.56 ± 0.07	3.46 ± 0.36	0.29 ± 0.06	0.73 ± 0.13	0.04 ± 0	0.11 ± 0.03	0.1 ± 0.003	0.46 ± 0.16
Herd 3	2.55 ± 0.31	3.56 ± 0.21	1.27 ± 0.62	0.41 ± 0.08	0.07 ± 0.01	0.14 ± 0.02	0.53 ± 0.03 **	0.34 ± 0.14

Data for herds and overall are means ± SEM. ** $P < 0.001$, different to herds 1 and 2.

Table 3. Linear regression analyses of *in vivo* collected FF and plasma and animal parameters

	Glucose (FF)	**Lactate (FF)**	**TG (FF)**	**NEFA (FF)**	**Glucose (Plasma)**	**Lactate (Plasma)**	**TG (Plasma)**	**NEFA (Plasma)**
Lactate (FF)	**r^2 = 0.78 P = 0.004**							
TG (FF)	r^2 =0.058 P = 0.57	r^2 = 0.045 P = 0.615						
NEFA (FF)	**r^2 = 0.48 P = 0.057**	r^2 = 0.26 P = 0.197	**r^2 = 0.48 P = 0.058**					
Glucose (plasma)	r^2 = 0.15 P = 0.34	r^2 = 0.24 P = 0.218	r^2 = 0.07 P = 0.526	r^2 < 0.001 P = 0.972				
Lactate (plasma)	r^2 = 0.047 P = 0.61	r^2 = 0.007 P = 0.849	**r^2 = 0.58 P = 0.028**	**r^2 = 0.005 P = 0.074**	r^2 = 0.164 P = 0.32			
TG (plasma)	r^2 = 0.006 P = 0.86	r^2 = 0.001 P = 0.951	r^2 = 0.27 P = 0.19	r^2 = 0.005 P = 0.863	r^2 = 0.129 P = 0.383	r^2 = 0.066 P = 0.54		
NEFA (plasma)	r^2 = 0.078 P =0.50	r^2 = 0.2 P = 0.266	r^2 = 0.076 P = 0.507	r^2 = 0.007 P = 0.848	r^2 = 0.192 P = 0.277	r^2 = 0.148 P = 0.346	r^2 = 0.001 P = 0.941	
Days PP	**r^2 = 0.711 P =0.008**	**r^2 = 0.921 P < 0.001**	r^2 = 0.004 P = 0.883	r^2 = 0.118 P = 0.406	r^2 = 0.318 P = 0.145	r^2 = 0.021 P = 0.732	r^2 = 0.041 P = 0.632	r^2 = 0.245 P = 0.213
% Milk fat	r^2 = 0.20 P = 0.26	r^2 = 0.121 P = 0.398	**r^2 = 0.305 P = 0.09**	r^2 = 0.304 P = 0.157	r^2 = 0.007 P = 0.845	r^2 = 0.038 P = 0.644	r^2 = 0.094 P = 0.459	r^2 = 0.068 P = 0.534
% Milk protein	r^2 = 0.04 P = 0.63	r^2 = 0.001 P = 0.95	**r^2 = 0.417 P = 0.084**	r^2 = 0.193 P = 0.277	r^2 = 0.232 P = 0.227	r^2 = 0.349 P = 0.123	r^2 = 0.094 P = 0.461	r^2 < 0.001 P = 0.988
BCS	r^2 = 0.3 P = 0.17	r^2 = 0.128 P = 0.384	r^2 = 0.105 P = 0.432	r^2 = 0.186 P = 0.286	r^2 = 0.133 P = 0.375	r^2 = 0.313 P = 0.15	r^2 = 0.018 P = 0.753	r^2 = 0.022 P = 0.681
Milk yield	r^2 = 0.144 P = 0.355	r^2 = 0.013 P = 0.79	r^2 = 0.04 P = 0.636	r^2 = 0.03 P = 0.683	r^2 = 0.064 P = 0.546	r^2 = 0.014 P = 0.782	r^2 = 0.271 P = 0.186	r^2 = 0.03 P = 0.681
Feeding (kg)	r^2 = 0.082 P = 0.485	r^2 = 0.056 P = 0.571	r^2 = 0.001 P = 0.938	r^2 = 0.006 P = 0.859	r^2 = 0.057 P = 0.569	r^2 = 0.001 P = 0.933	r^2 = 0.136 P = 0.368	r^2 = 0.388 P = 0.1

Shaded boxes = significant ($P < 0.05$) or trending to be significant ($P < 0.1$) relationships; grey = negative and black = positive relationships.

Table 4. Regression analyses of embryo development and animal parameters

	FF-IVM				**Plasma-IVM**			
	Cleavage	**Total Blastocysts**	**Expanded Blastocysts**	**Hatched Blastocysts**	**Cleavage**	**Total Blastocysts**	**Expanded Blastocysts**	**Hatched Blastocysts**
Glucose (FF)	$r^2 = 0.039$ $P = 0.29$	$r^2 = 0.006$ $P = 0.67$	$r^2 = 0.034$ $P = 0.32$	$r^2 = 0.057$ $P = 0.20$	$r^2 < 0.001$ $P = 0.87$	$r^2 = 0.004$ $P = 0.75$	$r^2 = 0.054$ $P = 0.27$	$r^2 = 0.016$ $P = 0.51$
Lactate (FF)	$r^2 = 0.064$ $P = 0.17$	$r^2 = 0.0002$ $P = 0.95$	$r^2 = 0.087$ $P = 0.11$	**$r^2 = 0.13$** **$P = 0.045$**	$r^2 < 0.001$ $P = 0.89$	$r^2 = 0.16$ $P = 0.69$	$r^2 = 0.062$ $P = 0.18$	$r^2 = 0.013$ $P = 0.55$
TG (FF)	$r^2 = 0.026$ $P = 0.39$	$r^2 = 0.015$ $P = 0.51$	$r^2 = 0.033$ $P = 0.32$	$r^2 < 0.001$ $P = 0.94$	$r^2 < 0.001$ $P = 0.90$	**$r^2 = 0.096$** **$P = 0.095$**	$r^2 = 0.043$ $P = 0.27$	$r^2 = 0.001$ $P = 0.85$
NEFA (FF)	$r^2 = 0.0003$ $P = 0.92$	$r^2 = 0.028$ $P = 0.36$	$r^2 = 0.004$ $P = 0.73$	$r^2 = 0.026$ $P = 0.39$	$r^2 = 0.014$ $P = 0.53$	**$r^2 = 0.11$** **$P = 0.077$**	**$r^2 = 0.11$** **$P = 0.071$**	$r^2 = 0.011$ $P = 0.59$
Glucose (Plasma)	$r^2 = 0.031$ $P = 0.34$	**$r^2 = 0.15$** **$P =0.033$**	**$r^2 = 0.25$** **$P = 0.004$**	**$r^2 = 0.25$** **$P = 0.004$**	$r^2 < 0.001$ $P = 0.90$	$r^2 = 0.013$ $P = 0.55$	$r^2 = 0.016$ $P = 0.50$	$r^2 = 0.034$ $P = 0.33$
Lactate (Plasma)	$r^2 = 0.009$ $P = 0.62$	$r^2 = 0.029$ $P = 0.336$	$r^2 = 0.087$ $P = 0.11$	$r^2 = 0.004$ $P = 0.74$	$r^2 = 0.036$ $P = 0.32$	$r^2 = 0.072$ $P = 0.15$	$r^2 = 0.034$ $P = 0.33$	$r^2 < 0.001$ $P = 0.96$
TG (Plasma)	$r^2 = 0.04$ $P = 0.28$	$r^2 = 0.079$ $P = 0.13$	$r^2 = 0.023$ $P = 0.42$	$r^2 = 0.032$ $P = 0.33$	$r^2 = 0.004$ $P = 0.75$	$r^2 < 0.001$ $P = 0.97$	$r^2 = 0.001$ $P = 0.85$	$r^2 < 0.001$ $P =0.97$
NEFA (Plasma)	$r^2 = 0.002$ $P = 0.79$	$r^2 = 0.009$ $P = 0.62$	**$r^2 = 0.15$** **$P = 0.029$**	$r^2 = 0.030$ $P = 0.35$	$r^2 = 0.003$ $P = 0.79$	$r^2 = 0.053$ $P = 0.22$	$r^2 = 0.005$ $P = 0.71$	$r^2 < 0.001$ $P = 0.92$
Post Partum (days)	**$r^2 = 0.10$** **$P = 0.085$**	$r^2 = 0.0002$ $P = 0.94$	**$r^2 = 0.12$** **$P = 0.058$**	**$r^2 = 0.13$** **$P = 0.042$**	$r^2 < 0.001$ $P = 0.93$	$r^2 < 0.001$ $P = 0.96$	$r^2 = 0.043$ $P = 0.27$	$r^2 = 0.017$ $P = 0.50$
% Milk Fat	$r^2 = 0.045$ $P = 0.25$	$r^2 = 0.033$ $P = 0.33$	$r^2 = 0.005$ $P = 0.70$	$r^2 = 0.017$ $P = 0.49$	$r^2 = 0.017$ $P = 0.49$	$r^2 = 0.016$ $P = 0.51$	$r^2 = 0.004$ $P = 0.73$	$r^2 = 0.001$ $P = 0.61$
% Milk Protein	**$r^2 = 0.097$** **$P = 0.089$**	$r^2 = 0.07$ $P = 0.15$	$r^2 = 0.092$ $P = 0.10$	**$r^2 = 0.15$** **$P = 0.034$**	$r^2 = 0.003$ $P = 0.77$	$r^2 < 0.001$ $P = 0.88$	$r^2 = 0.010$ $P = 0.59$	$r^2 = 0.035$ $P = 0.33$
BCS	$r^2 = 0.003$ $P = 0.77$	**$r^2 = 0.13$** **$P = 0.045$**	$r^2 = 0.057$ $P = 0.20$	**$r^2 = 0.11$** **$P = 0.074$**	$r^2 = 0.002$ $P = 0.79$	$r^2 = 0.006$ $P = 0.67$	$r^2 = 0.005$ $P = 0.71$	$r^2 = 0.018$ $P = 0.48$
Milk Yield	$r^2 = 0.033$ $P = 0.33$	$r^2 = 0.004$ $P = 0.73$	$r^2 = 0.002$ $P = 0.80$	$r^2 =0.011$ $P = 0.57$	$r^2 = 0.015$ $P = 0.52$	$r^2 = 0.041$ $P = 0.28$	$r^2 = 0.017$ $P = 0.49$	$r^2 = 0.002$ $P = 0.81$
Feed (kg)	$r^2 = 0.013$ $P = 0.55$	$r^2 = 0.005$ $P = 0.71$	$r^2 = 0.033$ $P = 0.35$	$r^2 = 0.006$ $P = 0.68$	$r^2 = 0.007$ $P = 0.64$	**$r^2 = 0.17$** **$P = 0.031$**	$r^2 = 0.067$ $P = 0.18$	$r^2 =0.014$ $P = 0.56$

Shaded boxes = significant ($P < 0.05$) or trending to be significant ($P < 0.1$) relationships; grey = negative and black = positive relationships

Table 5. Summary of the relationships between animal parameters and FF and plasma.

		Post Partum (Days)	% Milk Fat	% Milk Protein	BCS	Milk Yield	Feeding	FF	Plasma
FF	Glucose	-						- Lactate - NEFA	
	Lactate	+							
	TG		-	-				+ NEFA	- Lactate
	NEFA								- Lactate
Plasma	Glucose								
	Lactate								
	TG								
	NEFA								
Embryo Development	FF-IVM	-		-	+			- Lactate	- Glucose
	Plasma-IVM						-	+ TG + NEFA	+ NEFA

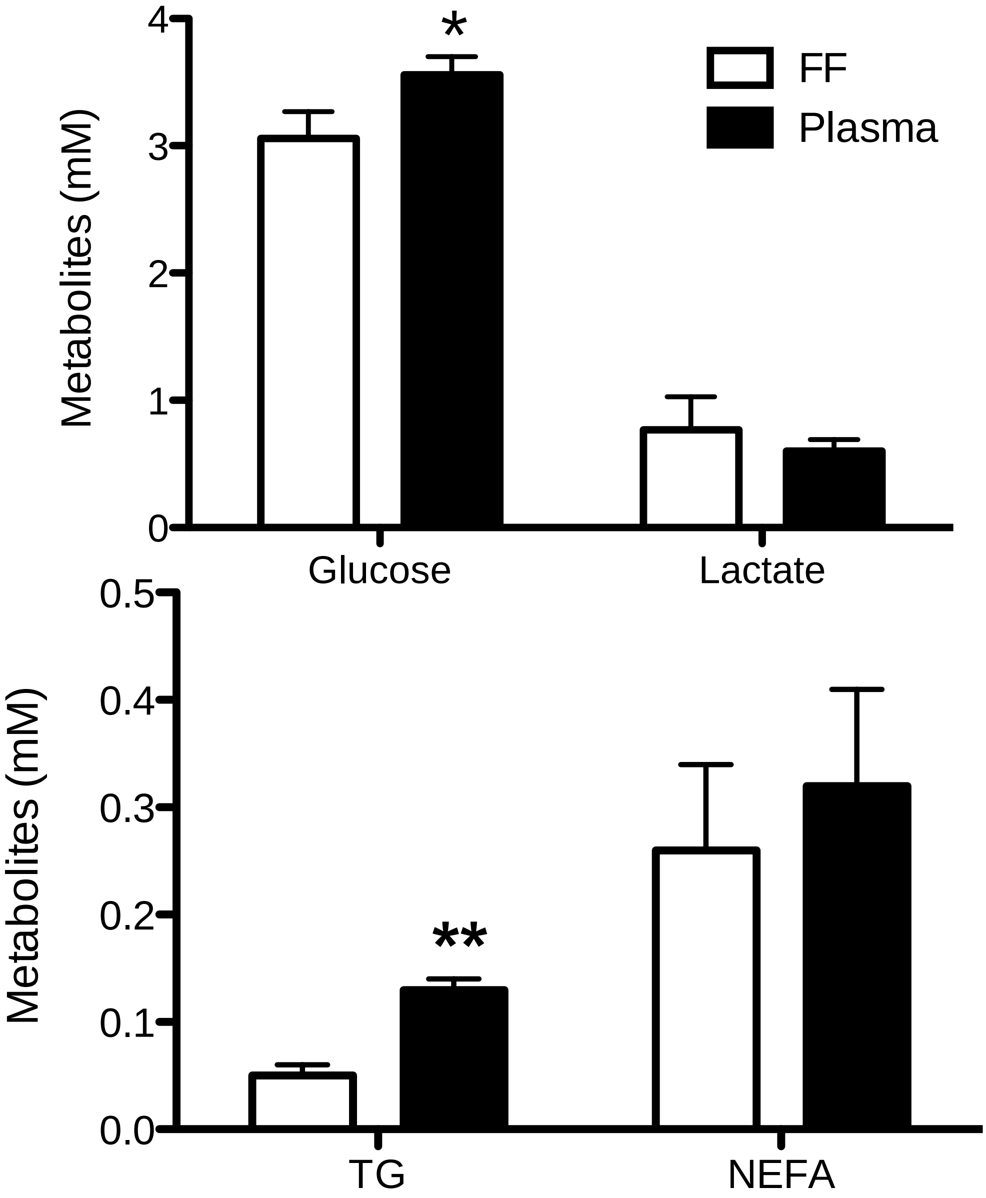
4
3
2
1
0
Metabolites (mM)
*
FF
Plasma
Glucose
Lactate
0.5
0.4
0.3
0.2
0.1
0.0
Metabolites (mM)
**
TG
NEFA

Figure 1.

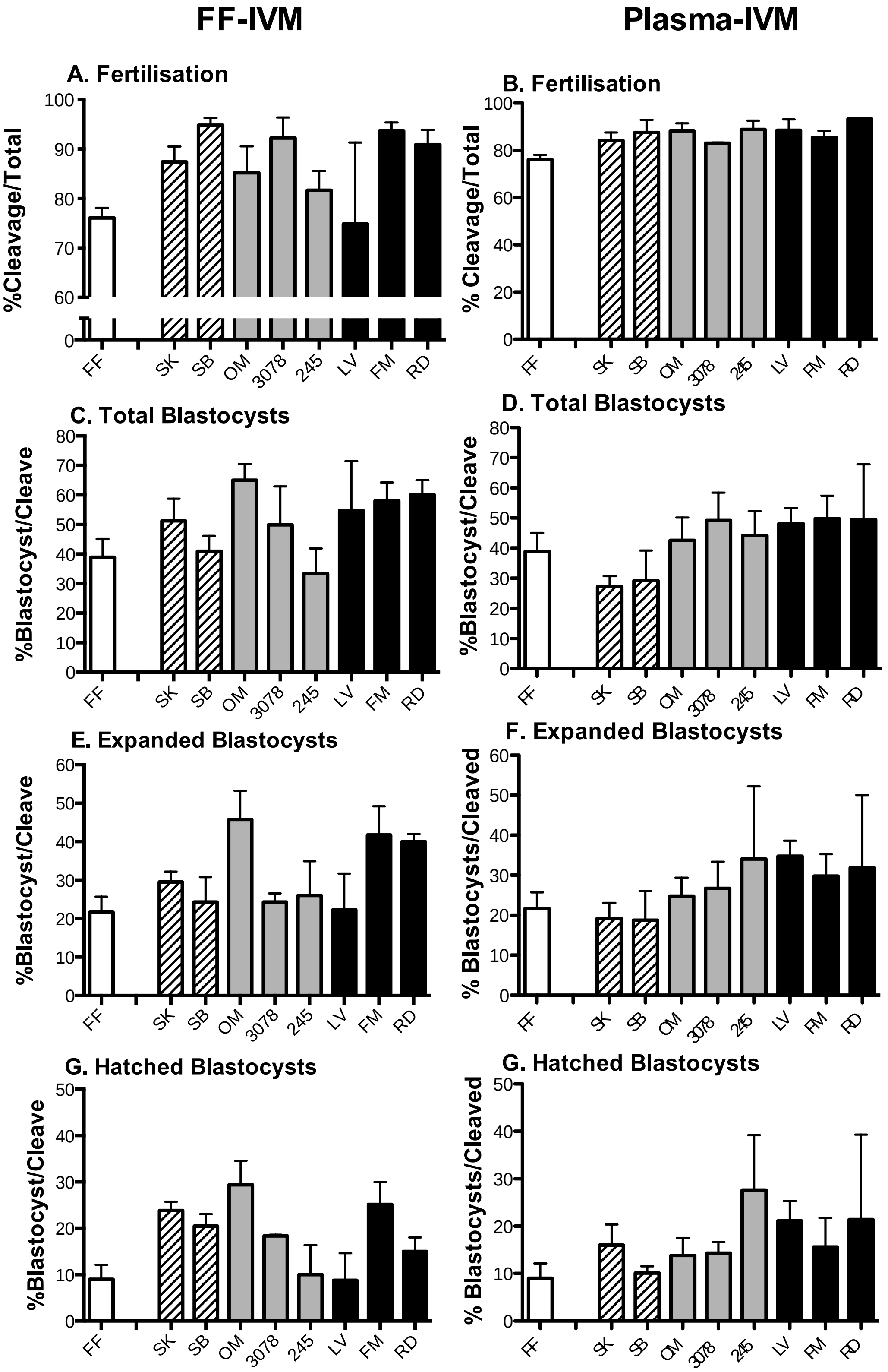
FF-IVM
Plasma-IVM
A. Fertilisation
%Cleavage/Total
100
90
80
70
60
0
B. Fertilisation
% Cleavage/Total
100
80
60
40
20
0
C. Total Blastocysts
%Blastocyst/Cleave
80
70
60
50
40
30
20
10
0
D. Total Blastocysts
%Blastocyst/Cleave
E. Expanded Blastocysts
%Blastocyst/Cleave
F. Expanded Blastocysts
% Blastocysts/Cleaved
G. Hatched Blastocysts
%Blastocyst/Cleave
G. Hatched Blastocysts
% Blastocysts/Cleaved
FF
SK
SB
OM
3078
245
LV
FM
RD

Figure 2.