

# Metabolomics and fertility in cattle: A promising predictor

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## Summary

During her lifetime, the ovarian environment of female domestic farm animals is subject to biochemical changes brought about by multiple factors, including husbandry practices, production demands and disease, which may ultimately impair oocyte quality and subsequent embryo development. The lactating dairy cow appears to be particularly challenged in this respect and many investigations have been carried out to characterize the underlying physiology and to identify biomarkers and indicators in readily accessible body fluids. The use of metabolomics-based techniques has identified biomarkers in follicular fluid and is being expanded for oocyte and embryo selection.

## Introduction

Fertility can be defined as the natural capability to produce offspring. In cattle, a number of metrics exist by which “fertility rate” is reported, including the number of days open, or calving to conception interval, and pregnancy rate to first and/or subsequent services. Regardless of the variation in measures of reproductive success, pregnancy is the endpoint and it is the culmination of precisely ordered, well-orchestrated events, which commence with the timely resumption of ovarian activity post-calving. The onset of cyclicity should initially result in the selection and growth of a healthy follicle that encloses a competent oocyte, and ultimately in, oestrus, ovulation, fertilization and uterine attachment by a viable embryo (Leroy *et al.* 2011). The concomitant development of a functional corpus luteum should provide an appropriate environment, through optimal progesterone secretion, in which the embryo can grow and develop (Diskin & Morris 2008). The decline in fertility in high-yielding dairy cows has been reviewed extensively (Lucy 2007, Wathes *et al.* 2007, Diskin & Morris 2008, Diskin *et al.* 2011, Wathes 2012). The lactating dairy cow appears to be challenged in particular by high-energy requirements for milk production simultaneous with inadequate feed intake, which results in the cow falling in to a state of negative energy balance (NEB), which has a dramatic impact on her fertility (Leroy *et al.* 2008a,b, Walsh *et al.* 2011). Although, characterized by a substantially smaller body of literature, there is also evidence of declining fertility in beef females, primarily evidenced by a decrease in calves per cow per year, and an increase in calving interval and age at first calving. The main factors influencing reproductive efficiency in beef cattle include age at puberty and first conception, duration of post-partum anoestrus and total lifetime productivity (Burns *et al.* 2010). Evidence suggests that the switch to later maturing genetics is increasing the age and weight of heifers at puberty; selection for low residual feed intake results in selection of leaner heifers that reach puberty at older ages and calve later in their first and subsequent

calving seasons (Berry & Crowley 2012, Randel & Welsh 2013). Additionally, genetic selection for growth and carcass traits within beef breeding programs has had a negative genetic effect on cow fertility (Crowley *et al.* 2011).

### Where is the problem?

**Oocyte/embryo:** Evidence for a contribution of poor oocyte quality to infertility comes primarily from embryo transfer studies which have reported higher pregnancy rates in lactating dairy cows after embryo transfer compared with AI (Putney *et al.* 1989, Ambrose *et al.* 1999, Rutledge 2001, Al-Katanani *et al.* 2002, Vasconcelos *et al.* 2006, Demetrio *et al.* 2007). These findings are substantiated by data from nonsurgical flushing of unstimulated dairy cows, which suggest that, a significant proportion of embryos degenerate before the blastocyst stage (reviewed by Sartori *et al.* 2010). For example, in three studies by Cerri *et al.* (2009a,b,c), the proportion of viable embryos recovered on Days 6-7 was approximately 50%. Given that fertilization rate is estimated at 85-95%, a 50% viable embryo recovery rate suggests that a significant proportion of embryos are lost as early as Day 7. The data from *in vitro* studies in UCD examining the effect of lactation on oocyte quality are more conflicting. For example, Sniijders *et al.* (2000) reported lower cleavage and blastocyst rates following *in vitro* embryo production (IVP) of oocytes recovered from dairy cows with a higher genetic merit for milk production compared to developmental rates of oocytes from cows of average genetic merit. In contrast, Rizos *et al.* (2005) reported no difference in the proportion of good quality oocytes undergoing fertilization and development to the blastocyst stage between lactating cows and heifers. Similarly, Matoba *et al.* (2012), failed to demonstrate an effect of metabolic status postpartum on oocyte ability to undergo IVF and develop to the blastocyst stage *in vitro*.

**Embryo/Reproductive tract:** The findings of the studies listed above do not eliminate a role for the embryo or reproductive tract environment; a sub-optimal uterine environment is likely to be a major contributor to the higher incidence of early embryonic death found in repeat-breeder cows (Hill & Gilbert 2008). Two recent studies carried out in UCD compared embryo development rates in lactating and non-lactating animals. Both studies reported lower rates of embryo development to the blastocyst stage on Day 7 in the lactating animals (Rizos *et al.* 2010, Maillou *et al.* 2012), highlighting the suboptimal uterine milieu for embryo development in lactating animals. Similar models have been described by Thompson *et al.* (2012) and Green *et al.*, (2012). The sub-optimal environment is most likely due to a reduced ability to mount an effective immune response leading to a higher incidence of persistent uterine infection in dairy cows (Sheldon *et al.* 2006, Pyorala 2008, Wathes *et al.* 2009). Histological examination of the post-partum endometrium revealed that the shallow stroma is characterized by lipid accumulation and formation of foam cells, likely a result of tissue damage after calving, bacterial infection and an influx of monocytes (Wathes *et al.* 2013).

**Metabolic status of the transition/postpartum cow:** Fluctuating concentrations of metabolites in the early postpartum period, associated with high milk production, have been strongly correlated with poor reproductive inefficiency in commercial dairy herds (Lucy 2001). Decreasing (glucose, insulin, IGF-I) or increasing (non-esterified fatty acids (NEFA), ketone bodies) circulating metabolites during nutrient partitioning associated with low body condition score (BCS) have been associated with alterations to the steroidogenic and transcriptomic profiles of ovarian follicles during their development, compared to non-lactating heifers (Bender *et al.* 2010, Walsh *et al.* 2012a,b). These differences are characterized by reduced dominant follicle estradiol and progesterone synthesis during differentiation and luteinization, and altered

expression profiles of transcripts associated with steroid biosynthesis (Walsh *et al.* 2012b), immune cell function and chemotaxis (Walsh *et al.* 2012a). Indeed, Garverick *et al.*, (2013) recently reported that postpartum dairy cows that became pregnant to first insemination had lower serum NEFA concentrations and greater plasma glucose concentrations compared to those that did not become pregnant. In addition, using logistic regression analysis, the authors were able to identify that NEFA and glucose concentration ratio on Day 3 postpartum had the greatest predictability of the probability of pregnancy in lactating dairy cows.

It is postulated that a better understanding of the metabolic effects of various aetiologies of subfertility may aid the development of targeted therapeutics or lead to the identification of non-invasive biomarkers for diagnostic and prognostic purposes (Baskind *et al.* 2011). The collection of low-molecular weight compounds (< 1500 Daltons) in an organism or biological sample is defined as the 'metabolome' (Wishart 2007). Low-molecular weight metabolites represent the intermediates or end products of the cell's regulatory processes; their individual profile is referred to as a 'metabolic fingerprint' (Kell 2005). Since the metabolome is related to an organism's genotype, physiology and environment, it provides a powerful tool to assess the physiological state and to assist in the identification of possible biomarkers for fertility research (Baka & Malamitsi-Puchner 2006, Sinclair *et al.* 2008).

### Metabolomics studies of follicular fluid

#### *The effect of lactation*

The metabolic alterations induced by postpartum NEB have been associated with anoestrus, poor follicle growth, poor-quality oocytes, inadequate corpus luteum function, reduced steroidogenesis and compromised immune function. The oocytes that are ovulated 2–4 months after calving, at a time when breeding is normally carried out in seasonal systems, have completed their growth phase when NEB is at its most severe. Some studies have indicated that increased concentrations of NEFA and  $\beta$ -hydroxybutyrate (BHB) in follicular fluid adversely affect oocyte quality (Leroy *et al.* 2004, Leroy *et al.* 2005). In addition, NEFA have been shown to reduce steroidogenesis and proliferation in follicular thecal cells (Vanholder *et al.* 2005). Leroy *et al.* (2005) determined the NEFA concentration and composition in follicular fluid of high-yielding dairy cows in relation to serum early and late postpartum and subsequently added the 3 predominant NEFA (oleic, palmitic, stearic) in follicular fluid during oocyte maturation *in vitro*. Both palmitic and stearic acid had a negative effect on meiotic maturation, fertilization and blastocyst formation. Marei *et al.* (Marei *et al.* 2012) found that bovine oocyte maturation in the presence of added linoleic acid influenced the distribution and function of mitochondria in the cytoplasm: The authors reported that the redistribution of oocyte mitochondria from a peripheral to a diffuse pattern was delayed and the proportion of oocytes presenting clusters of mitochondria in the perinuclear region was reduced, in addition, mitochondrial inner membrane potential was decreased, while reactive oxygen species levels were increased. Van Hoeck *et al.* (2011) demonstrated that exposure to high concentrations of the NEFAs, oleic acid, palmitic acid and stearic acid, during oocyte maturation appeared to reduce resulting embryo quality. Exposure to elevated NEFA concentrations resulted in blastocysts with altered *DNMT3A*, *SLC2A1* and *IGF2R* mRNA expression, lower cell number and increased apoptotic cell ratio in comparison to control blastocysts.

Applying metabolomic profiling to further investigate the potential of this technology as a predictor of cow fertility or oocyte quality, the metabolomic profiles of blood serum and follicular fluid from post partum lactating dairy cows and heifers were analysed and compared

(Bender *et al.* 2010). Samples were collected from cows and heifers over three phases of the oestrous cycle, corresponding to stages of follicle development: newly selected dominant follicles, preovulatory follicles prior to oestrus and peri-ovulatory follicles and were analysed using gas chromatography mass spectrometry (GC–MS)-based metabolomics. Follicular fluid samples from cows contained higher levels of total saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA). In particular, the concentrations of SFA, such as palmitic acid and stearic acid, were dramatically higher in the cow follicular fluid, whereas concentrations of the polyunsaturated omega-3 fatty acid, docosahexaenoic acid, were higher in heifer follicular fluid. In contrast, although the metabolic profile of serum from the lactating dairy cows was, as expected, characterized by lower insulin, IGF-1 and glucose concentrations and higher BHB concentrations compared to heifers, total SFA and MUFA levels were not different. Although each fatty acid that was different in cow serum compared to heifer serum was also different in the follicular fluid, their concentrations and ratios were generally not the same and there were additional differing fatty acids in the follicular fluid, revealing the particularly unique lipid composition of follicular fluid and highlighting the impact of the metabolic activity of follicular cells on its composition (Bender *et al.* 2010). Thus extrapolating serum metabolomic data to draw inferences on the ovarian environment and predict oocyte quality, while highly desirable is not readily achievable.

More recently, we profiled the follicular metabolome of cows at regular timepoints post partum (O'Doherty *et al.* 2014). Regardless of stage post partum the most abundant fatty acids in the follicular fluid were linoleic acid, oleic acid and stearic acid. Interestingly, analysis of bovine, ovine and porcine oocyte fatty acid composition also identified these and palmitic acid as the most abundant (McEvoy *et al.* 2000). Temporal differences were identified in the concentrations of several fatty acids and amino acids, although there were no distinct fatty acid profiles for lactating cows at particular time points post partum (O'Doherty *et al.* 2014). The abundance of valine and leucine increased significantly in follicular fluid with increasing days postpartum, whereas concentrations of creatinine and ornithine decreased. Alterations to branched chain amino acids levels have previously been implicated in insulin resistance and the development of metabolic syndromes, such as type II diabetes, hyperglycaemia, hypertension and obesity (Adeva *et al.* 2012, Nagata *et al.* 2013), conditions that are associated with poor fertility. Therefore, the profiles of branched chain fatty acids in the follicular fluid of postpartum cows may be reflective of their compromised metabolic status and may potentially indicate the competence of the oocyte within.

#### *Follicular metabolomic bio-markers: In vitro fertilization*

The selection of oocytes or embryos with the highest developmental potential is critical to successful in vitro embryo production (IVP) routines. Follicular fluid is superfluous to the IVP regime and provides a substrate for non-invasive assessment of oocyte quality (Revelli *et al.* 2009). Using an established individual oocyte IVP system, which permits the tracking of bovine oocytes as they progress through IVP, but avoids the low development rates associated with single oocyte culture (Matoba *et al.* 2010), we recently analysed the steroidal and metabolomic profiles of bovine follicular fluid from 6-8 mm antral follicles from which a competent oocyte had been retrieved compared to fluid from follicles associated with oocytes of low developmental potential (Matoba *et al.* 2013). Although there was no association of follicular testosterone, progesterone or oestradiol concentrations with the oocyte's ability to form a blastocyst in

vitro, the profiles of quantified aqueous metabolites in follicular fluid were different between oocytes that formed blastocysts and oocytes that degenerated. In agreement with a previous report (Sinclair *et al.* 2008), follicular fluid L-alanine, glycine and L-glutamate concentrations were positively correlated with and therefore potentially predictive of, blastocyst formation, whereas urea was negatively correlated. Several studies have described the beneficial effects of alanine (Cetica *et al.* 2003) and L-alanine and glycine (Lee & Fukui 1996) on embryonic development. Follicular fluid associated with competent oocytes was significantly lower in palmitic acid and total fatty acids and significantly higher in n-3 PUFA linolenic acid than follicular fluid from incompetent oocytes. Interestingly, higher linolenic acid was also noted in follicular fluid from heifers compared to lactating cows in the study highlighted above. In contrast, the detrimental effects of increased palmitic acid in follicular fluid, or, following addition to in vitro maturation (IVM), have been described above. Similarly, metabolomic profiles of follicular fluid from women undergoing in vitro fertilization (IVF) treatment has also revealed a negative correlation between levels of saturated fatty acids (Haggarty *et al.* 2006), particularly lignoceric acid, palmitic acid and arachidic acid (O’Gorman *et al.* 2013), with developmental outcome, and a positive correlation of docosahexaenoic acid (DHA) and of stearic acid with developmental outcome (O’Gorman *et al.* 2013). Multivariate statistical analysis of the data from different studies concerning the follicular metabolome indicate the high predictive ability of aqueous metabolites (Matoba *et al.*, 2013) and fatty acids (O’Gorman *et al.* 2013), according to the model and species used.

#### *Follicular metabolomic bio-markers: High and low cow fertility model*

Most recently, we have applied metabolomic profiling of follicular fluid to search for biomarkers of cow fertility (Moore *et al.* 2013), using a genetic model of Holstein dairy cow fertility established in Teagasc Moorepark (Cummins *et al.* 2012a,b,c). The model was composed of two groups of cows with similar genetic merit for milk production traits, but with extremes of good (Fert+) or poor (Fert-) genetic merit for fertility traits. The metabolomes of follicular fluid of the first wave dominant follicle and blood serum on d 7 of the oestrous cycle was compared in the Fert+ and Fert- cows. Following analysis, there was clear separation of the Fert- and Fert+ cows based on the concentrations of follicular fluid fatty acid, highlighting the potential of this technology as a predictor or biomarker of cow fertility. Interestingly and in keeping with the earlier mentioned findings of Bender *et al.* (2010), although the serum fatty acid profiles were also different, their predictive power was not as robust, again highlighting the unique metabolic milieu of the ovarian follicle. A summary of the key follicular fluid and serum metabolites associated with oocyte competence and cow fertility are presented in Table 1.

### **Conclusion**

The follicular fluid metabolome provides a greater potential for predicting cow fertility and oocyte competence than other readily accessible body fluids such as milk and blood. However, caution must be exercised when interpreting the results, as the data are model specific.

**Table 1:** Summary of metabolomic data associated with oocyte competence or cow fertility

Matrix	Association	Positive association	Negative association	Reference
Follicular fluid	Oocyte competence	DHA (C22:6n3) Stearic acid (C18:O)	Palmitic acid (C16:O) Linoleic acid (C18:2n6) Arachidic acid (C20:O) Total PUFA	O’Gorman <i>et al.</i> , 2013
		Linolenic acid (C18:2n6)  L-alanine Glycine L-Glutamate	Palmitic acid (C16:O) Total SFA	Matoba <i>et al.</i> , 2013
	Cow Fertility		Palmitic acid (C16:O) Stearic acid (C18:O)	Leroy <i>et al.</i> , 2005
			Myristic (C14:0) Palmitoleic (C16:1) Palmitic (C16:0) $\gamma$ -Linolenic (C18:3n6) Linoleic (C18:2n6)	Bender <i>et al.</i> , 2010
			Myristoleic (C14:1) Heptadecenoic (C17:1) Myristic (C14:0) $\gamma$ -Linolenic (C18:3n6) Arachidic acid (C20:O)	Moore <i>et al.</i> , 2013
Blood		DHA (C22:6n3) DGLA (C20:3n6) Total PUFA	Palmitic acid (C16:O) Linoleic acid (C18:2n6) (n-6) PUFA	Bender <i>et al.</i> , 2010
			Total NEFA	Garverick <i>et al.</i> , 2013

DHA:Docosahexanoic acid; PUFA: Polyunsaturated fatty acids; SFA: saturated fatty acids; DGLA: Dihomo- $\gamma$ -linolenic acid; NEFA: Non esterified fatty acids

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