

ATHENRY RESEARCH REPORT

Negative energy balance and gene expression in dairy cow metabolic and reproductive tissues

During the early *post-partum* interval high yielding dairy cows undergo extensive physiological and biochemical modifications in order to re-establish metabolic homeostasis and to counteract the adverse affects of negative energy balance (NEB). Energy balance (EB) therefore has a profound effect on tissue function which can compromise cow health and fertility and this is likely to be mediated by alterations in gene expression. To gain new insights into these events gene expression was examined in liver tissue obtained from cows managed to achieve a mild (MNEB) or severe negative energy balance (SNEB). As previously reported there was a 3.6 fold ($P<0.05$) difference in energy deficit on the day of slaughter between the SNEB and MNEB cows (research report 2005). This difference in energy balance was further confirmed by divergence in systemic concentrations of metabolites and IGF-1 (research report 2005). Following microarray analysis, a total of 524 genes were found to be significantly differentially expressed ($P<0.05$) of which 199 were up regulated and 325 down regulated in SNEB compared to MNEB cows. Of these, 222 represented expressed sequence tags and hypothetical proteins, the remaining 302 were categorised according to named Gene Ontology (GO) biological processes (Fig.28).

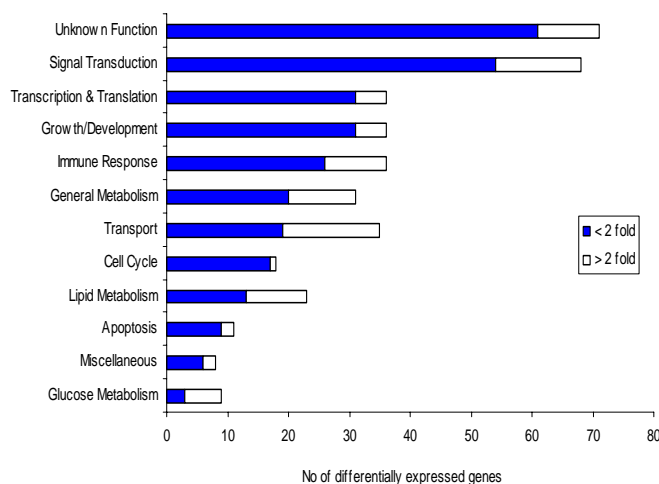


Figure 28. Categorisation of differentially expressed genes.

To validate the microarray data a random selection of 20 genes were subjected to real time RT-PCR analysis. Gene expression patterns were found to be similar using both procedures. Cows in SNEB had increased systemic concentrations of non-esterified fatty acids (1.40 vs 0.34 mmol/l), β -hydroxybutyrate (BHB) (3.7 vs 0.53 mmol/l) and hepatic triacylglyceride concentration (5.8 vs 1.5 mM) ($P<0.05$). This was accompanied by increased expression of genes involved in lipid transport (FABP5, ApoA1) and oxidation (VLCAD, AngP4, CPTB). Genes involved in inflammatory and acute phase response (IL-8, S100A12, SAA3, SERPINA3) were up regulated in SNEB cows. Excessive accumulation of lipids is known to induce cellular trauma and this is likely to account for the up regulation of immune and stress response genes in the SNEB cows. There was a pronounced down regulation of genes involved in cellular growth in SNEB cows. Moreover a negative regulator of cellular proliferation (HGFN) was up regulated in SNEB cows. High levels of lipid by products are also known to inhibit cellular proliferation and this may explain the decreased expression of cell growth associated genes in SNEB cows. It is therefore possible that reducing hepatic lipid accumulation during the early *post-partum* period may help reduce inflammatory response and improve cellular proliferation, thereby, hastening adaptation and recovery from NEB.

SNEB cows also had reduced mRNA expression of IGFBP-2 and -6 in the oviduct ($P<0.05$), and elevated expression of IGFBP-5 ($P<0.05$) when compared with MNEB cows. These effects were corroborated by *in situ* hybridisation using optical density measurements for IGFBP-2 ($P<0.05$) and -6 ($P<0.01$) but not -5 ($P=0.447$). All other components of the IGF system remained unchanged between the two treatment groups. When data from all animals was pooled, IGFBP-2 mRNA expression in the oviduct correlated positively with mean plasma glucose ($r=0.692$; $P<0.05$) and negatively with BHB ($r=-0.678$; $P<0.05$). Conversely, IGFBP-5 decreased with mean plasma glucose ($r=-0.734$; $P<0.02$) and increased with BHB ($r=0.727$; $P<0.05$). These results highlight a unique pattern of IGFBP expression associated with NEB, which may affect IGF bioavailability and oviduct function.

RMIS No. 5234

McCarty, S.D.¹, Morris, D.G., Diskin, M.G., Kenny, D.¹, Murphy, J.², Wathes, C.³, Sreenan, J.M. and Fitzpatrick, R.

¹UCD School of Agriculture, Food Science and Veterinary Medicine, Belfield, Dublin

²Teagasc Moorepark, Co. Cork

³Royal Veterinary College, London UK

Repeatability of embryo survival beef heifers

Work is continuing on this project to identify animals of “low” and “high” fertility. To date there is no evidence to suggest that follicle and endocrine variables are different in heifers categorised as low or high fertility. A total of 10 “low” and 14 “high” fertility heifers have been slaughtered on either days 7 or 14 of their oestrous cycle. Uterine endometrial, hepatic and muscle samples have been harvested for gene expression studies.

Endometrial tissue was collected from animals of high and low fertility (day 7 of the oestrous cycle) and frozen at -80°C . Total RNA was isolated from fragmented frozen endometrial tissue ($n=6$) using trizol reagent and chloroform and subsequently precipitated using isopropanol. The quantity and quality of the RNA was determined by spectrophotometric analysis and using the Agilent 2100 Bioanalyser (Figure 29). RNA was purified using the RNeasy[®] mini kit (Qiagen Ltd, Crawley, UK).

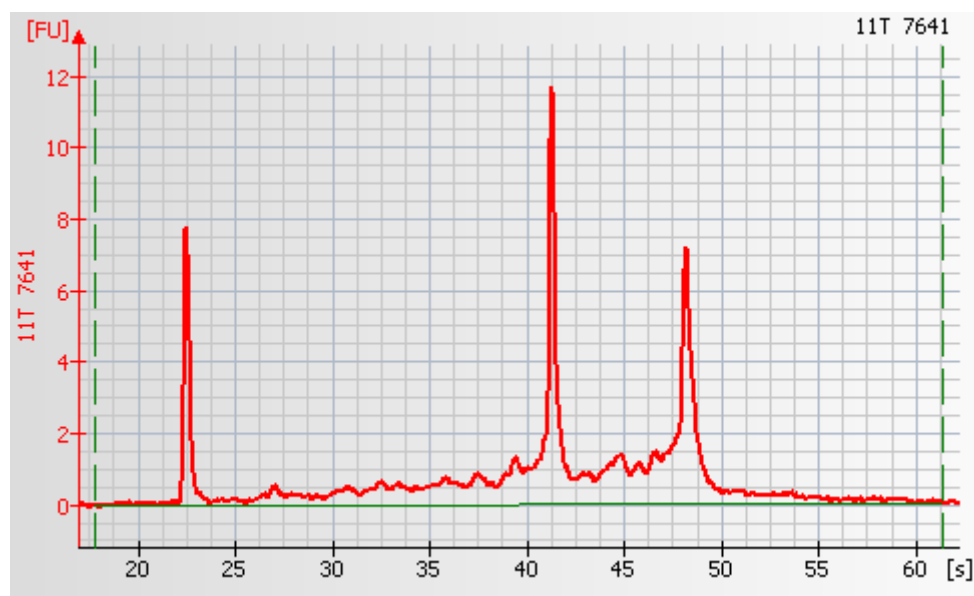


Figure 29. An example of electrophoretogram of endometrial total RNA from animals of high and low fertility using the Agilent 2100 Bioanalyser.

Diskin, M.G., Fitzpatrick, R., Waters, S. and Morris, D.G.

RMIS No. 5235

Identification of proteins that regulate uterine function in the cow insulin-like growth factor binding protein (IGFBP) expression in the bovine uterus throughout the oestrous cycle

Insulin-like growth factor I and II (IGFs) produced primarily by the liver but also by many other tissues exert their metabolic and proliferative effects by binding to specific cell surface receptors. Receptors for the IGF's are present on embryos and on the endometrium of the bovine uterus where the IGFs are thought to have important autocrine and paracrine functions with regard to embryo growth and development during pre-implantation and pregnancy. The bio-availability of the IGFs is directly regulated by a family of six IGFBPs which indirectly regulate the biological activity of the IGFs by modulating their interaction with receptors. Uterine IGFBPs are known to be regulated by progesterone in the human and previous studies have shown a positive linear and quadratic association between early embryo survival and the concentration of systemic progesterone in dairy cows. Furthermore, uterine gene expression is sensitive to small changes in progesterone on day 7 of the oestrous cycle. However, there is no information available on the pattern of IGFBP protein expression in the bovine uterus during the oestrous cycle or how this is modulated by the systemic concentration of progesterone. So the objectives of this study were firstly, to characterise the expression of IGFBPs in the bovine uterus during the oestrous cycle and, secondly, to determine the relationship between the IGFBPs and systemic progesterone concentrations on day 7 of the oestrous cycle.

Spontaneously cycling, lactating Holstein-Friesian dairy cows at least 50 days *post-partum* were used. In experiment 1, uterine flushings were collected non-surgically from the uterine horns, ipsilateral and contralateral to the *corpus luteum* on days 3, 7, 11 and 15 of the oestrous cycle (n=6 per day). In experiment 2, flushings were collected from cows on day 7 only (n=26). All flushings were centrifuged and stored at -80°C until analysis. Uterine flushings were analysed for IGFBPs in 30µg of protein by non-reducing SDS-PAGE followed by western ligand blotting onto a PVDF membrane using a semi-dry system. The blotted membrane was blocked with BSA and then probed with biotinylated IGF-II followed by extravidin-peroxidase. The IGFBPs were detected by chemiluminescence using the ECL-plus system. The blots were imaged and bands were quantified using a CCD camera system and software (Alpha Innotech). Approximate molecular weights of IGFBPs were extrapolated from molecular weight markers run on each gel. The concentration of each IGFBP was calculated from the integrated density values and a standard curve run with each gel. Total protein was determined using the Bradford assay (Bio-Rad). Data were analysed using a REML based mixed effects model using PROC MIXED in SAS (SAS, 2003). Pearson correlation coefficients between IGFBPs were determined using PROC CORR.

In experiment 1, western ligand blot analysis using the biotinylated IGF-II resulted in the detection of specific bands with molecular weights representing IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5 (Figure 30) in uterine fluid on days 3, 7, 11 and 15 of the oestrous cycle with IGFBP-3 representing 60-70 % of the total binding. IGFBP-2, IGFBP-3 and IGFBP-4 were detected in plasma across days 3, 7, 11 and 15 of the oestrous cycle using western ligand blotting. There was no significant effect of day or side on uterine IGFBP concentrations. There was, however, a significant day by side interaction with the concentration of IGFBPs on the ipsilateral side greater than that on the contralateral side for IGFBP-2 (P<0.05), 3 (P<0.01) and 5 (P<0.01) on day 15 (Figure 31). Total concentrations of IGFBPs were also higher on the ipsilateral side compared to the contralateral side on day 15 (P<0.01) and the concentration of all IGFBPs were positively correlated (P<0.05). Uterine and blood plasma IGFBP concentrations were compared and the concentration of IGFBP-2 and -3 were found to be higher in the uterus across all days of the cycle when compared to concentrations in plasma. In experiment 2, although a wide range of plasma progesterone concentrations were used, a significant relationship between the concentration of IGFBP-2, -3, -4 or -5 and the concentration of systemic progesterone was not found (P>0.05).

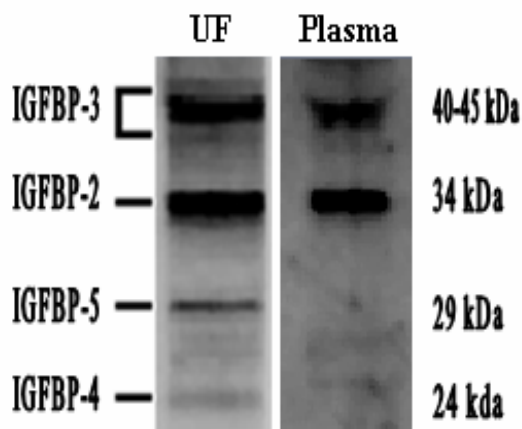


Figure 30. Western ligand blot of IGFBPs from uterine flushings (UF) and plasma following SDS-PAGE. Approximate molecular weights of IGFBPs are shown.

The results of this study show that the IGFBP-2, -3, -4 and -5 are expressed in the bovine uterus across days 3, 7, 11 and 15 of the oestrous cycle. However, there was no evidence of a significant relationship between the concentration of any of the IGFBPs and the concentration of systemic progesterone. A significant difference was, however, observed between the ipsilateral and contralateral uterine horns on IGFBP-2, -3 and -5 on day 15 of the oestrous cycle indicative of a local controlling mechanism on these binding proteins. This is consistent with possible modification of the uterine environment and modulation of uterine function, during a critical period of change in the development of the embryo. Furthermore, the marked differences in IGFBP concentrations between the uterus and plasma indicate synthesis or transportation of the proteins into the uterus, highlighting their importance in the pre-implantation uterine environment.

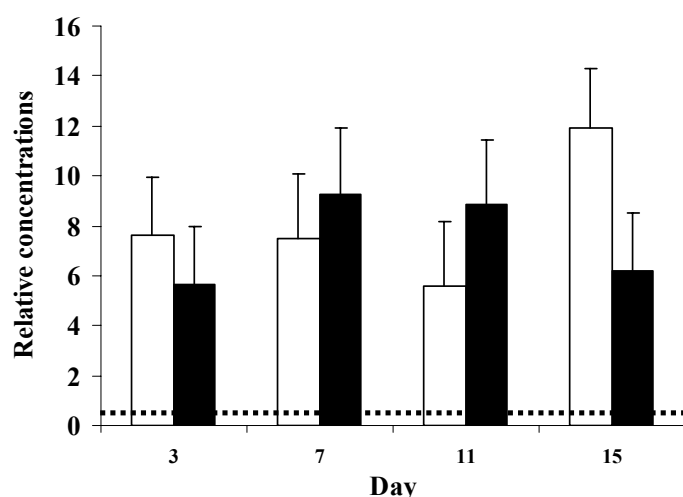


Figure 31. Relative uterine concentrations of IGFBP-3 (least square means \pm s.e.m) in the uterine horns ipsilateral and contralateral to the corpus luteum on days 3, 7, 11 and 15 of the oestrous cycle (open bars = ipsi; closed bars = contra). Dashed line indicates the corresponding mean plasma concentrations of IGFBP-3.

Morris, D.G., Costello, L.M.¹ and Diskin, M.G.

RMIS No. 5236

¹Department of Physiology, NUI Galway

Effect of dietary ω -3 polyunsaturated fatty acid supplementation on hormone and metabolite concentrations and corpus luteum size in beef heifers

Nutrition plays a fundamental role in reproduction. There is emerging evidence that dietary supplementation with ω -3 polyunsaturated fatty acids (PUFA) increases cow fertility although the mechanism(s) involved is unclear. It has been suggested that PUFA supplementation may affect (i) ovarian follicle size and number and consequently oestradiol (E_2) concentration, and, or (ii) corpus luteum (CL) size and progesterone (P_4) concentration. The objective of this study was to investigate the effect of dietary supplementation with the ω -3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on systemic E_2 , P_4 and cholesterol concentrations and CL size in heifers.

Heifers (n=40) were blocked on liveweight (479.35 ± 8.35 kg) and randomly assigned within block to one of four dietary treatments. All animals were individually fed a concentrate (5.33 kg dry matter (DM)) and straw (1.24 kg DM) based ration supplemented with one of four levels of an EPA/DHA supplement (EPA: DHA; 2: 1) to provide (1) 0g (Control - C); (2) 62g (T2); (3) 129g (T3) or (4) 273 g (T4) of dietary EPA plus DHA on a DM basis. All diets were isolipid and isonitrogenous (14% crude protein). Following an acclimatisation period, diets were offered for a 45 day experimental period. Animals were oestrous cycle- synchronised and blood samples were collected for P_4 and E_2 measurement on the day of oestrus and again on days 4, 7, 10, 14 and 16. CL size was measured on day 7 post-oestrus. PUFA and cholesterol concentrations were measured on day 16. Fatty acid methyl esters (FAME) were separated by gas chromatography. P_4 and E_2 were measured by radioimmunoassay. CL diameter was measured during ultrasonography. Data were analysed using repeated measures ANOVA with treatment and day and treatment x day interaction as variables in the model (SAS v.9.1, 2006).

The effects of diet on plasma EPA, DHA and cholesterol concentrations are shown in Table 66. There was a positive linear effect of dietary PUFA on plasma EPA ($P < 0.0001$) and both positive linear ($P < 0.01$) and quadratic ($P < 0.05$) components to the effect on plasma DHA. Plasma cholesterol was similar for C, T2 and T3, and higher for T4 compared with either C or T2. There was no treatment x sample day interaction for plasma E_2 , and no effect of treatment ($P < 0.05$) on E_2 on any of the sample days. The effect of dietary PUFA on CL size is also shown in Table 66. CL diameter was greater ($P < 0.05$) on T3 and T4 compared with either C or T2. The effect of dietary PUFA on plasma P_4 is shown in Figure 32. On day 14 progesterone concentration was higher on T4 than on C and T2 ($P > 0.01$) but did not differ between other treatment comparisons ($P > 0.05$). There was no relationship between plasma P_4 and CL size on day 7 ($P > 0.05$), however, there was a tendency towards a positive relationship between CL size on day 7 and plasma progesterone concentrations on day 14 ($P = 0.06$).

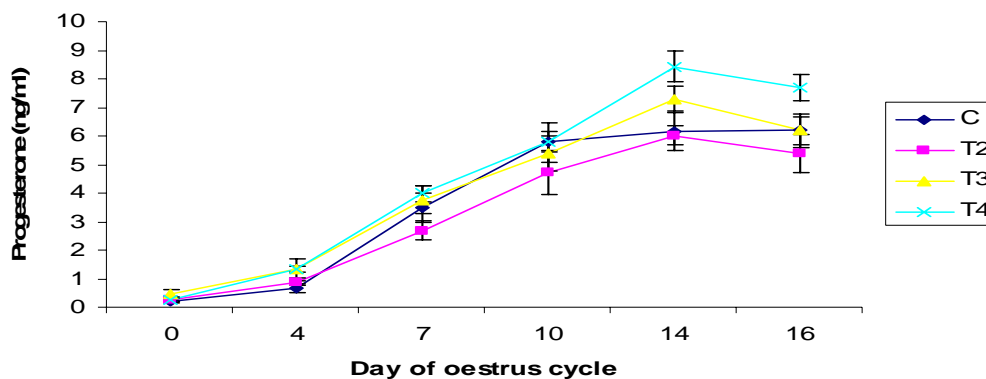


Figure 32. Effect of dietary treatment and day of oestrous cycle on systemic concentrations of progesterone.

Table 66: Effect of dietary PUFA treatment on CL diameter on day 7 and on plasma PUFA and cholesterol concentrations on day 16 (mean \pm SEM)

Treatment	C	T2	T3	T4
CL size (mm)	17.49 \pm 1.17 ^a	19.81 \pm 1.17 ^{a,b}	25.67 \pm 1.10 ^c	23.41 \pm 1.05 ^{b,c}
Plasma EPA (g/100g FAME)	4.43 \pm 0.89 ^a	10.56 \pm 0.97 ^b	16.97 \pm 0.97 ^c	24.10 \pm 0.97 ^d
Plasma DHA (g/100g FAME)	1.05 \pm 0.13 ^a	1.76 \pm 0.14 ^b	1.84 \pm 0.14 ^b	1.91 \pm 0.14 ^b
Plasma cholesterol (mmol/l)	4.63 \pm 0.24 ^a	4.32 \pm 0.38 ^a	5.39 \pm 0.40 ^{a, b}	6.14 \pm 0.39 ^b

^{a, b, c}Within row values are different (P < 0.05).

Increasing dietary ω -3 PUFA elevated plasma concentrations of both EPA and DHA. There was some evidence of an effect of ω -3 PUFA supplementation on P₄ concentrations on day 14, the critical time for maternal recognition of pregnancy. This increase may be mediated through increases in substrate concentrations and/or CL size.

RMIS No. 5358

Childs, S.¹, Sreenan, J. M., Stanton, C., Hennessy, A. A.², Kenny, D. A.¹ and Diskin, M. G.

¹UCD School of Agriculture, Food Science and Veterinary Medicine, Belfield, Dublin

²School of Biotechnology, Dublin City University, Dublin

Relationships between feed, rumen fluid, blood plasma, follicular fluid and endometrial tissue concentrations of the omega-3 (ω -3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in cattle

The essential ω -3 polyunsaturated fatty acids (PUFA) play critical roles in biological systems, including reproduction. However, long-chain PUFA undergo extensive biohydrogenation in the rumen resulting in poor transfer from diet to tissue in cattle. Clearly, it is essential not only to protect PUFA sources in the rumen but to also establish transfer efficiencies for target tissues. Follicular fluid, the environment of the developing oocyte and uterine endometrium, the environment of the developing embryo and the site of synthesis and secretion of prostaglandin F₂ α , the hormone of luteolysis, are the tissues of greatest interest in the context of improving cow fertility. The objective of this study was to examine the effect of feeding a partially rumen-protected source of EPA/DHA on the incorporation of EPA and DHA into blood plasma, rumen fluid, follicular fluid and the endometrium of heifers in order to develop a feeding model for reproduction studies.

Heifers (n=40) heifers were blocked on liveweight (479.35 \pm 8.35 kg) and randomly assigned within block to one of four dietary treatments. All animals were individually fed a concentrate (5.33 kg dry matter (DM)) and straw (1.24 kg DM) based ration and were supplemented with one of four levels of an EPA/DHA supplement (EPA: DHA; 2: 1) to provide (1) 0g (Control - C); (2) 62g (T2); (3) 129g (T3) or (4) 273 g (T4) of dietary EPA plus DHA on a DM basis. All diets were isolipid and isonitrogenous (14% crude protein). Diets were fed for 45 days at the end of which all the heifers were slaughtered. Jugular venous blood samples for plasma (PL) were collected on the day prior to slaughter for PUFA analysis. Immediately following slaughter, rumen fluid (RF), follicular fluid (FF) and uterine endometrial tissue (ET) samples were collected, flash frozen in liquid nitrogen and stored at -80°C until analysed for fatty acid concentration. Fatty acid methyl esters (FAME) were separated by gas chromatography. Data were analysed using ANOVA (SAS v.9.1, 2006) with linear and stepwise multiple regression procedures used as appropriate.

The effects of dietary PUFA treatment on the EPA and DHA fatty acid methyl ester (FAME) concentrations of RF, PL, FF and uterine ET are shown in Figures 33 and 33, respectively.

There was no effect of treatment ($P < 0.05$) on EPA concentrations in RF. There was a positive linear effect of treatment on concentrations of EPA in PL ($P < 0.0001$), FF ($P < 0.05$) and ET ($P < 0.0001$). The effect of treatment on DHA concentrations in RF was linear ($P < 0.01$) with a tendency towards a quadratic component ($P = 0.07$). There were both positive linear ($P < 0.01$) and quadratic ($P < 0.05$) components to the effect of treatment on PL concentrations of DHA. There were linear ($P < 0.01$) and quadratic ($P < 0.01$) effects of dietary PUFA on FF concentrations of DHA while the effect on ET concentration was both positive and linear ($P < 0.05$). Blood plasma concentrations of EPA ($r^2 = 0.69$) and DHA ($r^2 = 0.28$) were the best predictors of their respective concentrations in uterine endometrium.

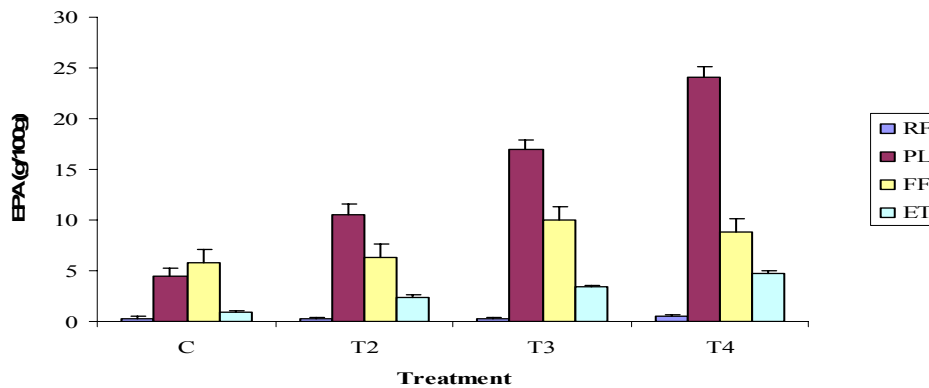


Figure 33. Effect of dietary PUFA treatment on the EPA concentrations of rumen fluid, blood plasma, follicular fluid and endometrial tissue.

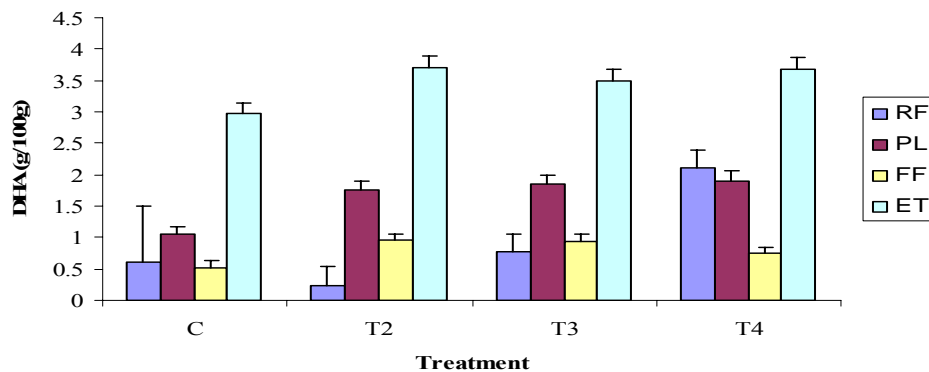


Figure 34. Effect of dietary PUFA treatment on the DHA concentrations of rumen fluid, blood plasma, follicular fluid and endometrial tissue.

Tissue concentrations of ω -3 PUFA can be manipulated through dietary supplementation and blood plasma concentration may be a useful predictor of concentrations of PUFA in some key reproductive tissues.

RMIS No. 5358

Diskin, M.G., Childs, S.¹, Sreenan, J.M., Stanton, C., Hennessy, A. A.² and Kenny, D. A.¹

¹UCD School of Agriculture, Food Science and Veterinary Medicine, Belfield, Dublin

²School of Biotechnology, Dublin City University, Dublin

Omega-3 fatty acid intake and immune response indicators in cattle

Recent non-ruminant studies suggest consumption of polyunsaturated fatty acids (PUFA) of the omega-3 (ω -3) series can alter immune response. There is a lack of information on ruminants, but one recent study in grazing beef calves suggested that dietary fishoil acted as an immune stimulant. The current study was carried out to examine the effect of level of dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on haematological variables as potential indicators of immune function in cattle.

Athenry Research Centre

Heifers (n=31) blocked on weight and body condition score (BCS) were randomly allocated to one of four isolipid and isonitrogenous (14% CP) diets consisting of a concentrate (6 kg DM) and straw (1.5 kg DM) ration, supplemented to provide 0g (n=6); 59g (n=6); 118g (n=8) or 236 g (n=11), of a combined EPA/DHA supplement on a DM basis. Haematology variables including haemoglobin concentration (HGB); red blood cell count (RBC); hematocrit (HCT); mean corpuscular volume (MCV); mean corpuscular haemoglobin (MCH) and red blood cell distribution width (RDW) along with white blood cell count (WBC), granulocyte (GR), monocyte (MO) or lymphocyte (LY) number were measured in blood samples taken 34 days prior to and again 30 and 43 days post allocation to diets. Interferon gamma (IFN γ) output was measured following stimulation of blood lymphocytes *in vitro* with Concavalin-A (ConA) or Phytohaemoagglutinin-A (PHA). Normally distributed data were analysed using repeated measures ANOVA (PROC MIXED, SAS, v. 8.2) and non-parametric variables were analysed using the Kruskal-Wallis test following a rank transformation.

Diet did not affect HGB, RBC, HCT, MCV, MCH or RDW though most were affected by sampling day. Neither diet nor day of sampling affected platelet numbers or baseline WBC, GR, MO or LY. These latter variables were higher in the EPA/DHA supplemented animals, but there was no effect of level of supplementation. There was no effect of supplement on IFN γ output in mitogen-stimulated lymphocytes. Supplementation with EPA and DHA apparently does not affect immunologically related blood variables in heifers.

RMIS No. 5358

Diskin, M.G., Childs, S.¹, Earley, B., Twigge, J.², Kenny, D. A.¹ and Sreenan, J. M.

¹UCD School of Agriculture, Food Science and Veterinary Medicine, Belfield, Dublin

²Nutreco Ruminant Research Centre, Boxmeer, Netherlands

Uterine gene expression in cows of high and low fertility

Cow conception rate is a major component of reproductive and production efficiency in beef and dairy herds. There is evidence of repeatable differences between cows in conception rate and of genetic variability for conception rate. Furthermore, it has been shown that conception rate or embryo survival rate is critically dependent on an optimum uterine environment prior to implantation. The critical period is the first two weeks after fertilisation when the embryo is totally dependent on the uterine fluid for its nutrition. The objective of this study is to (i) establish the uterine gene expression basis for difference in embryo survival rate, and (ii) to produce data that may help identify genetic markers for embryo survival rate/conception rate in cattle which may be exploitable at industry level. The new knowledge generated will enhance our understanding of cow fertility and will quantify the extent of gene expression differences existing for an important trait like uterine function.

Endometrial tissue was collected from animals of high and low fertility (day 7 of the oestrous cycle) and frozen at -80°C. Total RNA was isolated from fragmented frozen endometrial tissue (n=6). High quality RNA was subjected to microarray analysis. RZPD's gene expression analysis service was employed to hybridise the RNA samples to the Affymetrix 23K GeneChip® Bovine Genome arrays. Biotin labelled endometrial RNA from heifers of high or low fertility was hybridised to the arrays. The hybridisation process was successful. Complementary bioinformatics will be performed to identify the differential gene expression profiles in cows of high and low fertility.

Waters, S., Diskin, M.G. and Morris, D.G.

RMIS No. 5517

An examination of the molecular mechanisms controlling the tissue accumulation of conjugated linoleic acid (CLA) in cattle

Supplementation of cattle diets with a blend of oils rich in ω -3 PUFA and linoleic acid can have a synergistic effect on the accumulation of ruminal and tissue concentrations of vaccenic acid (VA), the main substrate for Δ -9 desaturase catalysed *de novo* tissue synthesis of the cis 9, trans 11 isomer of conjugated linoleic acid (CLA). The objective of this study was to employ real time RT-PCR technology to investigate the influence of dietary level and duration of feeding of ω -3 PUFA on Δ -9 desaturase gene expression in muscle of beef cattle.

Forty-eight continental cross young beef bulls were blocked according to liveweight and randomly allocated within block to one of four isolipid and isonitrogenous diets contained 6% soyaoil, in combination with either 0 (CON), 1 (FO1), or 2 (FO2) per cent of a high ω -3 fishoil product. Animals on CON, FO1 and FO2 were fed for 100 days prior to slaughter while the fourth treatment group (FO2(50)) were offered FO2 for 50 days prior to slaughter. Palmitic acid was added to each diet as appropriate to give 8% added lipid in the dry matter. Samples of *M. longissimus dorsi* were harvested within 30 min. *post mortem* and stored at -80°C. Muscle concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), VA and CLA were measured using gas chromatography. Total RNA was isolated from fragmented frozen muscle tissue using TRIzol reagent. RNA quantity was determined by absorbance at 260 nm. RNA quality was assessed using the Agilent Bioanalyzer 2100. RNA was reverse transcribed to generate cDNA. Quantitative real time RT-PCR reactions were performed to measure the relative expression of Δ -9 desaturase. β -actin was used as a reference gene following evaluation of a number of 'housekeeping' genes using the 'GeneNorm' software package. All amplified PCR products were sequenced to verify their identity. The $2^{-\Delta\Delta CT}$ method was used to determine fold change in relative gene expression between control and each of FO1, FO2 and FO50, respectively. In addition, to analyse the effect of level and duration of feeding ω -3 PUFA, the relative gene expression of FO1 and FO2 were compared as were FO2 and FO2(50), respectively. Data were analysed using mixed models methodology (PROC MIXED, SAS 2001) and fat concentration of the muscle was used as a covariate in the analysis. Univariate and stepwise multiple regression analyses was performed to establish relationships between tissue fatty acid concentrations and relative gene expression of Δ -9 desaturase using PROC REG and PROC STEPWISE (SAS v 9.1).

Muscle concentrations of eicosopentanoic acid (EPA), docosohexanoic acid (DHA) and VA were higher ($P < 0.01$) in all fishoil supplemented groups compared with CON, though there was no effect ($P > 0.05$) of treatment on the concentration of cis 9, trans 11 CLA. Expression of mRNA for Δ -9 desaturase was decreased 2.6 ($P < 0.05$), 4.6 ($P < 0.01$) and 4.1 ($P < 0.05$) fold, respectively in FO1, FO2 and FO2(50) compared with CON (Table 67). Increasing the level of fishoil in the diet from 1% to 2%, resulted in a tendency towards a reduction in the expression of Δ -9 desaturase mRNA ($P=0.09$). Increasing the duration of supplementation by 50 days i.e. FO2 v FO2(50) did not affect the expression of mRNA for this enzyme ($P=0.24$). Regression analyses displayed a negative relationship between VA, EPA, DHA and DPA and Δ -9 desaturase gene expression ($P < 0.05$). There was a strong tendency towards a negative relationship between linolenic acid and Δ -9 desaturase mRNA abundance ($P = 0.07$). EPA and DHA alone accounted for most of the variation in Δ -9 desaturase gene expression following STEPWISE multiple regression analysis. There was no effect of tissue fat concentration on Δ -9 desaturase gene expression ($P=0.55$).

Table 67: Effect of level and duration of dietary ω -3 PUFA supplementation on Δ -9 desaturase mRNA relative gene expression in muscle

Contrasts	Fold change of Δ -9 desaturase gene expression	<i>P</i> value
FO1 v CON	2.6 Down	0.030
FO2 v CON	4.6 Down	0.001
FO2(50) v CON	4.1 Down	0.030
FO1 v FO2	2.0 Down	0.090
FO2 v FO2(50)	1.3 Down	0.240

The results of this study show, for the first time, that dietary ω -3 PUFA supplementation inhibits gene expression of Δ -9 desaturase in the muscle of beef cattle. It was established that even 50 days of supplementation was sufficient to down regulate Δ -9 desaturase gene expression. These findings have important implications for the development of dietary strategies to augment the concentrations of both CLA and ω -3 PUFA in beef.

Development of a bovine intermuscular adipocyte cell line

The simultaneous increase in tissue concentrations of the human health promoting molecules, ω -3 polyunsaturated fatty acids (PUFA) and conjugated linoleic acid (CLA) has been hampered by apparent negative effects of ω -3 PUFA on tissue CLA synthesis. The objective of the current study was to develop a bovine intermuscular adipocyte cell line which could provide a cost effective, reproducible method to investigate the biochemical and molecular mechanisms of tissue fatty acid synthesis and accretion in beef cattle.

Intermuscular adipose tissue was obtained from the *M. longissimus thoracis* in a licensed abattoir within 20 min. of slaughter. Adipose tissue was stored in phosphate buffered saline containing Penicillin (5000IU/ml) and Streptomycin (5mg/ml) on ice during transport to the laboratory. Adipocytes were isolated whereby adipose tissue was cut into approximately 1cm² pieces and treated with PBS containing glucose (1.5g/100ml), bovine serum albumin (0.1g/100ml) and collagenase (0.25g/100ml) for 1 hour at 37°C with agitation. Following centrifugation, the upper adipose layer was removed and transferred to 25cm² culture flasks. The flasks were filled with DMEM/F12 culture medium (1:1 mix) supplemented with FCS (10%). Culture flasks were inverted and cultured under standard culture conditions for five days to allow for attachment. On day 5 fresh culture medium was added (10ml/flask) and the flask placed into culture in an upright position. The ability of the tissue to accumulate lipid was examined using light microscopy. Culture medium was removed and cells fixed with paraformaldehyde (10%). Lipid droplets were stained using Sudan IV and nuclei counterstained with Haematoxylin. To validate the adipocyte cell line the expression of various markers of fat metabolism were identified by reverse transcriptase-PCR. Adipocytes were typsinised and cells collected by centrifugation at 1500 rpm for 15 mins. The supernatant was removed and the pellet was immersed and stored in RNA later at -20°C. Total RNA was isolated from adipocytes using TRIzol reagent and chloroform and subsequently precipitated using isopropanol. RNA quantity was determined by absorbance at 260 nm. RNA quality was assessed using the Agilent Bioanalyzer 2100. RNA was DNase-treated and reverse transcribed to generate cDNA. Primers were designed to amplify specific fragments (of approximately 200 bp) of the following genes which are markers of fatty acid metabolism including fatty acid synthase (FAS), fatty acid binding protein (FABP), aP2 and CD36 as well as the enzyme Δ -9 desaturase. RT-PCR was performed to determine their expression in the adipocyte cell line.

Isolated cells were shown to accumulate lipid in culture (Figure 35) over time. Lipid droplets were clearly seen after 10-12 days in culture.

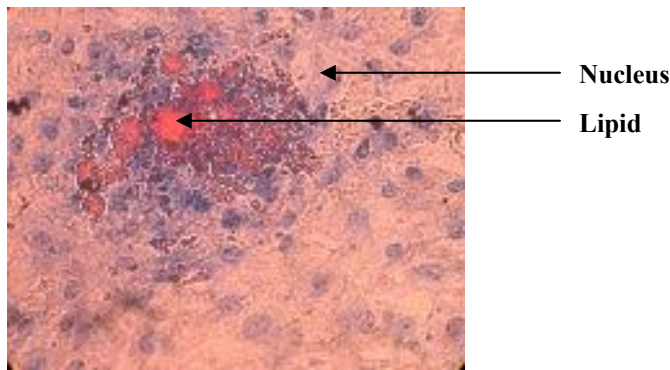


Figure 35. Isolated adipose cells. Fat droplets stained red with Sudan IV and nuclei counterstained with haematoxylin.

High quality total RNA was successfully isolated from adipocytes with 18S/28S ratios of 1.8. Markers of fat metabolism were shown to be expressed in the adipocyte cell line using reverse transcription-PCR (lanes 2-5, Figure 2). In addition, the Δ -9 desaturase was also confirmed (lane 6, Figure 36).

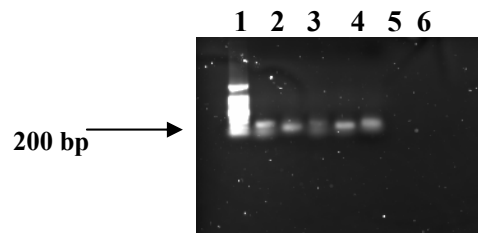


Figure 36. Reverse transcriptase PCR analysis of bovine adipocyte cells line of marker genes involved in fatty acid metabolism. Lane 1; 100 bp ladder, Lane 2; Fatty acid synthase, Lane 3; CD36, Lane 4; ap2, Lane 5; Fatty acid binding protein, Lane 6; Δ -9 desaturase.

A biologically functional bovine intermuscular adipocyte cell line has been developed and validated. The cell line was confirmed to display *in vivo* adipocyte characteristics as determined by both histological and molecular methods. This is an important resource for future studies examining the molecular regulation of lipogenesis in cattle and tissue accretion of the human health promoting molecules, ω -3 PUFA and CLA in beef.

Waters, S., Morris, D.G., Diskin, M.G. and Kenny, D. A.

RMIS No. 5541

Uterine gene expression of cows with high and low polyunsaturated fatty acid concentrations

The modern high-producing dairy cow is sub-fertile. Early embryo loss, within the first 16 days of gestation, is the greatest contributor to reproductive wastage, though the causes for this are not well understood. Nutrition plays a fundamental role in reproduction and specifically there is emerging evidence that supplemental polyunsaturated fatty acids (PUFA) can increase cow fertility. The objective of this study was to determine the effect of dietary

Athenry Research Centre

supplementation of ω -3 PUFA on the expression of mRNA for key uterine endometrial genes involved in prostaglandin biosynthesis.

Reproductively normal crossbred beef heifers (n=48) were individually fed a straw and barley/beet pulp based concentrate and supplemented with a rumen protected source of either saturated fatty acid (palmitic acid; low PUFA) or high ω -3 PUFA (High PUFA 273g dry matter) per head per day for 45 days. Both diets were isolipid and isonitrogenous. Following slaughter, uterine endometrial tissue from both groups was harvested and stored at -80°C . Analysis of fatty acid methyl esters in endometrial tissue was carried out using gas chromatography. Based on endometrial fatty acid concentration, total RNA was isolated, using TRIzol reagent, from fragmented frozen endometrial tissue of animals having either high (n=7) or low (n=7) EPA and DHA concentrations. RNA quantity was determined spectrophotometrically. RNA quality was assessed using the Agilent Bioanalyzer 2100. RNA was reverse transcribed to generate cDNA. Primers were designed to amplify specific fragments of the following 10 genes known to be involved in the prostaglandin biosynthetic pathway, phospholipase C, phospholipase A₂, cyclooxygenase I and II, prostaglandin E synthase, prostaglandin F synthase, 15-hydroxy prostaglandin dehydrogenase and peroxisome proliferator activated receptors α , δ , and γ . Quantitative real time RT-PCR reactions were performed to measure the relative expression of these genes. Ubiquitin was used as a reference gene following evaluation of a number of 'housekeeping' genes. All amplified PCR products were sequenced to verify their identity. Gene expression results were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. Statistical significance of differences between the groups was tested using PROC GLM, SAS.

Combined EPA and DHA concentrations in endometrial tissue were more than two-fold higher in the high compared to low group ($P < 0.05$), while EPA concentrations alone were more than four-fold higher in the high group ($P < 0.01$) (data not shown). Concentrations of arachidonic acid, the main substrate for $\text{PGF}_{2\alpha}$ synthesis, were lower in tissues from the high group (0.296 mg g^{-1} versus 0.188 mg g^{-1} ; $P < 0.001$). Expression of mRNA for prostaglandin E synthase (PGES) and the peroxisome proliferator activated receptors, PPAR α and δ was higher in animals fed the high compared with low PUFA diet ($P < 0.05$) (Figure 37). Mean phospholipase A₂ (PLA₂) gene expression tended to decrease by 2.1 fold in animals fed the high PUFA diet, ($P = 0.08$). The other 6 candidate genes were not differentially expressed in animals fed the high PUFA diet.

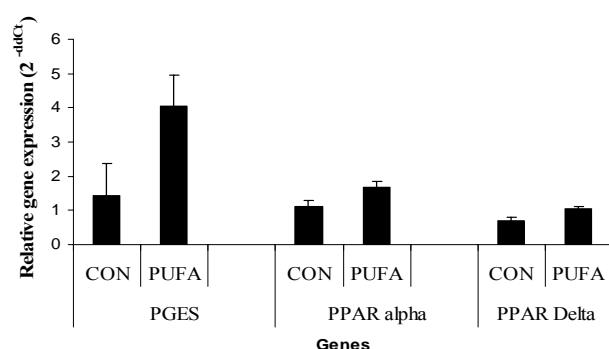


Figure 37. Effect of dietary supplementation of ω -3 PUFA on mRNA expression of endometrial genes coding for proteins involved in $\text{PGF}_{2\alpha}$ biosynthesis.

Changes in the expression of key uterine endometrial genes in response to ω -3 PUFA supplementation suggests a possible mechanism by which PUFAs may influence uterine function and in turn embryo survival in cattle.

Total RNA was isolated from fragmented frozen endometrial tissue (n=7) from animals with high and low systemic and endometrial ω -3 PUFA concentrations were subjected to microarray analysis. RZPD's gene expression analysis service was employed to hybridise the RNA samples to the Affymetrix 23K GeneChip® Bovine Genome arrays. Biotin labelled endometrial RNA from heifers with high or low endometrial PUFA concentrations was hybridised to the arrays. Complementary bioinformatic analysis of gene expression results was performed using R (Bioconductor). 584 genes were identified as being differentially regulated by PUFA supplementation in endometrial tissue. Validation of these genes is currently on-going.

Waters, S., Morris, D.G. and Diskin, M.G.

RMIS No. 5679

The association between circulating metabolic hormones during the early *post partum* period and subsequent fertility in dairy cows

Reproductive performance is a major factor affecting reproductive efficiency in cows. There is increasing evidence energy balance during the immediate *post partum* period has a significant effect on subsequent reproductive performance. The objective of this study was to determine the relationship between blood concentrations of insulin, IGF-I, beta hydroxyl butyrate, glucose, non esterified fatty acids and urea during the immediate *post partum* period and subsequent conception rate in dairy cows. A secondary objective was to determine the repeatability of these variables during the early postpartum period in dairy cows.

This study was carried out over a six month period January to September working in conjunction with 7 cooperating herds (herds A-G) providing a total of 371 spring calving dairy cows. Cows were blood sampled (coccygeal vessel) once weekly over a 4 week period (week 1-week 4) after calving. The mean calving date was 13th March. Prior to turnout 6 herds were fed grass silage supplemented with 4 – 7 kg of concentrates while the remaining herd was fed a total mixed ration containing 60% silage 40% maize, this was further supplemented with 6 kg of concentrates. Following turnout cows were supplemented with 2-4 kg concentrate herds across all herds. The mean turnout date for all herds was April 11th. The breeding period averaged 16 weeks. Cows that had a difficult calving, were lame or had suffered other diseases were removed from the trial. Body condition score was assessed on a scale of 0 – 5 in increments of 0.25 with 0 being emaciated and 5 being grossly over conditioned and were assessed by a single technician initially on week 1 and again on week 4.

Blood samples were collected via the coccygeal vein into lithium heparin vacutainers. The samples were collected into iced water, centrifuged at 2500 g for 15 minutes at 4°C and plasma was extracted and stored in sterile 7 ml vials at – 20°C until assayed. Milk samples were collected for progesterone three times weekly from Herd A during the evening milking commencing at day 15 *post partum* and continuing until day ???days *post partum*. Each sample was of the representative of the entire milking. Samples were collected in 30 ml universal plastic containers containing 1 Lactab Mark III preservative tablet which contains 30mg potassium dichromate and 20 mg salt. Samples were held in iced water and placed in storage at 4 °C until assayed for progesterone.

Milk concentration of progesterone was determined by enzyme immunoassay, using a commercial EIA. The intra- and inter-assay coefficients of variation for low, medium and high control samples were 15.4, 9.87 and 9.8%, and 10.9, 4.55 and 5.63%, respectively. The minimum detectable concentration of the assay was 0.385 ng/ml.

Athenry Research Centre

Insulin was analysed using a solid phase time resolved fluoroimmunoassay. The interassay and intraassay coefficients of variation were 3.46 and 8.04 %, respectively. Total plasma IGF-1 was measured in its using a non extracted two site immunoradiometric assay. The intra- and inter-assay coefficients of variation for low and high control samples were 4.57, 4.23% and 7.86, 5.31% respectively. Glucose, NEFA, BHBA and Urea concentrations were analysed using concentrations using appropriate kits and an ABX Mira auto analyser.

Cows were inseminated following an observed spontaneous oestrus. The outcome of each insemination was determined by transrectal ultrasonography at 30 to 50 post-insemination using an ALOKA SSD 500 V scanner with 7.5 MHz transducer. The relationship the independent variables (metabolite, metabolic hormone) and the depended variables conception rate to first service and for all service combined were evaluated using Logistic regression (Proc Logistic, SAS). Terms for herd were included in the model.

There was no association ($P>0.05$) between plasma concentrations of either urea, BHB or NEFA during weeks 1-4 *post partum* and conception rate to first service or conception rate for all service combined. Similarly, there was no association between means concentrations of each of these variables, or changes in these variables from week 1 to 4 *postpartum* and conception rate to first service or conception rate for all service combined ($P>0.05$). Significant associations were recorded between IGF-I and glucose and during weeks 1-4 *post partum and* conception rate to first service or conception rate for all service combined and these results are summarised in Tables 68-71.

Table 68: Relationship between IGF-1 during the early *post partum* period and conception rate to 1st service

Week	Odds Ratio	CI	P-value
Week 1	1.004	0.999-1.010	P= 0.14
Week 2	1.008	1.001-1.014	P= 0.02
Week 3	1.005	1.000-1.010	P= 0.05
Week 4	1.004	1.000-1.009	P= 0.06
Δ IGF-1	1.004	0.998-1.010	P= 0.22
Week 1-4			
Mean IGF-1	1.006	1.000-1.012	P= 0.05
Week 1-4			

Table 69: Relationship between IGF-1 during the early *post partum* period and conception to all services

Week	Odds Ratio	CI	P-value
Week 1	1.012	1.000-1.025	P= 0.04
Week 2	1.011	1.000-1.022	P= 0.06
Week 3	1.006	0.997-1.015	P= 0.17
Week 4	1.008	0.999-1.017	P= 0.09
Δ IGF-1	1.002	0.993-1.011	P= 0.62
Week 1-4			
Mean IGF-1	1.012	1.000-1.024	P= 0.04
Week 1-4			

Table 70: Relationship between glucose during the early *post partum* period and conception rate to 1st Service

Week	Odds Ratio	CI	P-value
Week 1	1.622	0.945-2.785	P= 0.07
Week 2	1.866	1.023-3.405	P= 0.04
Week 3	2.221	1.085-4.549	P= 0.03
Week 4	1.202	0.603-2.395	P= 0.60
Δ Glucose Week 1-4	0.673	0.396-1.144	P= 0.14
Mean Glucose Week 1-4	2.577	1.135-5.853	P= 0.02

Table 71: Relationship between glucose during the early *post partum* period and conception to all services

Week	Odds Ratio	CI	P-value
Week 1	1.297	0.562-2.995	P= 0.54
Week 2	1.44	0.606-3.425	P= 0.40
Week 3	3.025	1.114-8.208	P= 0.03
Week 4	3.79	1.353-10.638	P= 0.01
Δ Glucose Week 1-4	0.1926	0.915-4.054	P= 0.08
Mean Glucose Week 1-4	3.644	1.043-12.783	P= 0.04

There was no association ($P>0.05$) between plasma concentrations of insulin during weeks 1-4 *post partum* and conception rate to first service ($P>0.05$) but there was an association between plasma insulin and conception rate for all service combined (Table 72).

Table 72: Relationship between insulin during the early *post partum* period and conception rate to all services combined

Week	Odds Ratio	CI	P-value
Week 1	1.165	0.981-1.385	P= 0.08
Week 2	1.308	1.036-1.650	P= 0.02
Week 3	1.250	1.002-1.558	P= 0.04
Week 4	1.168	0.996-1.413	P= 0.11
Δ Insulin Week 1-4	0.996	0.889-1.115	P= 0.94
Mean Insulin Week 1-4	1.481	1.030-1.989	P= 0.01

Repeatability estimated for the 6 variables are presented in Table 73.

Table 73: Repeat ability estimates for different metabolites and metabolic hormones in dairy cows

Week	No Records	No Cows	Repeatability	Variance
IGF-1	4	368	0.63	0.031
Insulin	4	368	0.34	0.009
BHB	4	371	0.45	0.016
NEFA	4	371	0.38	0.011
Glucose	4	371	0.44	0.015
Urea	4	371	0.46	0.018

Athenry Research Centre

Most of the studies to date have examined the relationship between metabolic hormones and metabolites during the period concurrent with breeding and have generally found either no or weak relationship between them and conception rate. This study shows a significant association between plasma concentrations of IGF-I and glucose during the early *post partum* and conception rate to first service and conception rate for all service combined. Interestingly, there was no association between changes in plasma concentrations of IGF-I and conception rate to first service or conception rate for all service combined. This would suggest that metabolic events during the early *post partum* period have significant carry over effects on conception rate. This study also shows that IGF-I had a moderate to high repeatability and is therefore well conserved within animals.

Diskin, M.G., Lynch, C. and Kenny, D. A.¹

RMIS No. 5546

¹UCD School of Agriculture, Food Science and Veterinary Medicine, Belfield, Dublin

