

**Studies relating to Polyunsaturated Fatty Acid (PUFA) Supplementation and Fertility in Cattle**

**End of Project Report**

**Project 5358**

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## Summary

Reproductive inefficiency has a significant impact on the economic performance of both dairy and beef herds, particularly in seasonal calving systems. Nutrition plays a fundamental role in reproduction. Furthermore, there is emerging evidence that supplemental dietary polyunsaturated fatty acids (PUFA) may increase cow fertility independent of their role as energy substrates. A number of studies have reported enhanced reproductive performance in dairy cows following dietary supplementation with sources of n-3 PUFA. However the possible mechanisms involved have not been identified and there is some inconsistency in the published literature on this topic. The objective of the research reported was to conduct a holistic examination of the effects of dietary long-chain n-3 PUFA supplementation on metabolic and reproductive responses in cattle. Such information is essential for the appropriate formulation of diets to enhance cow reproductive performance and in particular embryo survival.

The first study carried out examined the effects of diets enriched with either n-3 or n-6 PUFA on systemic metabolite and hormone concentrations and ovarian function. Dietary fatty acid concentrations were manipulated through inclusion of either whole soyabeans to provide increased linoleic acid (n-6 PUFA) or fish oil as a source of n-3 PUFA. These feeding regimes increased plasma concentrations of n-6 and n-3 PUFA respectively compared with a non supplemented control. With the exception of an increase in plasma cholesterol following fat supplementation ( $P < 0.05$ ), there was no effect of diet on metabolite concentrations ( $P > 0.05$ ). Despite the increase in cholesterol, there was no effect of diet ( $P > 0.05$ ) on systemic concentrations of either  $P_4$  or  $E_2$ . Following an oxytocin challenge administered on days 15 and 16 of the oestrous cycle, n-6 PUFA intake was shown to increase ( $P < 0.05$ ) prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) measured as its more stable metabolite, 13, 14-dihydro-15-keto  $PGF_{2\alpha}$  (PGFM). However, there was no effect of n-3 PUFA supplementation on PGFM concentrations ( $P > 0.05$ ).

The second study examined the effect of incremental dietary supplementation with a concentrated source of n-3 PUFA on systemic and tissue fatty acid concentrations as well as on selected reproductive variables. Heifers were offered a straw and concentrate diet supplemented with one of four levels of a partially rumen protected n-3 PUFA enriched fish oil supplement in order to supply one of four levels of EPA and DHA combined *viz.* 0, 65, 140 or 275g. On days 15 and 16 of a synchronised oestrous cycle animals were challenged with oxytocin and PGFM response was measured. Animals were slaughtered on day 45 after the start of supplementation and endometrial tissue, rumen fluid and follicular fluid samples were collected for fatty acid analysis. Incremental addition of the n-3 PUFA resulted in increases in EPA and total n-3 PUFA in all tissues analysed. Increasing n-3 PUFA supplementation increased circulating cholesterol concentrations ( $P < 0.001$ ) and the diameter of the CL on day 7 post oestrus ( $P < 0.001$ ) but not on day 17/18, the day of slaughter ( $P > 0.05$ ). Overall, there was no effect ( $P > 0.05$ ) of diet on plasma  $P_4$  or  $E_2$  concentrations. Furthermore, there was no effect of n-3 PUFA supplementation on plasma concentrations of PGFM. Plasma concentrations of IGF-1 were increased ( $P < 0.05$ ) with increasing n-3 PUFA intake.

The third study examined the effect of dietary n-3 PUFA supplementation on embryo recovery rate and embryo quality in superovulated heifers. Animals were fed either a

low (control) or high n-3 PUFA diet. Non-surgical embryo recovery was carried out on day 7 post insemination. Recovered embryos were graded according to IETS guidelines. Blood samples were collected for the measurement of fatty acids, metabolites, insulin and IGF-1. mRNA expression for six genes involved in apoptosis, differentiation and implantation, and lipid metabolism was analysed. Supplementation with n-3 PUFA increased plasma n-3 PUFA concentration ( $P < 0.05$ ) and reduced n-6:n-3 PUFA ratio ( $P < 0.05$ ). The concentration of EPA and total n-3 PUFA was increased ( $P < 0.05$ ) and the concentration of arachidonic acid decreased ( $P < 0.05$ ) in uterine flushings following n-3 PUFA supplementation. Dietary n-3 PUFA did not affect plasma metabolites, insulin or IGF-1 ( $P > 0.05$ ). Similarly, there was no effect of treatment on superovulation rate, embryo recovery rate or embryo quality, ( $P > 0.05$ ) or on the mRNA expression of the genes examined ( $P > 0.05$ ).

Overall, the results of these studies show that strategic dietary supplementation with PUFA can alter the concentrations of these fatty acids in blood and in key reproductive tissues. Supplementation with n-6 PUFA increased systemic  $\text{PGF}_{2\alpha}$ , measured as PGFM, and could be beneficial for the promotion of earlier uterine involution in the immediate postpartum period and consequently for earlier resumption of oestrous cycles. Supplementation with n-3 PUFA increased systemic cholesterol and IGF-1 and corpus luteum size and any or all of these could, at least to some extent, mediate the positive effects on reproductive performance reported in previous studies. However, in contrast to the results of some previous studies, despite achieving significant increases in PUFA in blood and in key reproductive tissues we found no evidence of an effect of dietary n-3 PUFA supplementation on systemic  $\text{PGF}_{2\alpha}$  concentrations measured as PGFM. Furthermore, the results presented clearly suggest that any potential effect(s) of dietary n-3 PUFA on reproduction in cattle is not mediated through changes in embryo development, embryo gene expression or survival rate.

## **Introduction**

The Irish dairy industry is seasonal, and requires a production system that synchronises peak milk yields with peak spring grass supply. To achieve efficiency in such a system requires high cow fertility and a compact calving pattern. However, over the last two to three decades reproductive wastage has been the primary cause of involuntary culling in dairy herds (Kelly and Whitaker 2001) and is a significant limiting factor in the financial performance of herds (Esslemont *et al.* 2001). Furthermore EU regulations that attempt to limit greenhouse gas emissions (Garnsworthy 2004) and nitrate and phosphorus use means that the practice of carrying excess cow numbers to accommodate poor cow fertility is extremely inefficient. Clearly the objective must be to maximise individual cow as well as herd fertility.

The reasons for low cow fertility are probably multifactorial (Kelly and Whitaker 2001) and include cow related factors such as periparturient and production mediated diseases and management related factors associated with pre- and post-partum nutrition, oestrus detection and insemination technique. In isolation or combined, these factors can contribute to delays in the resumption of postpartum cyclicity and weaker expression of oestrus resulting in reduced detection rates. Furthermore, despite published estimates indicating that normal fertilisation rates in moderate yielding cows and heifers are approximately 90% (Sreenan *et al.* 2001), there is now

emerging evidence of a reduction in fertilisation rate in high yielding cows that is also contributing to reproductive wastage (Moore and Thatcher 2006). Notwithstanding this, the most significant contributory factor to reproductive wastage is embryo loss and in particular the losses sustained between day 8 and 16 post insemination (Sreenan *et al.* 2001).

The reason(s) for the high level of early embryo loss in cattle and particularly in high genetic merit, high yielding, dairy cows are poorly understood (Sreenan *et al.* 2001). Several factors have been implicated albeit with a lack of supporting experimental evidence (Diskin *et al.* 2006). These factors include genetic improvement, inadequate nutrition, poor reproductive management, increased incidence of disease, particularly in the periparturient period, and poor welfare conditions (Lucy 2001) though the relative importance of these factors is unclear. It is clear that these factors interact and there are varying contributions arising from specific individual farm management strategies (Roche 2006). However, it is clear that to achieve a good level of cow fertility, appropriate nutritional management of the cow is important (Hess *et al.* 2005, Roche 2006).

The abrupt shift in nutritional requirements after parturition (Butler 2001) results in dairy cows experiencing an intense energy deficit due to the onset of copious milk synthesis coupled with a depressed appetite (Block *et al.* 2001). The modern high producing dairy cow partitions a significant quantity of the nutrients available to it towards milk production and away from body stores and reproduction (Roche 2006). Maternal metabolism at this time is almost completely devoted to the support of mammary metabolism (Block *et al.* 2001). While a degree of adipose tissue mobilisation is normal in early lactation (Bauman and Currie 1980), the energy demands of lactation in the high producing cow create a severe negative energy balance (Tamminga 2006). Prolonged negative energy balance is thought to have significant deleterious effects on post partum ovarian activity and subsequent conception rates (O' Callaghan and Boland 1999). Clearly, achieving high dry matter intake in the early postpartum period is crucial to normal resumption of ovulation and subsequent fertility (Roche *et al.* 2000). The supply of extra dietary rumen protected fat has been suggested as a possible approach to improve fertility in dairy cows (Tamminga 2006). Supplemental fats increase the energy density of the diet and attempt to meet the energetic demands of lactation. It is the preferred method for increasing dietary energy density as the alternative; increasing starch content, is often associated with deleterious effects on digestion, health and milk composition (Staples *et al.* 1998). Fat supplementation may play a role in ameliorating the negative energy balance experienced by the early postpartum cow, and therefore in improving subsequent reproductive performance. However, it has been suggested that specific dietary fatty acids and in particular polyunsaturated fatty acids may favour reproductive processes independent of possible energy effects and these include increased availability of the fatty acid precursors of steroid and eicosanoid synthesis (Mattos *et al.* 2000).

A major obstacle to the effectiveness of PUFA supplementation in cattle is the extensive ruminal hydrogenation of dietary PUFA to saturated FAs. To achieve significant increases in systemic or tissue PUFA concentrations, it is suggested that unsaturated fatty acid sources be protected or rendered rumen inert. In addition,

excessive quantities of lipid in the rumen can have negative effects on digestion which may compromise any potential benefits associated with supplementation.

The objective of these studies was:

- (i) To determine the effect of dietary enrichment with either n-3 or n-6 fatty acids on systemic metabolite and hormone concentrations, and ovarian function in heifers.
- (ii) To determine the effect of level of dietary n-3 PUFA supplementation on systemic and tissue fatty acid concentrations, and on selected reproductive variables in cattle.
- (iii) To determine the effect of level of dietary n-3 PUFA supplementation on embryo yield and quality in cattle

### **Study 1: Effect of Dietary Enrichment with either n-3 or n-6 Fatty Acids on Systemic Metabolite and Hormone Concentration and Ovarian Function in Heifers**

#### **Objective**

The objective of the study was to examine the effects of feeding diets rich in either n-3 (eicosapentaenoic; EPA, and docosahexaenoic acids; DHA), or n-6 (linoleic acid) PUFA on concentrations of reproductive and metabolic hormones, metabolites, ovarian follicle dynamics and corpus luteum diameter in cattle.

#### **Materials and Methods**

##### *Animals*

Reproductively normal nulliparous crossbred beef heifers (n = 24) were oestrous synchronised using two injections (PG1 & PG2) of a prostaglandin F2 analogue. Following response to PG1 animals were randomly assigned to one of three concentrate and straw based diets (n= 8 per diet). The concentrates contained either (i) no added lipid (CON); (ii) 2% added fat as supplemental whole raw soyabeans (WSB) or (iii) 2% added fat as supplemental fish oil (FO). The WSB and FO diets were formulated to provide approximately 150g/head/day of soya oil and fish oil respectively. All diets were formulated to be isonitrogenous (14% CP in total diet DM). Diets were offered for 32 days.

##### *Blood sampling*

Blood samples were collected by jugular venipuncture at appropriate times and intervals for measurement of plasma concentrations of progesterone (P<sub>4</sub>), oestradiol (E<sub>2</sub>), 13, 14 dihydro, 15-keto prostaglandin F<sub>2</sub> $\alpha$  (PGFM), fatty acids, cholesterol, glucose, triglycerides, urea, non-esterified fatty acids (NEFA) and beta-hydroxybuterate (BHBA).

To determine the effect of dietary treatment on plasma PGFM, the animals were administered an oxytocin challenge on days 15 and 16 of the synchronised oestrous cycle. Blood samples were taken at -60 and again at 0 min to measure basal PGFM concentrations. A 10 ml dose of oxytocin (50i.u.) was administered intravenously

immediately after the 0 time point sample. Subsequent blood samples were collected again at 15, 30, 45, 60, 75 and 90 min after oxytocin administration.

#### *Follicular dynamics and corpus luteum diameter*

Follicular dynamics and the diameter of the pre-ovulatory follicle were determined for each heifer via transrectal ovarian ultrasonography (7.5-MHz transducer; Aloka SSD-500, Aloka Ltd., Tokyo, Japan) from day 7 of the oestrous cycle until ovulation. The size of the corpus luteum was measured on day 7 post oestrus.

#### *Statistical analyses*

Data were checked for adherence to a normal distribution (PROC UNIVARIATE, SAS v9.1, 2002). Continuous data were analysed using two-way ANOVA with terms included for diet and block. Variables having more than one observation per subject were analysed using repeated measures ANOVA (PROC MIXED, SAS v9.1, 2002) with terms for diet, time period and their interaction included in the model and animal within diet set as the error term. Significant differences were compared using Tukey's option. A probability of  $P < 0.05$  was considered significant.

## **Results**

#### *Plasma concentration of fatty acids*

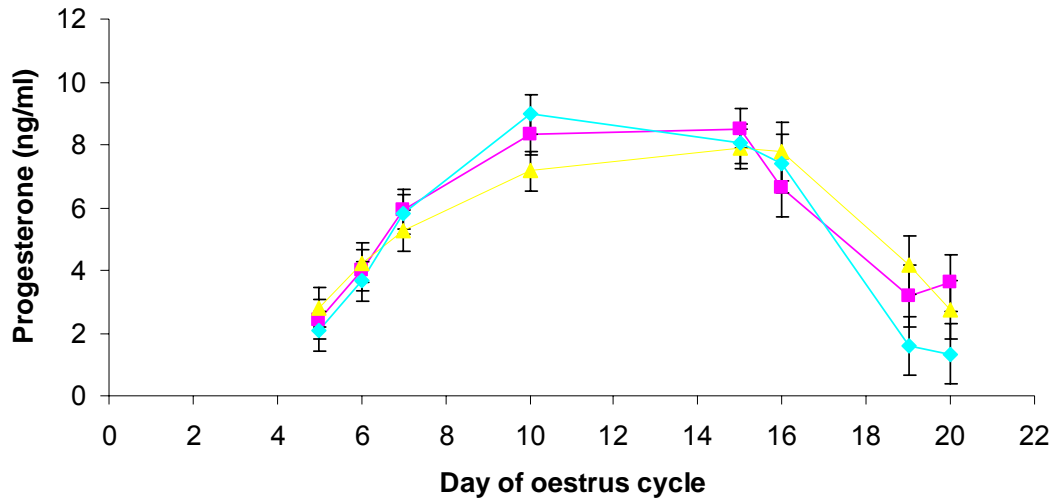
As expected, heifers fed the WSB diet had higher concentrations of the n-6 PUFA linoleic acid in comparison with the other two diets. Concentrations of the n-3 PUFA linolenic acid decreased with time on treatment in CON and WSB but increased in FO. Heifers fed the FO diet had higher ( $P < 0.0001$ ) concentrations of the n-3 PUFAs linolenic acid ( $P < 0.001$ ), EPA and DHA in comparison with the control diet, whereas eicosatrienoic acid was reduced in FO. The n-6 PUFAs arachidonic acid and t10, c12 CLA were both higher ( $P < 0.0001$ ) on the FO diet than on either control or WSB diets. There were diet x day of sampling interactions for linolenic acid and EPA where both FA increased with time on FO did not change ( $P > 0.05$ ) on either WSB or CON.

#### *Plasma metabolites*

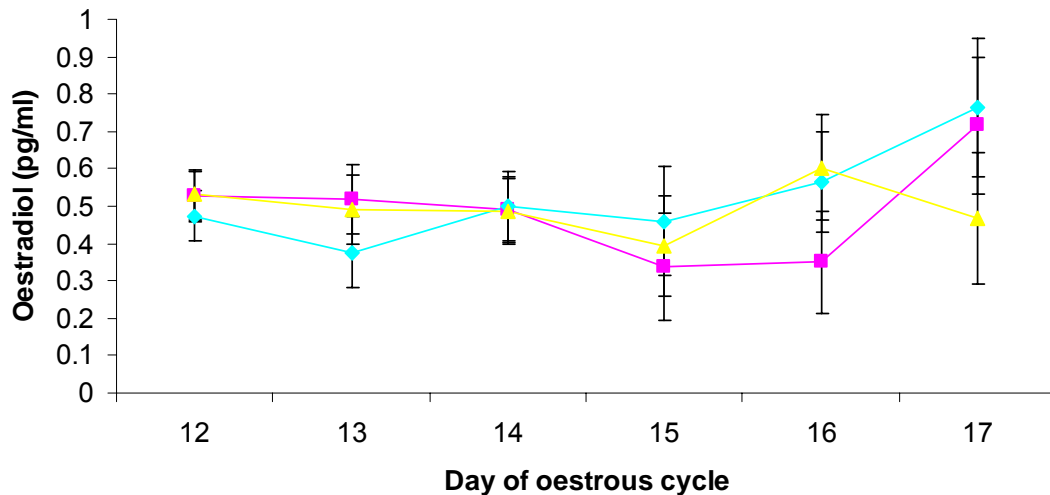
Plasma concentrations of triglycerides decreased with time on all diets whereas BHBA increased. Supplementation with PUFA increased cholesterol concentrations and also caused a time related change in NEFA: with increases with time on WSB ( $P < 0.05$ ) but no change in NEFA with time in either CON or FO ( $P > 0.05$ ). Heifers on WSB had the highest concentration of urea while plasma concentrations of glucose were not altered by diet ( $P > 0.05$ ).

#### *Plasma progesterone and oestradiol*

There was no diet x sample day interaction or effect of diet on plasma concentrations of either progesterone or oestradiol ( $P > 0.05$ ) on any day of the oestrous cycle recorded (Figures 1 and 2).



**Figure 1.** Plasma concentrations of progesterone (ng/ml) of heifers fed CON (■, n = 8), WSB (◆, n = 8) or FO (▲, n = 8) diets.

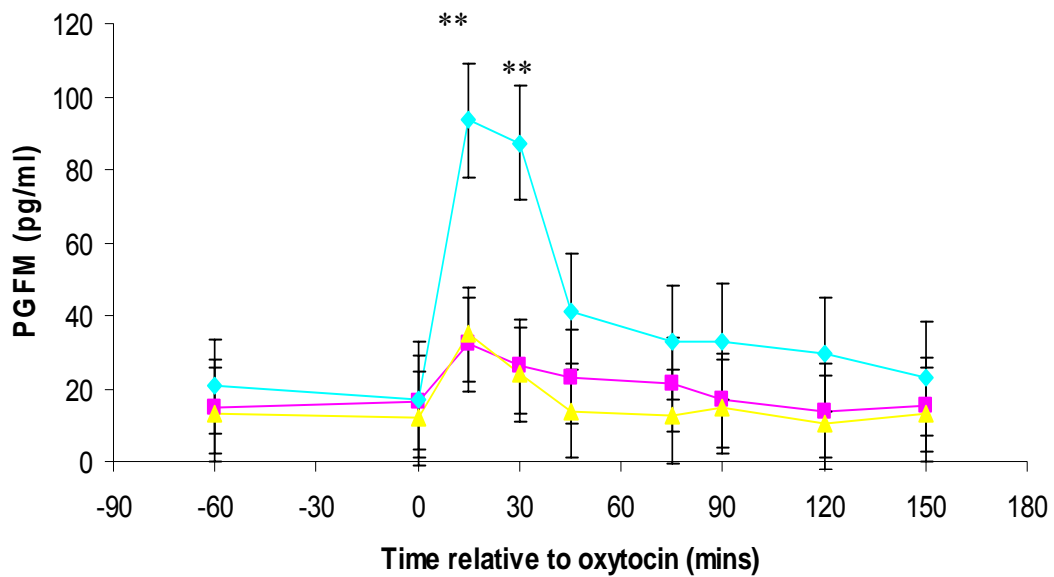


**Figure 2.** Plasma concentrations of oestradiol (pg/ml) of heifers fed CON (■, n = 8), WSB (◆, n = 8) or FO (▲, n = 8) diets.

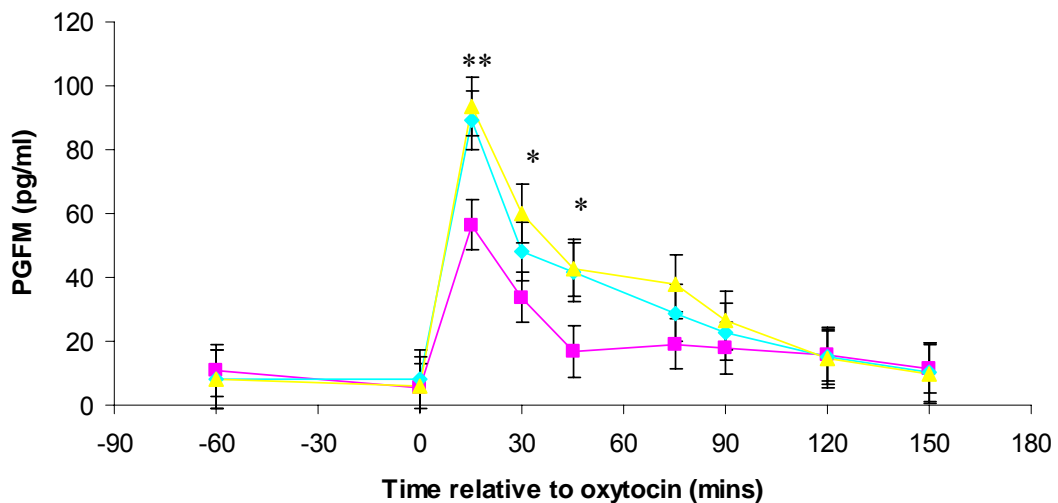
*Plasma 13, 14-dihydro-15-keto-prostaglandin F<sub>2α</sub>*

The effect of diet on plasma concentrations of 13, 14-dihydro-15-keto-prostaglandin F<sub>2α</sub> (PGFM) produced in response to an oxytocin challenge on days 15 and 16 of the oestrus cycle is shown in Figures 3 and 4. Although there were no ( $P > 0.05$ ) diet x day x time or diet x time interactions for concentrations of PGFM; there was, however, a diet x day of oestrus cycle interaction ( $P < 0.01$ ). On day 15, PGFM concentrations were higher ( $P < 0.01$ ) on WSB at 15 and 30 min post oxytocin compared with either CON or FO which were similar ( $P > 0.05$ ). On day 16, however, concentrations of PGFM at 15 min post oxytocin were similar ( $P > 0.05$ ) for FO and WSB, which were both higher ( $P < 0.01$ ) than CON while at 30 min post oxytocin concentrations for FO and WSB groups were again similar ( $P > 0.05$ ) but only FO was higher than CON ( $P < 0.05$ ). At 45 min post oxytocin on day 16, PGFM concentrations on FO and WSB were similar and both higher ( $P < 0.05$ ) than CON.





**Figure 3.** Plasma concentrations of prostaglandin  $F_{2\alpha}$  metabolite (PGFM) on day 15 of the oestrus cycle of heifers fed CON (■, n = 8), WSB (◆, n = 8) or FO (▲, n = 8) diets following correction for P4:E2 ratio (\*\*,  $P < 0.01$ ).



**Figure 4.** Plasma concentrations of prostaglandin  $F_{2\alpha}$  metabolite (PGFM) on day 16 of the oestrus cycle of heifers fed CON (■, n = 8), WSB (◆, n = 8) or FO (▲, n = 8) diets following correction for P4:E2 ratio (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).

*Oestrous cycle length, ovulatory follicle and corpus luteum diameter*

There was no effect of diet on the length of the oestrous cycle, or on the maximum diameter or growth rate of the pre-ovulatory follicle ( $P > 0.05$ ). The diameter of the CL measured on day 7 post oestrus was not affected by diet ( $P > 0.05$ ). Furthermore, there was no relationship between CL diameter measured on day 7 of the oestrous cycle and  $P_4$  concentrations on any day of the oestrous cycle ( $P > 0.05$ ).

## **Study 2: Effect of Level of Dietary n-3 Polyunsaturated Fatty Acid Supplementation on Systemic and Tissue Fatty Acid Concentrations and on Selected Reproductive Variables in Cattle**

### **Objective**

The objectives of this study were to investigate the effects of feeding a partially rumen protected, n-3 PUFA enriched fish oil supplement on uterine, ovarian, and metabolic responses of beef heifers, and to determine the relationship between feed and plasma concentrations of n-3 PUFA and their concentrations in some reproductive tissues.

### **Materials and Methods**

#### *Animals*

Reproductively normal nulliparous crossbred heifers (n = 40) were randomly assigned to one of four concentrate and straw based diets (n = 10 per diet). All animals were individually fed a barley straw (1.40 kg dry matter; DM), molasses (0.28 kg DM) and concentrate (5.50 kg DM) based ration, supplemented with one of four levels of a partially rumen protected n-3 PUFA enriched fish oil supplement (EPA: DHA; 1.5: 1) to provide (1) 0g (Control - **CON**); (2) 65g (**LOW**; 39g EPA, 26g DHA); (3) 140g (**MED**; 84g EPA, 56g DHA) or (4) 275g (**HIGH**; 165g EPA, 110g DHA) of EPA and DHA combined on a DM basis. Concentrations of fish oil in the total dry matter offered were 0, 1.04, 2.08 and 4.15% for the CON, LOW, MED and HIGH diets respectively. Rumen protection was achieved via encapsulation in a pH sensitive matrix which remains intact at rumen pH but breaks down at the lower pH in the abomasum releasing the constituents for absorption. All diets were formulated to be isonitrogenous (14% crude protein (CP) in total diet) and isolipid and were fed for a total of 45 days. Heifers were oestrous cycle synchronised using two injections of prostaglandin F<sub>2α</sub> analogue (PG), administered intramuscularly 11 days apart commencing on day 14 of the experimental period

#### *Blood sampling*

Blood samples were collected by jugular venipuncture at appropriate times and intervals for measurement of plasma concentrations of progesterone (P<sub>4</sub>), oestradiol (E<sub>2</sub>), insulin-like growth factor 1 (IGF-1), fatty acids, cholesterol, glucose, triglycerides, urea, non-esterified fatty acids (NEFA), and beta-hydroxybutyrate (BHBA).

#### *Oxytocin challenge*

To examine the effect of diet on plasma concentrations of 13, 14 dihydro, 15-keto prostaglandin F<sub>2α</sub> (PGFM), a metabolite of PGF<sub>2α</sub>, animals were administered oxytocin on days 15 and 16 of the oestrous cycle to stimulate uterine PGF<sub>2α</sub> synthesis and secretion. Jugular blood samples were collected -90 and again at -1 min relative to the administration of oxytocin in order to determine basal PGFM concentrations. A 10 ml dose of oxytocin (50 i.u.) was then administered intravenously (time point 0). Subsequent blood samples were collected at 15, 30, 45, 60, 90, 120, 150 and 180 min post oxytocin administration.

#### *Post-mortem sample collection*

Heifers were slaughtered on either day 45 or 46 of the experimental period which corresponded to either day 17 or 18 of the synchronised oestrous cycle. Endometrial tissue from the uterine horn ipsilateral to the corpus luteum was dissected from the myometrium. Follicular fluid was collected from the dominant follicle and rumen fluid samples were collected by filtering the fluid from a sample of the rumen contents.

#### *Corpus luteum diameter*

The diameter of the corpus luteum was determined on day 7 post oestrus and on the day of slaughter for each heifer. Measurements were carried out via transrectal ovarian ultrasonography using vernier callipers on day 7 post oestrus and on the day of slaughter respectively.

#### *Statistical analyses*

All data were checked for adherence to a normal distribution (PROC UNIVARIATE, SAS v9.1, 2002). Data were analysed using two-way ANOVA with terms included for diet and block. Variables having more than one observation per subject such were analysed using repeated measures ANOVA (PROC MIXED, SAS v9.1, 2002) with terms for diet, time period and their interaction included in the statistical model. Area under the curve for progesterone profiles was calculated for each treatment using the trapezoidal method. Significant differences were compared using Tukey's option. Linear and stepwise multiple regression procedures (PROC REG and PROC STEPWISE, SAS) were also used as appropriate. A probability of  $P < 0.05$  was considered significant while those  $< 0.10$  were considered to indicate a tendency towards statistical significance.

## **Results**

#### *Rumen fluid fatty acids*

There was no effect of diet on the concentrations of c9t11CLA ( $P > 0.05$ ). There were quadratic responses to n-3 PUFA supplementation for both linoleic ( $P < 0.001$ ) and to a lesser degree, linolenic acid ( $P = 0.01$ ). With the exception of the linear decline in stearic ( $P < 0.0001$ ) and the quadratic response in palmitic acid ( $P < 0.0001$ ) the concentrations of the other fatty acids generally increased following increasing intake of FO. There were linear increases in the concentrations of vaccenic acid, t10c12CLA, DPA and DHA ( $P < 0.0001$ ) and also myristic, oleic, eicosatrienoic and arachidonic acid, and EPA ( $P < 0.001$ ).

#### *Plasma concentration of fatty acids*

The linear increase ( $P < 0.001$ ) in total n-3 fatty acids and decrease ( $P < 0.0001$ ) in total n-6 were in keeping with the formulation objectives of the experiment. Furthermore, the n-3 PUFA EPA, DPA and DHA increased ( $P < 0.0001$ ) linearly with increasing dietary n-3 intake resulting in a linear reduction in the n-6: n-3 ratio ( $P < 0.0001$ ). There was no effect of diet ( $P > 0.05$ ) on the concentrations of myristic acid, c9t11CLA or linolenic acid (C18:3). Palmitic, vaccenic and linoleic acid concentrations decreased ( $P < 0.0001$ ) as the level of n-3 PUFA supplementation increased. There was a linear decline in concentrations of stearic acid ( $P < 0.001$ ) while concentrations of oleic, t10c12CLA and arachidonic acid increased linearly ( $P < 0.0001$ ) with increasing dietary n-3 PUFA supplementation.

#### *Uterine endometrial fatty acids*

The increases in the n-3 fatty acids EPA, DPA and DHA led to a linear increase in the concentration of total n-3 present in the tissue ( $P < 0.0001$ ). Similarly, the decline in the concentrations of linoleic acid and arachidonic acid led to a linear decline in total n-6 ( $P < 0.0001$ ) with a consequent decline in the ratio of n-6: n-3 PUFA ( $P < 0.0001$ ). There was no effect of diet on the endometrial concentrations of palmitic or stearic acid ( $P > 0.05$ ). The concentrations of myristic acid, vaccenic acid and c9t11CLA increased with increasing n-3 PUFA supplementation ( $P < 0.0001$ ). Concentrations of oleic acid ( $P < 0.05$ ) declined with increasing n-3 PUFA supplementation while the response of eicostrienoic acid (dihomo- $\gamma$ -linolenic acid) was quadratic ( $P < 0.01$ ).

#### *Follicular fluid fatty acids*

Increasing EPA ( $P < 0.0001$ ) and decreasing linoleic acid ( $P < 0.0001$ ) with increasing dietary n-3 PUFA led to an overall decrease in the n-6: n-3 PUFA ratio of follicular fluid. There was no clear effect of treatment on follicular fluid concentrations of myristic acid, palmitic acid, DPA or DHA. Similarly there was no effect of diet on the concentrations of stearic, eicostrienoic or arachidonic acid or t10c12CLA ( $P > 0.05$ ). There was a linear increase in the concentrations of vaccenic acid ( $P < 0.0001$ ) and c9t11 CLA ( $P < 0.05$ ) while oleic acid and linolenic acid ( $P < 0.05$ ) decreased with increasing n-3 PUFA supplementation.

#### *Relationship between plasma concentrations of PUFA and concentrations in endometrial tissue and follicular fluid*

Regression analysis was used to establish the relationship between concentrations of selected saturated fatty acids (SFA) and PUFA in plasma and their concentrations in endometrial tissue. The relationship between SFA concentrations in plasma and their concentration in either endometrial tissue or follicular fluid was weak. For most PUFA the relationship between their concentrations in plasma and both endometrium and follicular fluid was linear in direction. There were moderate to strong positive relationships between plasma concentrations of linoleic acid, EPA, total n-3 PUFA, total n-6 PUFA and the n-6: n-3 ratio and their concentrations in both endometrial tissue and follicular fluid.

#### *Plasma metabolites*

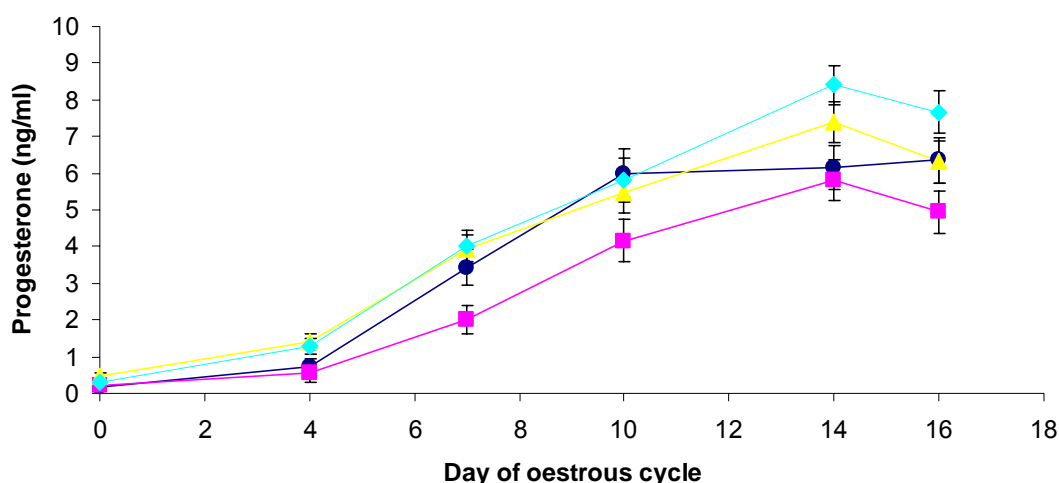
There was a linear increase in cholesterol in response to increasing n-3 PUFA supplementation. There was no effect of the supplementation regime on the concentrations of triglycerides, glucose or BHBA ( $P > 0.05$ ). Urea increased ( $P < 0.0005$ ) while NEFA decreased ( $P < 0.01$ ) linearly in response to increasing dietary n-3 PUFA intake.

#### *Plasma IGF-1*

The effect of diet on plasma IGF-1 concentrations is shown in Table 4.8. There were both linear ( $P < 0.01$ ) and quadratic ( $P < 0.05$ ) components to the effect of n-3 PUFA supplementation on the concentrations of IGF-1. On average IGF-1 was higher ( $P < 0.05$ ) in the n-3 PUFA supplemented versus non supplemented animals.

### Plasma progesterone

There was a treatment x day interaction for plasma concentration of progesterone ( $P < 0.05$ ). While there was no effect of dietary treatment ( $P > 0.05$ ) on the progesterone concentration on any day of the oestrous cycle measured, the overall mean concentration of progesterone, as measured by the area under the curve, was higher ( $P < 0.05$ ) on HIGH compared with LOW but there were no differences among other diet comparisons (Figure 5).



**Figure 5.** Plasma concentrations of progesterone (ng/ml) of heifers fed CON (●), LOW (■), MED (▲) and HIGH (◆) n-3 PUFA diets measured on days 0 (standing oestrus), 4, 7, 10, 14 and 16 of the oestrous cycle.

### CL diameter, cholesterol and plasma concentrations of progesterone

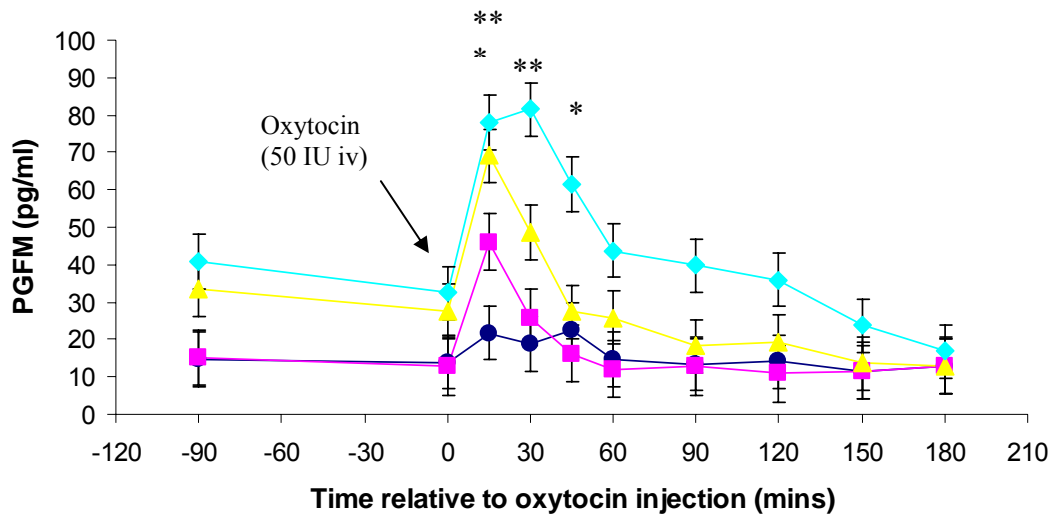
There was both a positive linear ( $P < 0.0001$ ) and a quadratic ( $P < 0.05$ ) response in CL diameter on day 7 to increasing dietary n-3 PUFA. The diameter of the CL increased up to the MED level of n-3 PUFA intake with no further increase in size ( $P > 0.05$ ) following the fourth dietary increment ( $17.5 \pm 1.17$ ;  $19.8 \pm 1.17$ ;  $25.7 \pm 1.17$  and  $24.1 \pm 1.05$  mm for CON, LOW, MED and HIGH respectively). CL diameter ( $25.2 \pm 3.48$ ;  $21.8 \pm 1.42$ ;  $24.3 \pm 1.42$  and  $23.2 \pm 1.42$  mm for CON, LOW, MED and HIGH respectively) on the day of slaughter did not differ between diets ( $P > 0.05$ ).

A regression analysis showed a positive but weak relationship between the diameter of the CL on day 7 and the concentrations of plasma progesterone measured on day 14 ( $R^2 = 0.10$ ;  $P = 0.06$ ). There was no relationship between CL diameter on day 7 and plasma progesterone concentrations on any of the other sample days ( $P > 0.05$ ). The diameter of the CL on day 7 of the oestrous cycle together with plasma concentrations of cholesterol on the day prior to slaughter (day 17) combined were the best predictors of variation in progesterone concentrations on day 14 ( $R^2 = 0.43$ ;  $P < 0.001$ ).

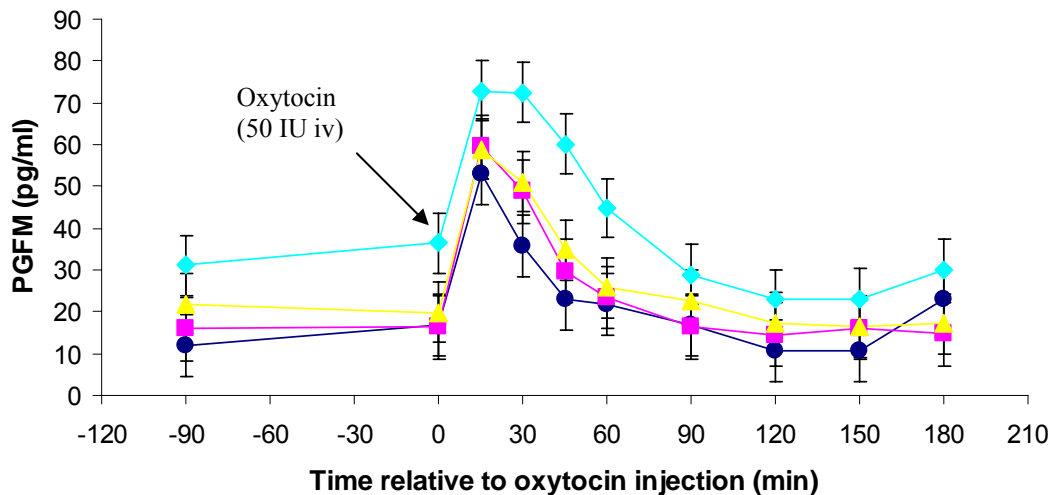
### Plasma 13, 14-dihydro-15keto-prostaglandin $F_{2\alpha}$

The effect of diet on the concentrations of 13, 14-dihydro-15-keto-prostaglandin (PGFM) produced in response to the oxytocin challenge on days 15 and 16 of the oestrous cycle is shown in Figures 6 and 7. The concentrations were affected by diet ( $P < 0.01$ ) and there was a diet x day interaction ( $P < 0.01$ ). On day 15, the

concentrations of PGFM on the HIGH diet were the greatest and were greater ( $P < 0.001$ ) at 15 min and 30 min post oxytocin compared with the CON group and greater than LOW group at 30 ( $P < 0.001$ ) and 45 min ( $P < 0.05$ ). The concentrations of PGFM for the MED diet were also greater than CON at 15 min post oxytocin administration ( $P < 0.05$ ). On day 16, while the pattern of secretion across treatments was similar to day 15, differences between diets were not statistically significant.



**Figure 6.** Plasma concentrations of prostaglandin  $F_{2\alpha}$  metabolite (PGFM) on day 15 of the oestrous cycle of heifers fed CON (●), LOW (■), MED (▲) and HIGH (◆) n-3 PUFA diets (\*\*  $P < 0.001$  - HIGH vs CON, HIGH vs LOW; \* $P < 0.05$  - HIGH vs CON, MED vs CON).



**Figure 7.** Plasma concentrations of prostaglandin  $F_{2\alpha}$  metabolite (PGFM) on day 16 of the oestrous cycle of heifers fed CON (●), LOW (■), MED (▲) and HIGH (◆) n-3 PUFA diets.

### **Study 3: Embryo Yield and Quality Following Dietary Supplementation of Beef Heifers with n-3 PUFA**

#### **Objective**

The objective of the study was to examine the effect of dietary n-3 PUFA supplementation of superovulated heifers on embryo yield and embryo quality based on a morphological assessment and relative gene transcript abundance.

#### **Materials and methods**

##### *Animals*

The oestrous cycles of reproductively normal crossbred beef heifers (n = 60) were synchronised using two injections of a 500µg prostaglandin F<sub>2α</sub> analogue (PG1 and PG2), administered intramuscularly 11 days apart. Following PG2, heifers in standing heat (n = 36) were randomly assigned to one of two diets (n = 18 per diet). All animals were individually fed a barley straw (1.40 kg dry matter, DM) and concentrate (5.50 kg DM) based ration, supplemented with either palmitic acid (Palmit 80<sup>®</sup>; saturated FA) as a control (**CON**) or with 334 g DM of a high, partially rumen protected, n-3 PUFA supplement (**n-3 PUFA**). The n-3 PUFA supplement was estimated to provide approximately 140 g combined of the long chain n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) based on the results of the study described in study 2. All diets were formulated to be isonitrogenous (14% crude protein (CP) in total diet) and isolipid.

##### *Blood sampling*

Blood samples were collected by jugular venipuncture at appropriate times and intervals for measurement of plasma concentrations of fatty acids, cholesterol, glucose, triglycerides, urea, non-esterified fatty acids (NEFA), beta-hydroxybuterate (BHBA), insulin and IGF-1.

##### *Embryo recovery*

Fifteen days after commencement of the dietary regimen, all heifers were oestrous cycle synchronised using two injections of Estrumate<sup>®</sup> (PG3 and PG4) administered intramuscularly 11 days apart. Superovulation treatment began on day 10 following PG4. All heifers received a total of 420 i.u. (12 ml) FSH administered i.m. twice daily. Luteolysis was induced by administering a further injection of PG (PG5) at 60 h after commencement of superovulation. The heifers were inseminated at 12 and 24 h after the onset of standing oestrus with frozen-thawed semen from a bull of known high fertility.

On day 7 post insemination embryo recovery was performed by non surgical flushing. The flushing medium was filtered through an Emcon<sup>®</sup> filter and the first 50 ml recovered from each uterine horn was collected into a sterile 50 ml tube for fatty acid analysis. Embryos were identified by stereomicroscopy and were graded according to International Embryo Transfer Society. Grade 1 morula, blastocysts and expanded blastocysts were isolated and deposited in 1.5 ml eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C pending gene expression analysis.

### *Quantitative real-time PCR (Q-RT-PCR)*

Relative transcript abundance was assessed by performing Q-RT-PCR for six genes known to be involved in embryo development and/or lipid metabolism: apoptosis regulator bax-alpha mRNA (BAX), bovine leukemia inhibitory factor (LIF), Connexin 43 (Cx43), E-cadherin (E-CAD), PPAR- $\alpha$ , PPAR- $\delta$ . The comparative Ct method was used for quantification of mRNA expression levels. Fold changes in the relative mRNA expression of target was determined by using the formula  $2^{-\Delta\Delta CT}$ .

### *Statistical analyses*

All data were checked for adherence to a normal distribution (PROC UNIVARIATE, SAS v9.1, 2002). Continuous data were analysed using two-way ANOVA with terms included for diet and block. Variables having more than one observation per subject were analysed using repeated measures ANOVA (PROC MIXED, SAS v9.1, 2002) with terms for diet, time period and their interaction included in the statistical model. Significant differences were compared using Tukey's option. Nonparametric data were analysed using the GENMOD procedure of SAS and a Poisson distribution was chosen with a log link function. The student's t-test was used to analyse the fold  $\Delta$  values in the gene expression analysis.

## **Results**

### *Metabolites, insulin and IGF-1*

An interaction was detected ( $P < 0.05$ ) between treatment and day of sampling for cholesterol, triglycerides, urea, BHBA and IGF-1 though the biological significance of this is unlikely to be of any concern. There was no difference between diets with regard to the systemic concentrations of any of the metabolites measured or on insulin or IGF-1 ( $P > 0.05$ ). IGF-I and urea concentrations were higher ( $P < 0.001$ ) on day 50 compared with pre-experimental concentrations on both diets but were unaffected ( $P > 0.05$ ) by n-3 PUFA supplementation.

### *Fatty acid concentration of plasma and uterine flushings*

The trends in the results were in general similar to those reported in the study described in study 2. There was no effect of diet on concentrations of either linoleic or arachidonic acid ( $P > 0.05$ ), however, there was a reduction in the concentration of linolenic acid ( $P < 0.05$ ). Dietary supplementation with n-3 PUFA led to an almost 12 fold increase in the plasma concentration of EPA ( $P < 0.0001$ ) and a 3.6 fold increase in the concentration of DHA ( $P < 0.001$ ). Consequently, supplementation with n-3 PUFA increased plasma concentration of total n-3 PUFA by 3.5 fold ( $P < 0.001$ ) but did not affect total n-6 PUFA concentrations ( $P < 0.05$ ). As a result, the ratio of n-6 to n-3 PUFA was reduced in the n-3 PUFA supplemented animals ( $P < 0.05$ ).

Supplementation with n-3 PUFA led to an almost 10 fold increase in the concentration of EPA ( $P < 0.001$ ) in uterine flushings and a greater than two-fold increase in total n-3 PUFA. Concentrations of myristic and linoleic acid also increased ( $P < 0.05$ ) while the concentration of the PGF<sub>2 $\alpha$</sub>  precursor, arachidonic acid, was reduced ( $P < 0.05$ ). There was no effect of diet on the concentrations of palmitic, stearic, oleic, linolenic, DPA or DHA ( $P > 0.05$ ) in uterine flushings.



### *Embryo yield and quality*

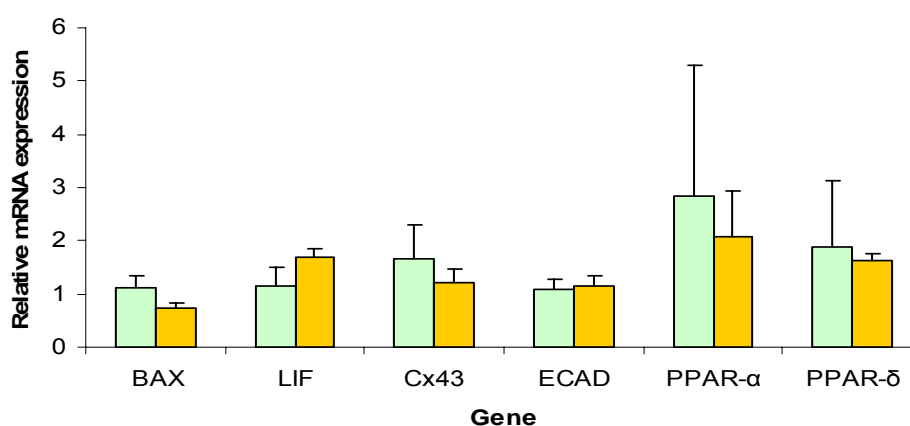
The effect of dietary n-3 PUFA supplementation on the estimated number of ovulations and on the yield and quality of embryos recovered is shown in Table 1. There was no effect of n-3 PUFA supplementation on the number of ovulations or on the total number of structures (including unfertilised, degenerate and morphologically normal embryos) recovered ( $P > 0.05$ ). Similarly, there was no effect of n-3 PUFA supplementation on the number of good quality (grade 1 and 2) embryos ( $P > 0.05$ ). However, the number of degenerate embryos recovered from heifers on the n-3 PUFA diet was reduced ( $P < 0.05$ ).

**Table 1.** Effect of dietary n-3 PUFA on mean superovulation response and embryo recovery and development.

	Diet		SED	P-value
	Control	n-3 PUFA		
No. of animals	16	17		
Estimated ovulations	17.50	15.06	2.387	NS
Total structures recovered	11.00	8.81	2.360	NS
No. non-fertilised	1.063	1.69	0.634	NS
No. of degenerate embryos	4.06	1.25	1.700	<0.05
No. of morula	0.94	1.81	0.995	NS
No. of early blastocysts	4.18	3.81	1.310	NS
No. of expanded blastocysts	0.69	0.25	0.331	NS
No. of transferable embryos (Grades 1 & 2)	6.60	5.88	1.679	NS
Proportion of transferable embryos	0.74	0.79	0.108	NS

### *Embryo gene expression*

The effect of n-3 PUFA supplementation on embryo gene expression is shown in Figure 8. There was no effect ( $P > 0.05$ ) of supplementation on the mRNA expression of any of the six genes studied viz., BAX, LIF, Cx43, E-CAD and PPAR- $\alpha$ , and PPAR- $\delta$ .



**Figure 8.** The effect of n-3 PUFA supplementation on a range of genes involved in apoptosis, gap junction formation, cell differentiation, implantation and lipid metabolism.

## Overall Discussion

Variation in the source of fatty acids, extent of rumen biohydrogenation, dry matter intake, milk production and associated variation in negative energy balance make interpretation of the published effects of PUFA on cattle fertility difficult to interpret. A major obstacle to the effectiveness of PUFA supplementation in cattle in terms of appreciably altering tissue concentrations is the extensive ruminal hydrogenation of dietary PUFA to saturated FAs. To achieve significant increases in systemic or tissue concentrations, it is suggested that unsaturated fatty acid sources be rumen protected or rendered rumen inert. In addition, excessive quantities of PUFA in the rumen can have negative effects on digestion which may compromise any potential benefits associated with supplementation.

The literature relating to ruminal hydrogenation of the long chain n-3 fatty acids is inconsistent. Extensive hydrogenation of EPA and DHA has been reported in *in vivo* studies (Scollan *et al.* 2001, Castaneda-Gutierrez *et al.* 2007), while *in vitro* work suggests that the effects of hydrogenation on PUFA with chain length in excess of 20 carbons are negligible (Ashes *et al.* 1992). However, despite this report, it is generally believed that in order to appreciably alter tissue concentrations of the long chain PUFA, some form of ruminal protection is necessary. In this context a pH resistant protection mechanism was used to protect the n-3 PUFA supplement used in studies 2 and 3 from ruminal hydrogenation. This allowed for the effective generation of higher plasma and tissue concentrations of the long chain n-3 PUFA, EPA and DHA, than would not be possible if feeding fish oil (similar to study 1 and many other published studies) or fishmeal at the equivalent percentage (2%) of dietary DM.

The pattern of the changes in fatty acid concentrations in plasma and the other reproductive tissues examined in these studies were consistent with previously published results. While Moallem *et al.* (2007) has previously reported fatty acid concentrations in follicular fluid, the study 2 is the first to report concentrations of the long chain n-3 PUFA, EPA and DHA in the follicular fluid of cattle. The majority of fatty acids in the follicular fluid were unaffected by the n-3 PUFA supplementation in study 2, however, EPA concentration increased in a linear and quadratic fashion while linoleic and oleic acid concentrations both decreased linearly. In a previous study by Zeron *et al.* (2001) it is suggested that increasing the concentration of PUFA in follicular fluid may enhance conception rate.

The results describing the effects of n-3 PUFA supplementation and the fatty acid concentrations of the uterine flushings presented in study 3 are also novel. The increase in EPA and total n-3 PUFA in the uterine flushings is consistent with incremental increases in these PUFA in endometrial tissue (study 2) and is suggestive of a potentially luteotrophic environment in the uterus following n-3 PUFA supplementation. This would result from changes in the prostaglandin biosynthetic pathway which, when utilising EPA as a substrate produce less luteotrophic eicosanoids (Mattos *et al.* 2003).

An increase in systemic progesterone could be important in the context of cow conception rate (Staples *et al.* 1998). However, despite the increases in cholesterol, precursor to progesterone synthesis, there was no statistically significant evidence of an increase in the concentration of systemic progesterone in study 1. Study 2 showed numerical increases in progesterone during the late luteal phase and also in overall

cycle progesterone production with n-3 PUFA supplementation. The published literature in relation to the effect of lipid supplementation on systemic progesterone is inconsistent and while the responses reported in study 1 are in agreement with the findings of some studies (Hightshoe *et al.* 1991), they are in contrast to the results reported in study 4 and others who reported increases (Lammoglia *et al.* 1997, Mattos *et al.* 2002, Wamsley *et al.* 2005), and others reporting decreases (Hawkins *et al.* 1995, Burke *et al.* 1996).

While plasma concentrations of IGF-1 are positively associated with body condition and nutrient intake (Staples *et al.* 1998), an increase in systemic concentrations of IGF-1 with increasing dietary n-3 PUFA was recorded in study 2, despite a reduction in both feed intake and performance on the HIGH n-3 PUFA diet in that study. There was no effect of n-3 PUFA supplementation on insulin or IGF-1 in study 3 but the level of n-3 PUFA offered was not as high as that which had resulted in increased IGF-1 concentrations in study 2. The absence of an effect on either insulin or IGF-1 in chapter 5, at the level of PUFA supplementation used, is consistent with Bilby *et al.* (2006). The increase in IGF-1 concentration recorded in study 2 could be beneficial as there is evidence of a positive association between IGF-1 and conception rate (Diskin *et al.* 2003) and reduced postpartum interval (Patton *et al.* 2007).

The results presented in studies 1 and 2 are not completely unexpected, as the literature relating to the effects of n-3 PUFA on prostaglandin biosynthesis is equivocal. The results described in studies 1 and 2 are consistent with previous reports (Filley *et al.* 2000, Fahey *et al.* 2002, Wamsley *et al.* 2005) in which no evidence for an effect of n-3 PUFA on prostaglandin synthesis was found. Inconsistencies in PGFM response to dietary PUFA may be the result of using oestradiol as a priming agent in oxytocin challenges. Furthermore, net inhibition of PGF<sub>2α</sub> by n-3 fatty acids may be dependent on the amount of n-6 fatty acids reaching the target tissue as suggested by a *in vitro* study by Caldari-Torres *et al.* (2006).

The third study measured the effect of dietary n-3 PUFA supplementation on embryo yield and on embryo quality based on both a morphological and genomic assessment. Polyunsaturated fatty acids function as regulators of gene expression, impacting on the metabolism of carbohydrate, protein and fat, as well as on the growth and differentiation of cells (Goff 2004). Consequently, as a further measurement of embryo quality, the relative gene transcript abundance in blastocysts recovered following dietary supplementation with n-3 PUFA was examined. Despite the effectiveness of the diet in terms of significantly increasing the n-3 PUFA concentration of both plasma and of uterine fluid there was no effect on superovulation response, embryo recovery rate, embryo quality or on the expression of a number of genes, including BAX, LIF, Cx43 and E-CAD that are well established in terms of their role in early embryo development. Neither was there any effect on peroxisome proliferator-activated receptors (PPARs) -α and δ which are involved in lipid metabolism and have also been implicated in development and reproduction in various animal models (Jump 2004).

While the increase in uterine EPA and decrease in arachidonic acid recorded, might have been expected to increase production of the 3-series prostaglandins and thus result in a more luteotrophic environment, with the exception of a reduced number of degenerate embryos following n-3 PUFA supplementation, there was no further

evidence of effects in terms of embryo development, embryo quality or embryo gene expression. The results suggest that any potential effects of dietary n-3 PUFA on the reproductive process in cattle are not mediated through effects on the embryo *per se*

From the results described, it can be concluded that:

1. Dietary supplementation with n-3 and n-6 PUFA can significantly increase the concentrations of these fatty acids in plasma and in reproductive tissues such as the uterine endometrium and follicular and uterine fluids.
2. Dietary supplementation with n-6 PUFA increases uterine secretion of  $\text{PGF}_{2\alpha}$  which may accelerate postpartum uterine involution and ovarian cyclicity providing the potential for improved conception rates.
3. Despite generating elevated concentrations of n-3 PUFA in uterine endometrial tissue following dietary n-3 PUFA supplementation, no effect on prostaglandin  $\text{F}_{2\alpha}$  biosynthesis was established.
4. Dietary n-3 PUFA supplementation can increase systemic concentrations of both cholesterol and IGF-1 as well as increasing the size of the corpus luteum. Enhancements in all of these variables have previously been associated with improved fertility in cattle.
5. Dietary supplementation with n-3 PUFA does not affect embryo recovery rate, embryo quality or alter the gene expression of the embryo. This suggests that effects of n-3 PUFA on fertility are not mediated through effects on the embryo *per se*.

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## **Publications arising from this study**

### **Scientific Papers:**

**Childs, S., C. O. Lynch, A. A. Hennessy, C. Stanton, D. C. Wathes, J. M. Sreenan, M. G. Diskin and D. A. Kenny, (2007).** Effect of dietary enrichment with either n-3 or n-6 fatty acids on systemic metabolite and hormone concentration and ovarian function in non lactating heifers. *In press "Animal"*.

**Childs, S., A. A. Hennessy, J. M. Sreenan, D. C. Wathes, Z. Cheng, C. Stanton, M. G. Diskin and D. A. Kenny, (2008).** Effect of level of dietary n-3 polyunsaturated fatty acid supplementation on systemic and tissue fatty acid concentrations and on selected reproductive variables in cattle. *In press "Theriogenology"*.

**Childs, S., F. Carter, C. O. Lynch, A. A. Hennessy, J. M. Sreenan, P. Lonergan and D. A. Kenny, (2008).** Embryo yield and quality, and embryo gene expression following dietary supplementation of beef heifers with n-3 PUFA. *Submitted to "Theriogenology"*

**Childs, S., B. Earley, A. A. Hennessy, C. Stanton, J. M. Sreenan and D. A. Kenny.** Effect of dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation on haematological variables in cattle. *In preparation, due to be submitted early 2008.*

**Coyne, G. S., D. A. Kenny, S. Childs, J. M. Sreenan and S. M. Waters, (2008).** Dietary n-3 polyunsaturated fatty acids alter the expression of genes involved in prostaglandin biosynthesis in the bovine uterus. *In press "Theriogenology"*.

### **Abstracts:**

**Childs, S., C. Lynch, N. Fox, M.G. Diskin, J.M. Sreenan and D.A. Kenny, (2005).** Effect of fatty acid enriched diets on reproductive parameters in cattle. *Proceedings of Irish Agricultural Research Forum, Tullamore, March 2005, p97.*

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