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Effect of dietary *n*-3 polyunsaturated fatty acid supplementation and post-insemination plane of nutrition on systemic concentrations of metabolic analytes, progesterone, hepatic gene expression and embryo development and survival in beef heifers

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Abstract

Nutrition, and particularly dietary energy intake, plays a fundamental role in reproductive function in cattle. There is some evidence that supplemental omega-3 dietary polyunsaturated fatty acids (*n*-3 PUFA) can exert positive effects on fertility. The objectives of this study were to evaluate the effect of dietary *n*-3 PUFA supplementation, post-insemination energy plane of nutrition and their interaction on embryo survival in cattle. Crossbred beef heifers (n=185) were individually offered barley straw *ad libitum* and 6 kg DM of concentrate supplemented with either a rumen-protected source of saturated fatty acid (palmitic; control, CON) or a partially rumen-protected *n*-3 PUFA-enriched supplement (*n*-3 PUFA). Estrous was synchronised using two injections of PG administered at 11-d intervals and following artificial insemination (AI = Day 0) 179 heifers exhibiting estrus were inseminated and assigned to one of two dietary treatments: (i) remain on their pre-insemination high dietary plane of nutrition (High) or (ii) restricted to 0.6 x estimated maintenance energy requirements (Low) in a 2 x 2 factorial design. The heifers were then maintained on their assigned diets until slaughter and embryo recovery on Day 16 (n = 92) or pregnancy diagnosis by ultrasound scanning at Day 30 post-AI (n = 87). Plasma concentrations of fatty acids, metabolites, insulin, progesterone (P4) and insulin-like growth factor 1 (IGF-1) were measured at appropriate intervals. Hepatic expression of mRNA for aldo-keto reductase *(AKR1C)*, cytochrome P450 2C *(CYP 2C)* and cytochrome P450 3A *(CYP 3A)* was examined. The *n*-3 PUFA supplementation increased plasma *n*-3 PUFA concentration ($P < 0.05$) and reduced *n*-6: *n*-3 PUFA ratio ($P < 0.05$). Plasma IGF-1 was higher for *n*-3 PUFA relative to the CON ($P < 0.05$) and for High compared with Low plane of nutrition post-AI ($P < 0.05$) groups. A low plane of nutrition post-AI increased plasma concentrations of progesterone from Days 7 to 16 after insemination ($P<0.001$) but reduced embryo length (P<0.001). Supplementation with n-3 PUFA reduced and tended to reduce hepatic expression of *CYP2C* (P=0.01) and *CYP3A* (P=0.08), respectively. However, while dietary n-3 PUFA supplementation and an abrupt reduction in nutrient status following insemination elevated plasma concentrations of n-3 PUFA and mid and late phase P4, respectively, there was no effect of either PUFA supplementation or post-insemination plane of nutrition on embryo survival.

Keywords: n-3 PUFA; Cattle; Embryo; Progesterone;

Introduction

Reproductive efficiency has a major impact on the economic performance of the global cattle industry and therefore strategies to alleviate declining fertility and enhance reproductive capacity are of critical importance [1]. In particular, there is clear evidence that nutrition plays a fundamental role in fertility with both concurrent and latent effects identified [1]. For example, over the past two decades there has been particular interest on the potential of dietary enrichment with *n*-3 polyunsaturated fatty acid (*n*-3 PUFA) to improve the fertility of both male and female cattle [2-5]. While there is little evidence for an appreciable influence on bull fertility [4], several studies have reported beneficial effects on aspects of reproductive function following dietary supplementation of female cattle with *n*-3 PUFA [6-9]. Fatty acid supplementation may act to specifically regulate some key reproductive processes including ovarian function [10], steroidogenesis [9, 11], oocyte competence [12], uterine prostaglandin F2α (PG) synthesis [13, 14] potentially leading to improved embryo survival. We have previously demonstrated that dietary enrichment with *n*-3 PUFA can alter the expression of key genes involved in prostaglandin biosynthesis in the uterus [13] and those associated with IGF signalling in both the liver and endometrium, suggesting a role in mediating metabolic and reproductive events [15]. In addition, Waters et al. [16] also identified effects on genes involved in maternal immune response and tissue remodelling following n-3 PUFA supplementation to beef heifers.

Improved conception rates following dietary inclusion of PUFA may be associated with greater embryo development [17]. Furthermore, the findings of Mattos et al. [18] showed that *n*-3 PUFA may act in concert with conceptus-derived interferon-τ to inhibit the release of PG, thus preventing the onset of luteolysis and facilitating the establishment of pregnancy [19]. Despite these data, work from our laboratory failed to establish an effect of *n*-3 PUFA on embryo yield or quality in cattle either *in vivo* [20] or *in vitro* [21].

Although the estimated absolute energy requirement to support reproduction is modest [22], the type and timing of the nutrient supply is highly specific and necessitates a focussed and targeted approach to improve reproductive efficiency. Indeed, abrupt fluctuations in energy intake during the peri-breeding period can negatively affect reproductive success [23] and embryo quality [24]. Dairy cow nutrition studies have traditionally increased dietary energy content in an attempt to improve reproductive performance [25]. However, this approach, in isolation, is inadequate and potentially counter-productive as increased dietary energy is typically partitioned towards enhanced milk production in the modern high genetic merit dairy cow, further aggravating the problem of poor reproductive performance.

Indeed, studies have shown a positive relationship between plane of nutrition, liver blood flow and as a consequence, the metabolic clearance rate of progesterone for both sheep and dairy cows [26, 27]. In addition, data from *in vitro* studies indicate that the hepatic enzymes cytochrome P450 2C, cytochrome P450 3A and aldo-keto reductase are pivotally involved in progesterone inactivation in bovine hepatocytes [28]. There is, however, no information available on the effect of dietary n-3 PUFA supplementation or indeed post-insemination plane of nutrition on activity or mRNA expression of these enzymes *in vivo*.

While many studies, including our own [2, 15, 16, 20] have adopted a methodological approach to investigating the effects of *n*-3 PUFA on key processes controlling reproduction, there are few published reports that have tested the hypothesis that these nutrients affect overall pregnancy outcome. Thus, using an energy-restricted heifer model as opposed to a lactating dairy cow model to avoid the well-documented confounding effects of differences in milk yield and energy balance on fertility, the specific objectives of this study were to examine the main effects of (i) *n*-3 PUFA supplementation, (ii) peri-insemination energy nutrition, and (iii) their interaction, on embryo survival and physiological indices of metabolic status in cattle.

Materials and Methods

Animals and feeding regime – Pre-insemination

This study was conducted under licence, at University College Dublin's Lyons Research Farm and the Teagasc Research Centre, Athenry, Co. Galway, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876, as amended by the European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. The management of the animals was the same in both facilities.

The experimental design is illustrated in Figure 1. As mentioned earlier, in an effort to counteract the possible confounding influences of variation in lactation yield and energy balance on reproductive processes, a nulliparous beef heifer model was employed in the present study. Reproductively normal nulliparous continental crossbred (Charolais and Limousin) heifers (n = 185) with a mean age of 22.6 ± 2.4 months, liveweight of 486 ± 60 kg and body condition score (BCS) of 3.17 ± 0.22 units were blocked on the basis of liveweight and BCS and randomly allocated within block to one of two dietary treatments. The concentrate-based ration (6 kg dry matter, DM) contained either (i) 334 g of a partially rumen-protected, eicosapentaenoic acid (EPA; C20:5*n*-3)/docosahexaenoic acid (DHA; C22:6 *n*-3) fish oil-based supplement (***n*-3 PUFA**; n=93 heifers) or 151 g of a 90% palmitic acid supplement (Palmit 80®; saturated FA) as a control (**CON**; n=92 heifers). Both supplements were provided by Trouw Nutrition (36 Ship Street Belfast, BT15 1JL, Northern Ireland). 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. The fish oil was derived from anchovy, sardine and salmon oil however, the oil was distilled in order to concentrate the EPA and DHA content. The dietary management of the heifers was similar to that described by Childs et al. [11] and is briefly described below.

Heifers were split-fed, initially receiving their entire daily allocation of supplementary lipid in the form of a 1.0 kg DM bolus feed at 09.00 h each morning, combined with 1.5 kg DM of a 24% crude protein (CP) ration (Balancer 1) to counteract the low crude protein CP content of the bolus rations. This regimen helped to ensure that heifers consumed the entirety of their daily lipid supplement allocation. Subsequently, at 12.00 h, the heifers were offered the remainder of their respective daily concentrate allocation in the form of 3.5 kg DM of a second 13% CP ration (Balancer 2), together with 1.5 kg DM barley straw.

Treatment diets were formulated to be isoenergetic (17 MJ GE/kg DM), isonitrogenous (140 g/kg DM) and isolipidic (20 g/kg DM) in the total diet (including forage), the latter ensuring that observed effects of *n*-3 PUFA, if any, were independent of their role as energy substrates. The ingredients and chemical composition of the concentrate rations and straw are presented in Supplementary Table 1 and the typical fatty acid concentrations of the diets fed have been reported by Childs et al. [2]. All heifers were housed indoors on concrete slats, with unrestricted access to fresh drinking water and fed individually using an electronic feeding system (Calan Inc., Northwood, New Hampshire, USA). During the experimental period, daily consumption of concentrate and straw was measured and recorded for each individual heifer and dry matter intake (DMI) was calculated.

Oestrous synchronisation and post-insemination experimental diets

Subsequent to receiving the respective diets for 14 days, oestrous was synchronised using two injections (PG1 and PG2, respectively) of a 500 μg PG analogue (Cloprostenol, Estrumate®, Schering-Plough Ltd., Welwyn Garden City, Hertfordshire, UK) administered intramuscularly 11 days apart. Pressure-activated heat detection aids (Kamar® Heatmount Detectors, San Diego, California) or scratch-cards (Estrotect™ Heat Detectors) were used as an aid for oestrus detection. All heifers were monitored for signs of oestrus 5 times daily (07.00, 11.00, 15.00, 19.00 and 23.00h), commencing 24 h after administration of PG2 and continuing for a further 96 h. Only heifers displaying standing oestrus (n=179) were artificially inseminated (AI) by one of two experienced operators using frozen-thawed semen from one high fertility bull. All inseminations were carried out within 12 h of standing oestrus.

On the day of insemination (Day 0), animals were blocked on the basis of bodyweight and BCS within the two PUFA treatments and allocated from within their original treatment group to one of two post-insemination diets: (i) remain on their pre-insemination high dietary plane of nutrition (High, n=88) or (ii) restricted to 0.6 x estimated maintenance energy requirements ([29]; Low, n=91). The latter group received a total of 2 kg DM concentrate daily, which included the same level of lipid supplement as that offered pre-insemination, together with 0.85 kg DM straw. The experiment was thus constructed as a 2 x 2 factorial design with four treatments (two pre- and four post-insemination treatment groups). This resulted in 44, 45, 47 and 43 heifers allocated to CON_Low, CON_High, n-3 PUFA_Low and n-3 PUFA_High dietary treatment groups, respectively. The heifers were maintained on their assigned diets until slaughter and embryo recovery on Day 16 post-insemination (n = 92) or pregnancy diagnosis by ultrasonic scanning at Day 30 (n = 87).

Animal performance

At initiation of the experimental period, all heifers were weighed prior to feeding on two consecutive days (Days -30 and -29). A similar protocol was followed at the end of the experiment providing two mean values, which were utilized to determine the start and end bodyweights respectively. In addition, live weight was recorded on a weekly basis and average daily gain was calculated using the linear regression of bodyweight on day of experiment. Body condition score was assessed fortnightly by the same technician using a 5-point scale with 0.25 intervals [30], with a score of 1 representing severely emaciated animals and a score of 5 representing over conditioned animals. A representative subsample of heifers from each treatment (n = 20/treatment group) were ultrasonically scanned (Aquila Vet real time ultrasound scanner, with a 3.5-Mhz transducer, Esaote Pie Medical, Pie Medical Equiptment B.V., Maastricht, Netherlands) for back fat depth measured at the third lumbar vertebra.

Feed sampling and analysis

Representative ration samples, relative to each individual batch of feed milled, were oven-dried in duplicate to a constant weigh at 55°C to ascertain an accurate fresh weight feeding level. In addition, weekly composite samples of concentrates and straw were stored at -20°C until analysed for DM, ash, crude protein (CP), fibre (crude fibre, CF, neutral detergent fibre, NDF, and acid detergent fibre, ADF), ether extract, and gross energy (GE). Samples were milled through a 1 mm screen using a hammer mill (Christy and Norris Process Engineers Ltd., Chelmsford, England). Residual dry matter was determined by oven drying at 104°C for a minimum of 16 h. Ash was determined after ignition of a known weight of ground material in a muffle furnace (Nabertherm, Bremen, Germany) at 550°C for 4 h. In conjunction with the technique of Van Soest et al. [31], fibre content (CF, NDF, ADF) of all samples was determined using a Fibertec extraction unit (Tecator, Hoganas, Sweden). Crude protein, defined as total nitrogen*6.25, was calculated using a Leco FP 528 nitrogen analyser (Leco Instruments, U.K. Ltd., Stockport, UK), as described by Sweeney [32]. Ether extract was determined using a Soxtex instrument (Tecator) while the GE of the samples was determined using a Parr 1201 oxygen bomb calorimeter (Parr Instrument Company, Moline, Illinois, USA).

Blood sampling and analysis

Blood samples were collected by jugular venipuncture prior to the commencement of the daily feeding regime. Samples were collected into 10 ml 170-IU lithium heparinised vacutainers (Becton Dickenson Vacutainer Systems, Plymouth UK) for plasma concentrations of IGF-1, insulin, fatty acids and metabolites on predetermined days during the experimental period. Retrospectively, samples analysed incorporated pre-supplementation

(Day -30), pre-insemination (Day -14) and post-insemination (Day 3 and Day 14) periods. Blood samples to measure concentrations of P4 were collected on the Days 0, 4, 5, 6, 7, 10, 14, 15 and 16 into 10 ml ethylenediamine tetracetic acid heparinized vacutainers (Becton Dickenson Vacutainer Systems). On collection, all blood samples were immediately stored in ice water and centrifuged at 1500 x g at 4ºC for 15 min. Finally, the plasma was pipetted into scintillation vials and stored at -20ºC until assayed.

Subsequent to an acid-ethanol extraction procedure, plasma IGF-1 was quantified by radioimmunoassay (RIA), as previously described by Ting et al. [33]. The mean inter-assay coefficients of variation (CV) for samples containing low (46.9 ± 1.54 ng/ml), medium (169.0 ± 11.85 ng/ml) and high (406.4 ± 2.14 ng/ml) IGF-1 concentrations were 3.3%, 7% and 0.5%, respectively. Intra-assay CV was 24.1% (low), 24.5% (medium) and 11.8% (high).

Concentrations of insulin in plasma was established by time-resolved fluoroimmunoassay (AutoDELFIA Insulin, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland; catalogue no. B080-101) and validated for bovine plasma [34]. The inter-assay CV for samples containing low (5.13 ± 0.62 pmol/l), medium (10.17 ± 0.88 pmol/l) and high (150.9 ± 7.04 pmol/l) insulin concentrations were 12.1%, 8.6% and 4.7%, respectively. Intra-assay CV was 12.0% (low), 8.7% (medium) and 4.7% (high).

Plasma concentrations of glucose, urea, triglycerides, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), and cholesterol, were determined using commercial biochemical assay kits (Olympus Diagnostics, Tokyo, Japan and Randox Laboratories Ltd., Co. Antrim, Northern Ireland) on an automated biochemical analyzer (AU400: Olympus Diagnostics, Tokyo, Japan).

Plasma concentrations of P4 were measured in duplicate using a ^{125}I - labelled progesterone antibody radioimmunoassay (Coat-A-Count Progesterone In Vitro Diagnostic

Test Kit™, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) with each sample measured in duplicate. The minimum detectable concentration for this assay was 0.171 ± 0.053 ng/ml. The inter-assay CV for samples containing low (0.49 ± 0.07 ng/ml), medium (1.94 ± 0.12 ng/ml) and high (7.01 ± 0.55 ng/ml) P_4 concentrations were 12.4%, 5.2% and 6.8%, respectively. Intra-assay CV was 9.6% (low), 3.8% (medium) and 4.1% (high).

Fatty acid analysis of feeds and plasma

In accordance with the extraction procedure described by Folch et al. [35], total lipids were isolated and purified from 6 g of ground fresh feed and 1 g of plasma using chloroform methanol (2:1 v/v). Subsequently, utilizing the method outlined by Park and Goins [36], sample methylation was performed by *in situ* transesterification with 0.5 N methanolic NaOH followed by 14% boron trifluoride in methanol. The fatty acid methyl esters (FAME) were separated using a CP Sil 88 column (100 m x 0.25 mm i.d., 0.20 μm film thickness; Chrompack, Middleburg, Netherlands) and quantified using gas liquid chromatography (GLC) (3400; Varian, Harbor City, CA, USA). Calibration of the GLC was performed with commercial fatty acid standards (Sigma-Aldrich Ireland Ltd.) and the internal standard utilized was heptadecanoic acid (C17:0; 99% purity - Sigma-Aldrich). The GC was fitted with a flame ionization detector (FID) and helium (37 psi) was used as the carrier gas. The injector temperature was maintained isothermally at 225ºC for 10 min and the FID was held at 250ºC. The initial column oven temperature was 140ºC for 8 min, which increased at a rate of 8.5 ºC/min to a final temperature of 200ºC, which was sustained for 41 min. A Minichrom PC system (VG Data System, Manchester, UK) was utilized to record and analyse the data, which was expressed as g/100g FAME.

Post-mortem sample collection (Day 16)

A representative sub-sample of heifers from within each of the four treatment groups were slaughtered on Day 16 post-AI. Specifically, 23, 22, 24 and 23 heifers from the, CON_Low, CON_High, n-3 PUFA_Low and n-3 PUFA_High dietary groups, respectively were harvested. The reproductive tracts were recovered from all heifers and transported on ice to the laboratory, which was within a 30 min journey. The uterine horns were trimmed free of excess tissue and flushed with 100 mL of phosphate-buffered saline (PBS), containing 5% fetal calf serum. Pregnancy was confirmed by locating a conceptus under a stereomicroscope. Conceptus length was measured using an optical callipers.

Hepatic tissue was sampled from all animals within 30 min of slaughter. All surgical instruments used for tissue preparation were sterilized and treated with RNA Zap prior to use (Ambion, Applera Ireland, Dublin, Ireland). Samples were washed thoroughly with sterile DPBS and immediately snap frozen in liquid nitrogen before subsequent storage at −80 °C.

Pregnancy diagnosis (Day 30)

In the remaining heifers (n = 89), pregnancy was determined by ultrasound scanning of the uterus using an Aloka SSD-500 V ultrasound scanner fitted with a 7.5-MHz transducer (Aloka Co. Ltd, Tokyo, Japan) at Day 30 after AI. A positive pregnancy diagnosis was based on the presence of a viable embryo with a visible heartbeat and clear amniotic fluid.

Liver Tissue Sampling, RNA isolation and purification

Total RNA was isolated from liver tissue samples using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. The quantity of the RNA isolated was determined by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, DE, USA). RNA quality was assessed on the Agilent Bioanalyser 2100 using the RNA 6000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd., Dublin, Ireland). RNA samples with RNA integrity numbers between 8 and 10 were deemed to be of sufficiently high quality. RNA quality was also verified by ensuring all RNA samples had an absorbance (A260/280) of between 1.8 and 2.

cDNA Synthesis, and Real-Time Quantitative PCR

One microgram of total RNA was reverse transcribed to cDNA, with random hexamers, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK), according to instructions supplied, and stored at −20°C. Real-time quantitative PCR (**RT-qPCR**) was used to measure expression of genes involved in the progesterone inactivation ((aldo-keto reductase 1C (*AKR1C),* Cytochrome P450 2C *(CYP2C)* and Cytochrome P450 3A (*CYP3A)*). The sequences of primers used for each gene were commercially synthesized (Sigma-Aldrich Ireland Ltd.) and are listed in Table 1. The PCR products generated by amplification were sequenced to verify their identity and confirm primer specificity (Eurofins MWG Operon, Ebersberg, Germany).

The stability of expression of candidate reference genes, β-actin (*ACTB*), ribosomal protein S9 (*RPS9*), and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH),* was investigated across all samples in the study. The resulting expression data were analyzed using geNorm software (version 3.5, Excel add-in, Microsoft, Redmond, WA) as described by Vandesompele et al. [37] to test the overall stability of the tested reference genes. The highest stability was achieved by including the 3 reference genes, achieving a combined *M* value of 0.29. All RT-qPCR reactions were performed using SYBR Fast Green mastermix (Applied Biosystems). Assays were performed in triplicate using the Applied Biosystems Fast 7500 v2.0.1 instrument with the following cycle parameters (95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s). Primer and cDNA concentrations were optimized for each gene. The efficiency of the reaction was calculated using a 5-fold dilution series of cDNA to generate a standard curve. Dissociation curves were examined for the presence of a single PCR product. All PCR efficiency coefficients were between 0.9 and 1.08 and therefore deemed acceptable. The software package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden) was used for efficiency correction of the raw cycle threshold values, interplate calibration based on a calibrator sample included on all plates, averaging of replicates, normalization to the reference gene, and the calculation of quantities relative to the highest cycle threshold value.

Statistical analyses

All data were analyzed using Statistical Analysis Systems software (SAS, Cary, NC, USA). Data were examined for adherence to normality using UNIVARIATE procedure of SAS and transformed if necessary by Box-Cox transformation analysis using the TRANSREG procedure in SAS. A mixed model ANOVA (PROC MIXED) was conducted with statistical models including terms for the fixed effects of (i) PUFA supplementation, (ii) post-insemination plane of nutrition and (iii) sampling day (i.e. for blood analytes), where appropriate, (iv) site (two experimental farms) as well as for (v) block (initially blocked to treatment on bodyweight and BCS). The Tukey test was used to determine statistical difference between mean comparative group values for each outcome variable. For binary variables such as conception rate, logistic regression was conducted to examine their relationship with specific continuous variables, using the LOGISTIC procedure of SAS. Statistical differences were denoted at $P < 0.05$ with values expressed as least square means ± standard error of the mean (SEM). Tendencies towards statistical significance were denoted for p-values ≥0.05 and <0.10.

Results

There was no interaction between either of the two nutritional factors and site of study for any of the measurements taken (P>0.05).

The effects of *n*-3 PUFA supplementation and post-AI diet on animal performance ADG (kg/d) and BCS change (units/d) during the pre- and post-AI periods, where appropriate, are presented in Table 2. There was no *n*-3 PUFA x post-AI diet interaction for either ADG or BCS measures. Average daily gain in the pre-AI period was higher ($P < 0.0001$) for non-supplemented heifers compared with *n*-3 PUFA-supplemented heifers. However, during the post-AI period ADG was similar for both *n*-3 PUFA treatment groups. Heifers on a high plane of nutrition post-AI had increased ADG ($P < 0.0001$) while those on the low plane of nutrition lost on average 1.19 kg per day. Neither pre- nor post-AI BCS was affected by *n*-3 PUFA supplementation. However, restricting energy supply post-AI led to differences in BCS gain manifested as heifers maintained on a high plane of nutrition gaining BCS while those on the low plane of nutrition lost, on average, 0.01 of a BCS unit per day.

The effect of treatment on plasma concentrations of metabolic hormones and metabolites are presented in Table 3. There were no three-way interactions detected for any of the plasma analytes measured. There was an *n*-3 PUFA x sample day interaction (P <

0.001) and also a strong tendency towards a post-AI diet x sample day interaction (P = 0.05) detected for plasma IGF-1. Although similar at the start of the study (Day -30), plasma IGF-1 concentrations for heifers offered *n*-3 PUFA increased during the experimental period and remained higher at all time-points compared to control heifers whose plasma concentrations of IGF-1 declined throughout the experimental period.

IGF-1 concentrations were similar for *n*-3 PUFA supplemented and non-supplemented heifers prior to AI. However, following AI, heifers subjected to metabolic constraints had decreased systemic concentrations of IGF-1 compared with their unrestricted counterparts.

There were no interactions between the main factors for plasma concentrations of insulin. Plasma insulin was lower (P < 0.0001) for heifers supplemented with *n*-3 PUFA compared with non-supplemented heifers on all sample days. Additionally, there was an effect of sample day (P = 0.006) on plasma concentrations of insulin with concentrations increasing from Day -30 to Day -14, while concentrations were lower for the post-AI period. There was no effect of post-AI nutrient intake on plasma concentrations of insulin.

There was no treatment x sample day interaction for plasma glucose nor was glucose affected by either *n*-3 PUFA or post-AI diet. However, glucose was affected by sample day, with higher levels on Day -30 compared with the subsequent sample days (P = 0.04). There was an *n*-3 PUFA x sample day interaction for plasma urea with a tendency (P = 0.08) for urea to be higher on Day 3 post-AI in PUFA-fed heifers but not on any of the other sample days.

We observed an *n*-3 PUFA x post-AI diet interaction for plasma concentrations of BHB. This was manifested as CON heifers offered a low post-AI diet having higher plasma BHB concentrations than their contemporaries offered a high plane of nutrition post-AI, while for PUFA-supplemented heifers this effect was reversed. Plasma BHB was also affected by sample day with concentrations higher on Days -14, 3 and 14 compared with Day -30.

There was a post-AI diet x sample day interaction for plasma concentrations of NEFA. While there was no pre-AI difference between heifers offered a High or Low post-AI diet, heifers with declining nutrient status had higher NEFA concentrations than those offered the high allowance following AI.

An *n*-3 PUFA x post-AI diet interaction was evident for plasma concentrations of cholesterol. This was manifested as heifers receiving *n*-3 PUFA and maintained on a high post-AI diet having increased concentrations of cholesterol compared with their counterparts receiving the low post-AI plane of nutrition (3.52 v 3.14 mmol/L) whilst in non *n*-3 PUFA supplemented heifers whose cholesterol concentrations were actually lower when maintained on the high compared with low post-AI diet (3.05 v 3.36 mmol/L). There was also an effect of day of sampling on plasma concentrations of cholesterol with concentrations increasing linearly across the experimental period ($P < 0.0001$).

There was an *n*-3 PUFA x sample day interaction detected for plasma concentrations of triglycerides. This was apparent as heifers supplemented with *n*-3 PUFA having lower concentrations of triglycerides on Day -14 compared with Day -30 which was not observed for non-supplemented heifers. Similarly, there was also a post-AI diet x day of sampling interaction for triglyceride concentrations manifested as concentrations of triglycerides decreasing for heifers on the low post-AI plane of nutrition while those offered the high plane of nutrition maintained their pre-AI concentrations of triglycerides.

The effect of *n*-3 PUFA supplementation, post-AI diet and sample day and their respective interactions on plasma concentrations of 35 fatty acids is presented in Table 4. Rather than describing the results for each individual fatty acid, the main results for a selection of fatty acids with potential effects on reproductive processes are shown. With the exception of palmitic acid (C16:0) there was generally no biologically significant interaction between the main effects or of the main effects themselves for the saturated fatty acids measured.

There was an *n*-3 PUFA x sample day interaction for concentrations of palmitic acid. This was manifested as concentrations of C16:0 being similar between *n*-3 PUFA supplemented and non-supplemented heifers at the start of the experiment but increasing in non-supplemented heifers (C16:0 dominant diet) at Day 3 with this difference maintained throughout the remainder of the experiment. There was no effect of post-AI diet on concentrations of palmitic acid.

There was an *n*-3 PUFA x sample day interaction for concentrations of the parent *n*-6 PUFA, linoleic acid. This was manifested as a lack of difference between *n*-3 PUFA fed groups at the start at the experiment with the concentration of linoleic acid increasing in the non-supplemented heifers on Day 3 and this difference being maintained with the progression of the experiment. There was also a main effect of post-AI diet with heifers fed the high diet having higher concentrations.

There was an *n*-3 PUFA x sample day interaction for concentrations of the parent *n*-3 PUFA, linolenic acid. This was manifested, similarly to linoleic acid, as a lack of difference between *n*-3 PUFA fed groups at the start at the experiment but also similar on Day 3 but the concentration of linolenic acid was higher in the unsupplemented heifers on Day 14. There was also a post-AI diet x day of sampling interaction for concentrations of linolenic acid.

This was apparent as a lack of difference between post-AI dietary energy groups at the start of the experiment and on Day 3 but the concentration of linolenic acid was higher in the unsupplemented heifers on Day 14.

A strong tendency (P = 0.06) towards an *n*-3 PUFA x day of sampling interaction was observed for concentrations of the *n*-6 PUFA, and the substrate for the 2-series prostaglandins, arachidonic acid (C20:4). This was manifested as a lack of difference between *n*-3 PUFA treatments on Days -30 and 3 but *n*-3 PUFA fed heifers had higher concentrations of C20:4 in plasma on Day 14 post-AI.

There was also a post-AI diet x sample day interaction for concentrations of arachidonic acid. This was apparent as a lack of difference between *n*-3 PUFA treatments on Days -30 and 3 but heifers fed a high post-AI diet had higher concentrations of C20:4 in plasma on Day 14 post-AI. An *n*-3 PUFA x day of sampling interaction was observed for concentrations of the *n*-3 PUFA, eicosapentaenoic acid (EPA; C20:5*n*-3). This was manifested as a lack of difference at Day -30 with EPA concentrations higher on days 3 and 14 in *n*-3 PUFA fed heifers.

There was a strong tendency towards an effect of *n*-3 PUFA supplementation on plasma concentrations of the *n*-3 PUFA, docosapentaenoic acid (DPA; C22:5) with concentrations higher in supplemented heifers (P = 0.05). Similar to EPA, an *n*-3 PUFA x day of sampling interaction was observed for concentrations of the *n*-3 PUFA, docosahexaenoic acid (DHA; C22:6 *n*-3). This was manifested as a lack of difference at Day -30 with EPA concentrations higher on Days 3 and 14 in *n*-3 PUFA fed heifers. There was also a post-AI diet x sample day interaction for concentrations of DHA as concentrations of DHA were higher on Day 14 in heifers fed the low energy diet but no differences were detected between groups prior to this.

The effects of nutritional treatment on plasma concentrations of P4, conception rate and embryo length are presented in Table 5. With the exception of a tendency on Day 0 (P = 0.05), there was no *n*-3 PUFA x post-AI diet interaction for plasma concentrations of P4 on any sampling day. Similarly, *n*-3 PUFA supplementation did not affect plasma concentrations of P4 on any day of the oestrous cycle on which it was measured. However, there was a post-AI diet x sample day interaction for plasma concentrations of P4 manifested as a higher concentration of P4 for heifers on the low compared with the high post-AI diet from Days 10 to 16 post-AI but not before this period (Table 5).

There were no two or three-way interactions between the main experimental factors (PUFA treatment, post-insemination diet, site of study) for pregnancy rate. Similarly, there was no difference in pregnancy rate between CON v *n*-3 PUFA (0.77 v 0.73, respectively; χ^2 = 0.40; P = 0.53) or High v Low post-AI (0.71 v 0.79, respectively; χ^2 = 1.596; P = 0.16) dietary groups (see Table 5).. For ease of comparison, mean pregnancy rate coefficients for the four individual treatments were 0.82, 0.69, 0.75 and 0.70 for CON_Low, CON_High, n-3 PUFA_Low and n-3 PUFA_High dietary treatment groups, respectively.,. Regression co-efficients (β_0: intercept; β_1: slope; R^2: co-efficient of determination) for the relationship between plasma concentrations of P4 on alternate days of the oestrous cycle post-AI and embryo length measured at slaughter on Day 16 post-AI are presented in Table 6. There was a strong tendency towards a positive relationship between plasma concentrations of P4 on Day 0 (Odds Ratio = 4.342; P = 0.057) and Day 7 (Odds Ratio = 0.82; P = 0.052) and subsequent pregnancy status. There were no two- or three- (*n*-3 PUFA, Post-AI diet, replicate) way interactions detected for pregnancy rate.

There was a positive though weak relationship between plasma concentrations of P4 on Days 4, 5, 7, and 15 with embryo length on Day 16 of pregnancy, while a negative relationship between these two variables was observed for Day 0 (Table 6). No relationship between concentrations of P4 on Days 6, 10, 14 or 16 and embryo length on Day 16 could be detected on any of the other sampling days employed.

Hepatic mRNA expression of *AKR1C* was not altered (P>0.05) by either to *n*-3 PUFA supplementation or post-AI plane of nutrition. There was no effect (P>0.05) of post-AI diet on the hepatic expression of *CYP2C* while *n*-3 PUFA supplementation reduced the mRNA expression of that gene (P=0.01). Similarly, post-AI plane of nutrition did not affect (P>0.05) the expression of *CYP3A* however, there was a strong tendency (P=0.083) towards a reduction in the expression of *CYP3A* in animals consuming diets supplemented with *n*-3 PUFA.

Discussion

This study investigated the interaction between dietary *n*-3 PUFA supplementation and post-insemination level of nutrition on metabolic indices, P4 and embryo survival in beef cattle. The main findings were (i) *n*-3 PUFA supplementation increased plasma concentrations of *n*-3 PUFA and reduced the *n*-6:*n*-3 PUFA ratio; (ii) there was an increase in plasma IGF-1 in heifers fed *n*-3 PUFA relative to the CON diet as well as on the High compared with the Low plane of nutrition post-AI group; (iii) declining nutrient status post-AI elevated plasma concentrations of P4 between Days 10 and 16 post-insemination, which in turn was positively associated with the length of day 16 embryos; (iv) Plane of nutrition following insemination did not affect transcript abundance for genes involved in the hepatic metabolism of P4, though there was evidence that dietary n-3 PUFA supplementation may down regulate genes involved in this process and (v) there was no effect of dietary *n*-3 PUFA or plane of nutrition on embryo survival.

Feeding diets fortified with *n*-3 PUFA (approximately 3% DM fish oil in concentrate) in a partially protected form did not affect DMI, with cattle consistently consuming their entire daily allocation, consistent with similar studies in beef heifers from our group [2, 20] and others [38]. In contrast, a negative relationship between dietary inclusion of fish oil exceeding 1% DM and subsequent intake has been documented in a number of studies [39, 40], most likely contributed to by a combination of modification to the ruminal environment and palatability issues.

A slight, though biologically insignificant, reduction (70 g per day) in the ADG of heifers supplemented with *n*-3 PUFA supplementation pre- but not post-AI was evident. However, this was not reflected in their body fat accretion (BCS change) which is consistent with previous reports [2, 20, 40]. As expected, heifers maintained on the high plane of nutrition post-insemination, experienced enhanced weight gain compared to their nutritionally-challenged counterparts.

Plasma metabolites can provide a point-in-time indication of the metabolic status of an animal. Glucose is an important energy source for the bovine ovary and the post-blastocyst stage embryo [41]. Consistent with our previous work, we failed to detect an effect of *n*-3 PUFA supplementation on systemic concentrations of glucose. In agreement, Grummer and Carroll [25] concluded that fat supplementation does not generally alter blood glucose and stable systemic glucose concentrations during fat supplementation and may indicate a reduction in hepatic gluconeogenesis. Despite the major differences in feed intake between the restricted and unrestricted heifers post-AI, there was no effect on plasma concentrations of glucose. This is in agreement with previous research carried out at this laboratory in which no differences were found between glucose concentrations in heifers restricted to 0.6 M in comparison to 1.2 M for 50 days [42].

Plasma concentrations of triglycerides were low in the current study and were consistent with those reported by Childs et al. [2, 11, 20]. In agreement with some reports [43] but not others [44], diet did not affect BHB concentrations in our study. In contrast to Childs et al. [20], we failed to observe any effect of *n*-3 PUFA supplementation on plasma concentrations of NEFA. This is consistent, however, with the findings of Moussavi et al. [45]. There was an increase in plasma concentrations of NEFA in the diet-restricted heifers, reflective of tissue lipid metabolism and consistent with others who have used the energy restriction model in heifers [23, 42]. Concentrations of insulin were not affected by *n*-3 PUFA supplementation, in agreement with Bilby et al. [46] and Childs et al. [2]. As one would expect, we did, however, observe a negative effect of dietary restriction post-AI on plasma concentrations of insulin.

Systemic urea was slightly higher in the high *n*-3 PUFA-fed heifers which is consistent with the findings of Childs et al. [20] but the magnitude of the increase was not biologically significant and is in line with the fact that the diets were isonitrogenous in nature. In contrast to Childs et al. [20] who reported an increase in plasma concentrations of cholesterol with incremental additions of fish oil to the diet, we failed to observe any such effects. As circulating cholesterol is the primary substrate for the synthesis of P4, the lack of difference is consistent with the observed similarity in plasma P4 profiles between supplemented and non-supplemented heifers.

IGF-1 functions as a mediator of cell growth, development and differentiation and has been positively associated with conception rate and a reduction of the post-partum interval in cattle [47]. Despite having slightly lower performance, systemic IGF-1 was higher in the cattle on the *n*-3 PUFA compared with the CON diet, in agreement with Childs et al. [20]. When comparing post-AI diet, however, as expected animals offered the high plane of nutrition had greater systemic concentrations of IGF-1, in agreement with previous work from our group [48]. .

The effect of *n*-3 PUFA supplementation on the plasma fatty acid profile in this study agrees with previous studies by our group [2, 11, 20] as well as those from other laboratories [50, 51]. A ten-fold increase in plasma EPA and an almost four-fold increase in DHA are consistent with the findings of Childs et al. [2, 20] and would be expected to result in biologically significant increases in these *n*-3 PUFA in reproductive tissues including follicular [20] and uterine [2] fluid as well as significant accretion within the luteal tissue [3] and the uterine endometrium [2]. We also know that such a plasma fatty acid profile is consistent with a less luteolytic environment towards the latter stage of the oestrous cycle, as outlined by Coyne et al. [13].

There is clear evidence in the literature of an association between circulating concentrations of P4 and conceptus development [52, 53] in heifers, mediated by P4-induced changes in the uterine endometrium [54, 55]. n-3 PUFA supplementation in the current study had no effect on plasma concentrations of P4, in contrast to the observations of Childs et al. [11] who reported evidence of an increase in overall P4 output as well as greater size of CL and cholesterol concentrations in animals fed a high *n*-3 PUFA diet. Indeed, the literature relating to effects of PUFA supplementation on systemic concentrations of P4 is inconsistent with reports of an increase [11], decrease [56-58] or no change [39, 45, 59-61]. The findings of Lopes et al. [62] suggest that feeding 0.1 kg/d of a rumen-inert PUFA supplement to ovariectomized, non-lactating, beef cows reduced hepatic P4 metabolism. Similarly, cows infused with a soybean oil emulsion (predominantly n-6 PUFA-based) displayed a reduced hepatic clearance of P4 [27, 50]. Moreover, greater serum P4 concentrations were also observed in beef cows receiving calcium salts of PUFA compared with SFA and control cows [63]. It is likely that different responses in P4 production to supplemental lipid is related to their specific fatty acid composition and potential contribution to systemic cholesterol availability.

In contrast to n-3 PUFA supplementation, there was a stark effect of post-insemination diet on plasma concentrations of P4 from Day 7 onwards. Heifers offered the restricted energy diet post-insemination had higher concentrations of P4 than their unrestricted counterparts. Not only can dietary lipid intake affected hepatic steroid metabolism but increased liver blood flow resulting from elevated feed intake in lactating dairy cows may increase steroid metabolism [27]. Our results are consistent with those of Sangsritavong et al. [27] who demonstrated lower systemic P4 as well as oestradiol concentrations in lactating and dry dairy cows fed a high compared with a low plane of nutrition. This is likely to be a consequence of increased hepatic enzymatic activity during bouts of increased metabolic load and feed intake and in particular increased expression of progesterone dehydrogenase [64]. However, in the current study, divergence in systemic concentrations of P4 between the restricted and non-restricted heifers only began to emerge after Day 7 and thus this may help to explain the lack of an effect of post-insemination plane of nutrition on embryo survival rate. The study of Dunne et al. [23] demonstrated a clear depression in embryo survival in heifers switched from a high pre- to a low post-insemination diet; they failed to observe an effect of post-insemination plane of nutrition on plasma concentrations of P4, though those authors did record a positive effect of concentration of P4 on Day 7 and overall embryo survival rate.

In an effort to provide some insight into the potential effect of n-3 PUFA and plane of nutrition on aspects of the biochemical regulation hepatic progesterone metabolism in cattle, we measured transcript of three key genes in this process. The contribution of cytochrome P450 2C and cytochrome P450 3A enzymes to progesterone inactivation in bovine hepatic cell cultures was estimated as 40 and 15%, respectively with aldo-keto reductase enzymes observed to contribute an additional 40% to progesterone inactivation [28]. We found that while a low post-AI plane of nutrition increased circulating progesterone, this was not accompanied by an effect on hepatic expression of transcripts for any of the inactivating enzymes examined. On the other hand, we observed a reduction in mRNA expression of *CYP2C* and a strong tendency towards lower transcript abundance for *CYP3A* in hepatic tissue from *n-3* PUFA supplemented heifers 16 days after insemination suggesting that more progesterone should be bioavailable. However, despite this, as discussed earlier, unlike our previous findings [11] this was not accompanied in the current study by an effect of n-3 PUFA on systemic concentrations of progesterone. While hepatic cytochrome P450 and aldo-keto reductase enzymes are known to play a pivotal role in the first step of steroid inactivation [28], other factors are involved in progesterone luteal production and hepatic decay will influence systemic concentrations of the hormone.

In the current study we found that supplementary dietary *n*-3 PUFA had no effect on embryo survival though it did result in a small reduction in the length of 16 day old embryos. The literature relating to effects of PUFA supplementation on conception rate and embryo survival is inconsistent with reports of positive [65, 66] or neutral effects [20, 67, 68]. Our current results substantiate findings from *in vitro* studies where inclusion of EPA (*n*-3) or arachidonic acid (*n*-6) to media during bovine oocyte maturation [69] or indeed incremental addition of EPA or DHA during *in vitro* culture [21] failed to affect embryo development. In contrast, Marei et al. [12] reported that supplementation of bovine oocytes with ALA (*n*-3) during maturation enhanced blastocyst yield and quality. Further, it has been shown that a low dose (1 µM) of DHA during *in vitro* maturation had a positive effect on oocyte development in comparison to a dose of 100 µM which had a negative effect [70]. Additionally, sheep studies by McEvoy et al. [71] reported significant increases in the number and diameter of good quality blastocysts, together with total cell counts in embryos cultured with serum from ewes receiving an intermediate (3% w/w) inclusion rate of long-chain PUFA fish oil (EPA and DHA). The same authors reported compromised embryo development following supplementation with dietary fish oil (3 or 6% w/w) compared with the non-supplemented control diet [71]. Similarly, the quality of bovine embryos was negatively affected by feeding donor lactating dairy cows with a rich source of *n*-3 fatty acids in the form of whole flaxseed compared with calcium salts of palm oil [67]. Thangavelu et al. [17] reported a reduced early embryonic development, as evidenced by fewer embryos beyond the morula stage when super-stimulated embryo donor cows were fed diets enriched with a saturated source of FA compared to those supplemented with unsaturated FA of both sunflower (LA) and flax (ALA) seed origin.

There was no effect of post-insemination plane of nutrition on embryo survival rate despite the large divergence in daily feed allowance employed. This is in contrast to the findings of Dunne et al. [23] who reported a 50 percentage-point reduction in embryo survival in heifers switched from a high pre- to a low post-insemination plane of nutrition. Similarly, Kruse et al. [24] recently offered non-superovulated heifers either a control (125% estimated maintenance energy requirements) or nutrient restricted diet (50-80% of estimated maintenance energy requirements) and observed that embryos from restricted heifers were both at a lesser stage of development and of poorer quality than those recovered from controls. Given that the heifers in our study were individually fed and managed under a controlled environment compared to those in the studies of both Dunne et al. [23] and Kruse et al. [24], which were managed as groups at pasture and a feedlot, respectively, our results are particularly surprising. Additionally, what is more surprising regarding the lack of effect on embryo survival rate is that unlike the results of Dunne et al. [23] who found no effect of dietary energy restriction post-AI on embryo size, we observed that dietary restricted heifers produced embryos that were over 2.5 fold shorter than their unrestricted contemporaries. Overall, however, these findings with nulliparous beef heifers should be viewed in the context that the origin of NEB in early pregnancy is different to that typically experienced by post-partum lactating cows.

4.1 Conclusions

In conclusion, *n*-3 PUFA supplementation had no effect on either embryo development or survival. The diets employed in this experiment were designed (as previous reports have verified) to provide more *n*-3 PUFA to reproductive and metabolic tissues than in any other previously published report from outside our own laboratory. However, in contrast to an earlier study from this laboratory, severely restricting dietary energy provision immediately post-insemination had no detrimental effect on embryo survival or development. Indeed, we show very clear evidence for an effect of plane of nutrition on plasma concentrations of P4, potentially mediated through altered hepatic blood flow but not through changes in catabolic enzymatic activity. Further research will be required to determine the effects, if any, on embryo development and survival following similar treatments in lactating cows.

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Conflict of Interest

None.

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Table 1. Oligonucleotide primer sequence information used for real-time quantitative PCR assays.

[1]Gene	Primer sequence (5′ to 3′)	Amplicon size (bp)	Accession number
Reference genes:			
RPS9	Forward: CCTCGACCAAGAGCTGAAG Reverse: CCTCCAGACCTCACGTTTGTTC	64	NM_001101152.1
ACTB	Forward: ACTTGCGCAGAAAACGAGAT Reverse: CACCTTCACCGTTCCAGTTT	123	BT030480
GAPDH	Forward: GATTGTCAGCAATGCCTCCT Reverse: CCATCCACAGTCTTCTGGGT	135	NM_001034034
Target genes:			
AKR1C	Forward: AGTCGGAGGAGCAAAACAGA Reverse: AATTTGGTGACCTCCACA	141	NM_001035367
CYP2C	Forward: TATGGACTCCTGCTCCTGCT Reverse: CATACTGCTGGGGACAAGGT	177	AY265992
CYP3A	Forward: GAAGCTGCAGGAGGAAATTG Reverse: CTCCCAGCAATTGGAAACAT	129	XM_015469393.1

[1]RPS9 = ribosomal protein S9; ACTB = β-actin; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase AKR1C = aldo-keto reductase 1C; CYP2C = Cytochrome P450 2C; CYP3A = Cytochrome P450 3A.

Table 2. Effect of *n*-3 PUFA (P) and post insemination (AI) energy allowance treatment on average daily gain (ADG; kg/d) and change in body condition score (ΔBCS: units/d) pre- and post-AI

	n-3 PUFA			Post AI Diet			Significance		
	High	Low	SEM	High	Low	SEM	P	AI	P x AI
Variable									
ADG (Pre-AI)	0.74	0.81	0.010				<0.0001	-	-
ADG (Post-AI)	-0.16	-0.13	0.020	0.90	-1.19	0.021	0.28	<.0001	NS
ΔBCS (Pre-AI)	0.002	0.005	0.001	0.002	0.005	0.001	0.11	-	-
ΔBCS (Post-AI)	-0.001	-0.002	0.001	0.009	-0.011	0.001	0.56	<.0001	NS

Table 3. Effect of n-3 PUFA supplementation treatment (P), post-insemination (AI) diet and sample day on plasma concentrations of metabolic hormones and metabolites

	PUFA			Post AI Diet			Day of Sampling (D)					Significance					
Variable	n-3 PUFA	CON	SEM	High	Low	SEM	-30	-14	3	14	SEM	P	AI	D	P x AI	AI x D	P x
IGF-1(ng/ml)	379.9	320.5	15.32	373.53	326.93	15.76	330.06	383.54	341.58	342.35	13.83	0.01	0.05	0.0004	NS	0.05	<0.0
Insulin (IU/ml)	4.01	6.06	0.375	5.11	4.96	0.375	5.13	5.93	4.81	4.28	0.463	<0.0001	NS	0.006	NS	NS	NS
Metabolites (mmol/L)																	
Glucose	4.31	4.42	0.085	4.36	4.37	0.084	4.47	4.3	4.37	4.31	0.07	0.39	0.97	0.04	NS	NS	NS
Urea	4.44	4.01	0.125	4.24	4.21	0.123	3.44	4.04	4.54	4.87	0.125	0.02	0.85	<0.001	NS	NS	0.0
BHB	0.17	0.18	0.006	0.17	0.17	0.006	0.14	0.19	0.2	0.17	0.006	0.35	0.89	<0.001	0.02	NS	NS
NEFA	0.42	0.47	0.025	0.38	0.52	0.025	0.38	0.38	0.43	0.59	0.03	0.11	0.0002	<0.001	NS	<0.0001	NS
Cholesterol	3.33	3.21	0.11	3.28	3.26	0.11	2.37	3.36	3.58	3.74	0.09	0.44	0.85	<0.001	0.03	NS	NS
Triglycerides	0.18	0.23	0.007	0.21	0.20	0.007	0.23	0.21	0.19	0.18	0.007	<0.001	0.38	0.0002	NS	0.0048	0.00

Table 4. Effect of n-3 PUFA supplementation treatment (P), post-insemination (AI) diet and sample day on plasma concentrations of fatty acids

	n-3 PUFA			**Post AI Diet**			**Day (D)**				**Significance**					
Variable	**n-3 PUFA**	**CON**	**SEM**	**High**	**Low**	**SEM**	**-30**	**3**	**14**	**SEM**	**P**	**AI**	**D**	**P x AI**	**P x D**	**AI**
C11_1_c10	1.20	1.04	0.082	1.20	1.04	0.083	0.99	1.17	1.19	0.093	0.1951	0.1901	0.2521	NS	NS	
C12_0	0.90	1.00	0.066	0.99	0.92	0.066	1.43	0.63	0.79	0.057	0.3063	0.4932	<.0001	NS	NS	
C12_1	0.60	0.80	0.085	0.70	0.72	0.085	0.46	0.77	0.90	0.105	0.0834	0.8395	0.018	NS	P<0.0001	
C13_0	0.61	0.83	0.104	0.84	0.59	0.104	0.59	0.64	0.92	0.105	0.1493	0.1063	0.0341	NS	NS	
C13_1_c12	0.61	0.49	0.086	0.50	0.60	0.087	0.46	0.76	0.43	0.104	0.3425	0.4133	0.0541	NS	NS	
C14_0	3.90	4.14	0.341	3.97	4.07	0.327	4.95	3.33	3.78	0.407	0.6288	0.8383	0.0238	NS	NS	
C14_1_t	0.79	1.05	0.051	0.98	0.86	0.051	1.30	0.75	0.71	0.067	0.0028	0.1186	<.0001	NS	0.039	
C14_1_c	1.36	1.47	0.069	1.43	1.40	0.069	1.74	1.26	1.23	0.103	0.24	0.713	0.0031	NS	NS	
C15_0	1.68	1.65	0.065	1.68	1.65	0.065	1.90	1.65	1.45	0.076	0.8135	0.7344	0.0006	NS	0.019	
C15_1_c10	0.29	0.29	0.050	0.30	0.29	0.050	0.20	0.36	0.31	0.060	0.915	0.878	0.1331	NS	NS	
C16_0	23.70	26.86	0.474	25.24	25.50	0.479	27.09	25.07	23.94	0.485	0.0004	0.7052	0.0001	NS	<.0001	
C16_1_t	0.71	1.03	0.064	0.93	0.81	0.064	1.06	0.73	0.82	0.094	0.0044	0.229	0.0612	NS	NS	

C16_1_c	1.09	1.58	0.089	1.21	1.46	0.090	1.44	1.15	1.41	0.087	0.0015	0.0684	0.0213	NS	0.001	<.
C17_0	0.46	0.40	0.021	0.42	0.45	0.021	0.58	0.45	0.27	0.026	0.0732	0.3037	0.0001	NS	NS	
C17_1_c10	0.31	0.30	0.024	0.31	0.30	0.024	0.39	0.28	0.25	0.030	0.7777	0.8955	0.0039	NS	0.004	
C18_0	13.34	14.64	0.360	13.42	14.56	0.360	15.82	13.31	12.84	0.423	0.0215	0.0392	<.0001	NS	NS	0.
C18_1_t13	0.47	0.04	0.069	0.25	0.26	0.069	0.04	0.45	0.27	0.064	0.0004	0.9085	<.0001	NS	<.0001	0.
C18_1_t11	0.84	0.39	0.146	0.66	0.56	0.144	0.35	0.65	0.83	0.173	0.0455	0.6339	0.1526	NS	NS	
C18_1_c9	4.62	6.22	0.249	4.97	5.87	0.249	6.47	4.69	5.10	0.239	0.0003	0.0217	<.0001	NS	<.0001	<.
C18_1_t9	0.38	0.32	0.049	0.42	0.28	0.049	0.36	0.27	0.43	0.062	0.357	0.0559	0.2034	NS	NS	
C18_2_c9__c12	11.20	15.37	0.534	14.13	12.43	0.534	13.62	13.14	13.09	0.571	<.0001	0.0373	0.7358	NS	0.002	
Linoleic_n_6	0.12	0.41	0.030	0.29	0.23	0.030	0.34	0.19	0.26	0.040	<.0001	0.1793	0.0581	NS	0.002	
C20_0	0.24	0.12	0.020	0.19	0.16	0.020	0.13	0.22	0.19	0.038	0.0006	0.2655	0.3132	NS	NS	
Linolenic_n_3	1.33	1.43	0.051	1.32	1.44	0.051	2.05	1.03	1.05	0.062	0.2267	0.116	<.0001	NS	0.008	0
		-														
		0.0003														
C20_1_c11	0.04	3	0.013	0.03	0.01	0.013	<0.0001	0.05	0.01	0.015	0.0421	0.2935	0.0365	NS	NS	
C18_2_t_t	0.36	0.40	0.037	0.41	0.35	0.037	0.45	0.30	0.37	0.053	0.4169	0.2975	0.2265	NS	0.035	
C20_3_n_6	0.66	1.64	0.090	1.20	1.10	0.090	1.37	1.04	1.04	0.092	<.0001	0.4651	0.0083	NS	<.0001	

C22_0	0.12	0.14	0.035	0.14	0.12	0.035	0.19	0.10	0.10	0.037	0.7182	0.7483	0.1138	NS	NS	
C20_3_n_3	0.16	0.07	0.044	0.08	0.15	0.044	0.01	0.22	0.11	0.054	0.1271	0.3056	0.031	NS	NS	
C20_4_n_6	2.59	2.73	0.135	2.42	2.90	0.135	2.50	2.46	2.99	0.117	0.4572	0.0224	0.0001	NS	0.058	0.
EPA	11.32	1.05	0.396	5.85	6.51	0.396	1.24	8.96	8.35	0.353	<.0001	0.2581	<.0001	NS	<.0001	
C22_4	0.12	0.05	0.030	0.05	0.12	0.028	0.10	0.11	0.04	0.036	0.0789	0.1009	0.3137	NS	NS	
DPA	2.52	2.01	0.173	2.03	2.50	0.173	2.17	2.58	2.05	0.165	0.0509	0.0733	0.0211	NS	NS	
DHA	2.34	0.66	0.090	1.34	1.66	0.090	0.51	2.23	1.76	0.103	<.0001	0.0221	<.0001	NS	<.0001	0.
Total FA	94.45	94.79	0.372	94.48	94.75	0.368	94.57	94.70	94.58	0.403	0.5314	0.6073	0.9631	NS	NS	

Table 5. Effect of n-3 PUFA supplementation, post-insemination (AI) diet and sample day following AI on pregnancy rate, embryo length and on plasma concentrations of progesterone

	PUFA (P)			**Post AI Diet (AI)**			**Significance (P-value)**		
	n-3 PUFA	CON	SEM	High	Low	SEM	P	AI	P x AI
Variable									
Pregnancy Rate	0.73	0.77		0.71	0.79		0.53	0.16	NS
Embryo Length	5.58	7.78	0.403	9.74	3.62	0.420	0.004	<0.001	NS
Day Post-AI	*Plasma Progesterone (ng/ml)*								
Day 0	0.48	0.43	0.252	0.43	0.48	0.251	0.877	1.00	0.05
Day 4	1.52	1.45	0.311	1.24	1.73	0.311	0.666	0.998	0.87
Day 5	2.74	2.85	0.309	2.48	3.11	0.310	0.993	0.994	0.50
Day 6	3.90	4.14	0.315	3.70	4.34	0.315	0.707	0.994	0.10
Day 7	5.12	5.50	0.252	4.76	5.86	0.252	0.237	0.165	0.30
Day 10	7.76	8.17	0.251	6.91	9.01	0.251	0.195	<0.0001	0.22
Day 14	9.35	9.71	0.303	8.66	10.40	0.302	0.641	0.006	0.62
Day 15	10.06	10.64	0.230	9.51	11.19	0.298	0.802	0.009	0.66
Day 16	10.08	10.07	0.298	8.57	11.58	0.301	1.00	<0.0001	0.76
AUC	81.04	81.10	3.212	74.70	87.34	3.210	0.993	0.006	0.149

Table 6. Linear regression coefficients (SE) for relationship between plasma concentrations of progesterone and embryo length on various days post insemination

Day	**β_0**	**β_1**	**R^2**	**P-value (β_1)**
0	8.54 (1.14)	-5.79 (2.88)	0.06	0.04
4	3.02 (1.62)	1.97 (0.78)	0.10	0.01
5	2.25 (1.82)	1.41 (0.53)	0.11	0.01
7	1.74 (2.06)	0.84 (0.33)	0.10	0.01
15	2.13 (2.40)	0.40 (0.21)	0.06	0.05

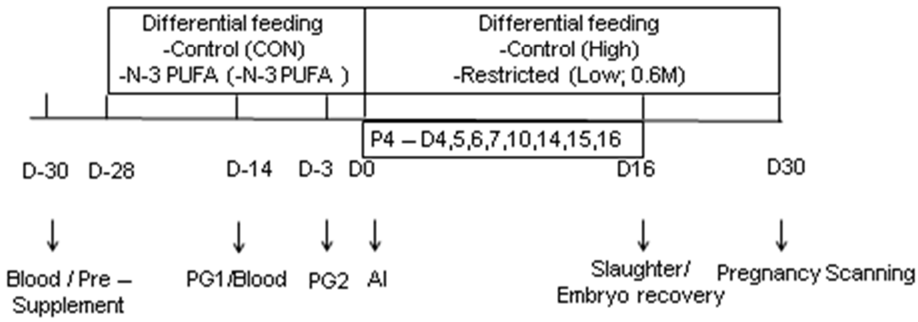

Figure 1. Experimental design. Animals were offered either a control (n=93) or n-3 PUFA (n= 92) supplemented diet for 28 days prior to AI. On the day of AI animals were further assigned within original dietary treatment to either a high or low plane of nutrition until Day 16 (slaughter; n=92) or 30 (pregnancy diagnosis; n= 87) of pregnancy.

Highlights

- Dietary supplementation of beef heifers with n-3 PUFA increased plasma concentrations of n-3 PUFA and IGF-1 and led to larger embryos 16 days after insemination. However this did not result in improved pregnancy rates.
- There was evidence for a reduction in the hepatic expression of some key genes coding for enzymes involved in progesterone degradation in heifers supplemented with n-3 PUFA.
- Offering a nutrient restricted diet directly after insemination reduced systemic concentrations of IGF-1 and embryo length, increased progesterone concentrations during the mid to late luteal phase but did not affect pregnancy rate