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**Effect of supplementation with different fat sources on the mechanisms involved
in reproductive performance in lactating dairy cattle**

RUNNING HEAD: FATTY ACIDS AND REPRODUCTION

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ABSTRACT

Supplementary fat positively influences reproductive performance in dairy cattle, although the mechanisms involved are not clearly defined. Our objective was to determine the effects of four different fat supplements on follicle development, plasma steroid hormone concentrations and prostaglandin (PG) synthesis in lactating dairy cattle. Forty-eight early lactation Holstein-Friesian cows (21 primiparous, 27 multiparous) were used in a completely randomised block design. Cows were fed the

35 same basal TMR diet and received one of four fat supplements: (i) palmitic acid (18:0
36 fatty acid; Control), (ii) flaxseed (rich in 18:3 n-3 fatty acid; Flax), (iii) conjugated
37 linoleic acid (a mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers; CLA), and (iv)
38 fish oil (rich in 20:5 and 22:6 n-3 fatty acids; FO). All lipid supplements were
39 formulated to be isolipidic; palmitic acid was added as necessary to provide a total
40 lipid supplement intake of 500 g/d. Cows were synchronised to be in oestrus on day
41 15 of dietary treatment. All antral follicles were counted, and dominant follicles,
42 subordinate follicles and corpora lutea were measured daily via transrectal ovarian
43 ultrasonography for one complete oestrous cycle. Blood samples were collected daily,
44 and selected samples were analysed for progesterone, oestradiol, insulin-like growth
45 factor-1, insulin, cholesterol and non-esterified fatty acids. Oestrus was synchronised
46 a second time, and liver and endometrial biopsies were collected on day 7 of the
47 oestrous cycle. Gene expression was evaluated for a number of genes involved in
48 prostaglandin synthesis (endometrium) and fatty acid uptake and utilisation (liver).
49 Fat supplementation had little effect on follicle development. Cows receiving
50 supplementary n-3 fatty acids had lesser plasma progesterone (P4) and smaller
51 corpora lutea than cows receiving the CLA or Control supplements. Effects of fat
52 supplementation on the endometrial expression of genes involved in PG synthesis
53 were minor. Hepatic expression of *SREBF1*, *ASCL1* and *FABP1* was reduced by FO
54 supplementation. Reduced plasma P4 in n-3 supplemented cows may lead to a
55 suboptimal uterine environment for embryo development and hence reduced fertility
56 compared to cows receiving the control or CLA supplements.

57
58 **KEYWORDS:** Fatty acids, reproduction, dairy cattle, progesterone.

1. INTRODUCTION

Dairy cow diets are supplemented with fat primarily to increase the energy density of the diet to enhance milk production, growth and reproduction. It was initially thought that feeding energy dense fat supplements in early lactation, when the dairy cow is experiencing a period of negative energy balance, would improve the energy status of the animal and consequently improve reproductive performance. When fat is fed in early lactation, however, cows either consume less feed or increase milk production and energy status is seldom altered [1,2]. It has since been suggested that any benefits of feeding fat may be independent of energy status, and may instead be due to specific effects on the pituitary gland, ovaries and uterus, mediated by the fatty acid composition of the fat source [3]. Potential improvements in dairy cow fertility with supplemental fat have generally been associated with increased dominant follicle diameter, improved oocyte and embryo quality, greater progesterone (P4) concentrations, and modulation of prostaglandin (PG) synthesis, collectively resulting in increased likelihood of conception [4,5,6].

Trans-10, *cis*-12 CLA, an n-6 polyunsaturated fatty acid (PUFA), is a potent inhibitor of milk fat synthesis, and has recently been demonstrated to decrease milk energy output, with subsequent improvements in energy balance and body condition score [7]. These improvements in energy balance have been shown to have a beneficial effect on reproductive performance [8]. Other potential mechanisms by which CLA might improve reproductive performance are yet to be fully elucidated.

Flaxseed oil is high in the essential n-3 PUFA α -linolenic acid, and has been shown to increase the size of the dominant follicle and reduce pregnancy losses [9]. Fish oil contains substantial amounts of the long chain n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Recent studies have highlighted the

potential to manipulate PG synthesis by adding n-3 PUFA supplements to the diet. Increasing the proportion of n-3 PUFA in the diet can result in increased synthesis of the less biologically active 3-series PGs at the expense of PGF_{2α} [10,11]. Studies conducted *in vitro* [12], and *in vivo* using beef heifers [13] demonstrated that n-3 PUFA supplementation can alter endometrial expression of genes regulating PGF_{2α} synthesis, potentially leading to a reduction in uterine PGF_{2α} production. Inhibition of uterine PGF_{2α} secretion may delay the regression of the corpus luteum (CL), and hence improve embryo survival.

The present study was conducted to determine the effects of four different fat supplements on follicle and CL development, steroid hormone concentrations and endometrial and hepatic gene expression in lactating dairy cows.

2. MATERIALS AND METHODS

2.1. Animals and treatments

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (1876) and the European Community Directive 86/609/EEC. Twenty one primiparous and 27 multiparous Holstein-Friesian cows were blocked on the basis of parity, calving date, average daily milk yield and body condition score (BCS), and randomly assigned to one of four dietary fat supplements at 38 days in milk (DIM) (+/- 11 days (S.D)): 500 g palmitic acid (Palmit 80, Trouw Nutrition, Belfast, UK) (Control); 200 g pure pressed flax seed (Flax 30, Bob Allen Feeds, Bandon, Ireland) containing 72 g α-linolenic acid (Flax); 100 g lipid-encapsulated conjugated linoleic acid (CLA) (Lutrell Pure, BASF, Ludwigshafen, Germany) containing 10 g each of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA (CLA); or 300 g of a partially rumen protected fish oil

110 supplement (Trouw Nutrition, Belfast, UK) containing 30 g each of EPA and DHA
111 (FO). All diets were balanced with Palmit 80 so that each treatment provided 500 g of
112 lipid per cow per day, and manually mixed with 1.5 kg of a specially formulated
113 concentrate to ensure palatability. This ration was fed in individual feed troughs and
114 consumed in a single meal following the morning milking.

115 The cows were housed in a free-stall barn from parturition until the end of the 70-day
116 treatment period, with the treatment groups sharing common accommodation space.
117 Individual dry matter intake (DMI) was measured daily throughout the trial using the
118 Griffith Elder Mealmaster feeding system (Griffith Elder Ltd, Suffolk, UK). Forage
119 mangers were mounted on electronic weighing scales, and cows had ad-libitum access
120 to the TMR, which was offered to allow for feed refusals of 5%. Refusals were
121 removed daily. Weekly samples of all feeds offered were dried and ground and
122 composited on a fortnightly basis for nutrient analysis. The dry matter, ash, crude
123 protein, neutral detergent fibre and oil content of the feeds were determined as
124 described by McNamara et al. [14]. The ingredient and nutrient composition of the
125 TMR and concentrate ration are reported in Table 1. Samples of the four fat
126 supplements used in the study were also collected and fatty acid content determined
127 by gas liquid chromatography, as described by Childs et al. [15] following lipid
128 extraction using a chloroform/methanol/water mixture [16], and methylation using
129 NaOCH₃/methanol and BF₃/methanol [17]. The fatty acid composition of the
130 supplements offered is shown in Table 2.

131 Cows were milked twice daily at 0730 and 1500, and milk yield (kg) was recorded
132 daily at the morning and evening milkings using electronic milk meters (Dairy
133 Master, Causeway, Co. Kerry, Ireland). Milk composition (fat, protein and lactose)
134 was determined on two days per week from successive evening and morning milk

samples using a Milkoscan 605 (Foss Electric, Hillerod, Denmark). The following equation was used to calculate solids corrected milk yield (SCM) [18]:

$$\text{SCM} = (12.3 \times \text{milk fat yield} + (6.56 \times (\text{milk protein yield} + \text{milk lactose yield})) - (0.0752 \times \text{milk yield}))$$

Body condition score was recorded every two weeks using a 1 to 5 scale (1 = emaciated, 5 = extremely fat) with 0.25 increments [19]. Energy balance (EBAL) was estimated as the difference between energy intake and the sum of energy requirements for maintenance and milk production, using the French net energy system [20]. This system uses unité fourragère lait (UFL) as the unit of net energy, which is equivalent to 1 kg of standard air-dried barley. The following equations were used to determine the energy required for maintenance and output in milk [21]:

$$\text{Energy required for maintenance (UFL/d)} = 1.4 + 0.6 \times \text{BW}/100;$$

$$\text{Energy requirement for milk (UFL/kg of milk)} = 0.0054 \times \text{FC} + 0.0031 \times \text{PC} + 0.0028 \times \text{LC} - 0.015; \text{ where FC = fat concentration (\%), PC = protein concentration (\%), and LC = lactose concentration (\%).}$$

Of the 48 cows on the trial, 9 cows failed to respond to synchrony treatment, and a further 5 cows did not ovulate at the subsequent spontaneous oestrus. In addition, two cows were removed from the study due to illnesses unrelated to the experimental treatments.

2.2. Synchronisation of the oestrous cycle

A protocol to synchronise oestrus was initiated on day 5 of dietary treatment with injection (i.m.) of GnRH (0.01 mg Buserelin (Receptal); Intervet, Dublin) and insertion of an intravaginal progesterone releasing device (Eazi-breed CIDR containing 1.38g P4, Pfizer Animal Health, Dublin, Ireland). On day 12 of dietary

treatment each cow received an injection (i.m.) of PGF_{2α} (Lutylase, Pfizer Animal Health), and the following day the CIDR was removed. All cows had tail paint applied at CIDR removal, and were observed for oestrus over the next 5 days. The schedule of all experimental procedures performed is presented in Table 3.

2.3. Monitoring ovarian activity

Follicles were counted and measured, and corpora lutea measured for each cow via daily transrectal ovarian ultrasonography (Aloka SSD-900, Aloka Ltd, Tokyo, Japan, 7.5 MHz transducer) from the day after CIDR removal until ovulation following the next spontaneous oestrus. In order to measure follicles and corpora lutea, images were frozen on screen during ultrasonography, and dimensions were measured with internal callipers on the Aloka SSD-990. The position of each large (≥ 10 mm diameter) follicle on the ovary was mapped daily to facilitate identification of the dominant follicle in each wave of follicular growth. Corpora lutea dimensions were recorded and used to calculate the average diameter and radius. Corpus luteum volume (V) was then calculated with the formula $V = 4/3 \times \pi \times \text{radius}^3$. For CL with a fluid-filled lacuna the volume of the lacuna was calculated and subtracted from the total volume of the CL.

2.4. Blood sampling and hormone and metabolite analysis

Blood samples were collected from each cow after the morning milking on the day of initiation of dietary treatment, on day 7 of dietary treatment, on the day after CIDR removal, and daily thereafter until the day of ovulation at the spontaneous oestrus following the synchronised oestrus. Blood samples were collected from the coccygeal vessels into lithium heparin vacutainers. Following collection, all blood samples were

185 centrifuged at 2000 x *g* for 15 min at 5 °C. The plasma was harvested and decanted
 186 into 1.5 mL tubes, sealed with an airtight cap and stored at -20 °C until further
 187 analysis.

188 All blood samples collected were analysed for plasma P4 concentration, and samples
 189 from days 0, 15, 25 and 35 of dietary treatment were analysed for insulin-like growth
 190 factor-1 (IGF-1), insulin, cholesterol and non-esterified fatty acid (NEFA)
 191 concentrations. Ultrasonography and heat detection records were used to select the
 192 blood samples analysed for oestradiol (E2) concentrations. Three consecutive samples
 193 from the day prior to synchronised oestrus, and 7 consecutive samples from 5 days
 194 prior to spontaneous oestrus were analysed for E2 concentration.

195 Plasma P4 and insulin concentrations were determined using solid-phase fluoro-
 196 immunoassays (AutoDELFIA, PerkinElmer Life and Analytical Sciences, Turku,
 197 Finland), with appropriate kits (Unitech BD Ltd., Dublin, Ireland). Plasma IGF-I
 198 concentrations were quantified by radioimmunoassay, following
 199 ethanol:acetone:acetic acid extraction as described by Butler et al. [22]. Plasma E2
 200 concentrations were determined as described by [23], using an E2 MAIA assay kit
 201 (Biostat Ltd, Stockport, UK). All hormone assays included a high, medium and low
 202 control, each treatment was equally represented in each assay, and all samples for a
 203 cow on a given treatment were completed in a single assay. Inter-assay coefficients of
 204 variation (CV) for P4 were 2.5% (high), 10.4% (medium) and 20.8% (low), and intra-
 205 assay CV for P4 were 7.0% (high), 8.3% (medium), 21.8% (low). Inter-assay CV for
 206 insulin were 7.9% (high), 13.1% (medium) and 21.3% (low), and intra-assay CV for
 207 insulin were 10.9% (high), 12.5% (medium) and 15.9% (low). Inter-assay CV for
 208 IGF-1 were 8.2% (high), 2.2% (medium) and 11.9% (low), and intra-assay CV for
 209 IGF-I were 9.7%, (high), 9.5% (medium) and 11.4% (low). Inter-assay CV for E2

were 12.7% (high), 9.8% (medium) and 25.7% (low), and intra-assay CV for E2 were 8.0 % (high), 8.9 % (medium) and 38.2 % (low). Plasma cholesterol and NEFA concentrations were determined by enzymatic colorimetry using appropriate kits (cholesterol kit supplied by Horiba ABX, Montpellier, France, NEFA kit supplied by Wako Chemicals, GmbH, Nissanstrasse, Germany).

2.5. Tissue biopsies

Following the synchronised cycle when cows were scanned daily, the cows were given a 10-day period of rest. The cows were then re-synchronised with two injections (i.m.) of PGF_{2α} (Lutylase, Pfizer Animal Health) after consecutive morning and evening milkings. Tail paint was applied to aid heat detection, and transrectal ultrasonography was used following observed oestrus to confirm ovulation. Liver and endometrial biopsies were collected 7 days after ovulation to examine gene expression at this stage of the oestrous cycle.

For the liver biopsy, a site between the 11th and 12th ribs was shaved, sanitised with Videne (Povidone-iodine, 7.5%; Ecolab, Leeds, UK) and methylated spirits, and anaesthetised with Willcain (Dechra Ltd, Shrewsbury, UK). An incision of approximately 1 cm was made through the skin and the biopsy instrument was used to pierce the intercostal muscles and peritoneum. The liver was located and a 1 to 1.5 g sample removed. The sample was immediately washed in saline, blotted dry, snap frozen in liquid nitrogen and stored at -80 °C. The incision site was sutured and treated topically with Duphacycline spray (Interchem, Naas, Ireland) and cows were given 10 mL of a subcutaneous antibiotic as a prophylactic (Excenel RTU, Pfizer Animal Health). For the endometrial biopsy, animals were given caudal epidural anaesthesia with Willcain, the external genital area was sanitized, and a biopsy tool

passed through the cervix and into the uterine horn ipsilateral to the CL by using transrectal manipulation. The open jaws of the biopsy basket (2×1 mm) were pressed against the endometrium and samples (approximately 100 mg) of endometrium removed. The samples were then washed in saline, blotted dry, snap frozen in liquid nitrogen, and stored at -80 °C. Animals were given 500 mg of an intrauterine prophylactic antibiotic (Metricure, Intervet, Bray, Co Wicklow, Ireland).

2.6. RNA extraction and cDNA synthesis

Total RNA was isolated from frozen tissue using TRI[®] Reagent and chloroform (Sigma-Aldrich Ireland Ltd. Dublin, Ireland). Homogenisation of the sample in TRI[®] Reagent was performed using a tissue lyser (Qiagen Ltd., Crawley, UK) and the RNA was subsequently extracted using chloroform and precipitated using isopropanol. Quantity and quality of total RNA was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA) and the Agilent Bioanalyser 2100 with the RNA 6000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd. Dublin, Ireland), respectively. RNA quality was verified by ensuring all RNA samples had an absorbance ($A_{260/280}$) of between 1.8 and 2 and RINs of between 7 and 10. RNA samples were treated with RQ1 RNase-free DNase (Promega UK Ltd., Southampton, UK) and purified using the RNeasy1 mini kit (Qiagen Ltd.). DNase-treated and purified total RNA was then reverse transcribed to cDNA, with random hexamers, using the High Capacity cDNA Reverse Transcription kit (Applied Biosciences, Ireland) according to the manufacturers instructions and stored at -20 °C for subsequent analyses.

2.7. Primer design and reference gene selection

All candidate and reference gene primers used to detect endometrial and hepatic gene expression (Table 4) in this study were designed using the Primer3 web based software program (<http://frodo.wi.mit.edu/primer3/>) [24], and obtained from a commercial supplier (Sigma Aldrich Ltd., Dublin, Ireland). Primer specificity was checked using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). All the primers were validated using a pooled cDNA sample. A standard curve was generated using serial dilutions of pooled cDNA. PCR products generated by amplification were sequenced to verify their primer specific identity (Biochemistry DNA Sequencing Facility, University of Cambridge). To determine the relative gene expression levels, suitable highly stable reference genes were required. In the current study, out of five tested, three reference genes for both endometrial (beta-actin (*ACTB*), ubiquitin (*UBQ*) and ribosomal protein S9 (*RPS9*)) and hepatic tissue (adenylate cyclase-associated protein (*CAPI*), proteasome 26S subunit, non-ATPase (*PSMD*) and *ACTB* were used to normalise gene expression data. The principle behind the selection of the reference gene is that the expression ratio of two perfect reference genes should be constant across all samples. The expression stability of the reference genes was validated with the software program, geNorm version 3.5, by calculating the gene expression stability measure (M value). Using three reference genes, the M values were 0.56 (*ACTB*, *UBQ*, *RPS9*) and 0.37 (*ACTB*, *PDSM2*, *CAPI*) for endometrial and hepatic RNA samples, respectively, both of which were below the default minimum coefficient of 1.5 as specified by the geNorm program [25].

2.8. Quantitative real time PCR (qPCR)

Following reverse transcription, cDNA quantity was determined and standardised to the required concentration for qPCR. Triplicate 20 µL reactions were carried out in 96-well optical reaction plates (Applied Biosystems, Warrington, UK), containing 1 µL cDNA (10-50 ng of RNA equivalents), 10 µL Power SYBR® Green PCR Master Mix (Applied Biosystems), 8 µL nuclease-free H₂O, and 1 µL forward and reverse primers (250-1000 nM per primer). Assays were performed using the ABI 7500 Fast qPCR System (Applied Biosystems) with the following cycling parameters: 95 °C for 20 s and 40 cycles of 95 °C for 30 s, 60 °C for 30 s followed by amplicon dissociation (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s). Gene expression levels were recorded as Ct values, i.e. the number of PCR cycles at which the fluorescence signal is detected above the threshold value and all samples were run in triplicate. Amplification efficiencies were determined for all candidate and reference genes using the formula $E=10^{(-1/\text{slope})}$, with the slope of the linear curve of cycle threshold (Ct) values plotted against the log dilution [26]. Primer concentrations were optimized for each gene and dissociation curves were examined for the presence of a single PCR product. The efficiency of the reaction was calculated using a 5-fold serial dilution of cDNA and generation of a standard curve. All PCR efficiency coefficients were between 0.9 and 1.1 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 Ct 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 Ct and log₂ transformation of the expression values for all genes.

2.9. Statistical analysis

All statistical analyses were carried out using SAS (SAS System Inc., Cary NC, USA). Daily measurements of milk yield, DMI and EBAL were collapsed into weekly means. A test for normality was performed on all the blood analyte data using the UNIVARIATE procedure of SAS. Insulin, IGF-1 and NEFA variables were log-transformed prior to analysis to generate a normal distribution. Milk production, milk composition, DMI, BCS, EBAL, blood analyte and CL volume data were analysed using mixed models with repeated measures, using the satterthwaite adjustment to calculate denominator degrees of freedom. The appropriate covariance structure for each repeated measures analysis was identified based on Akaike's Information Criterion (AIC) model fit statistic. Where appropriate, measurements made during the 3 wk prior to the initiation of dietary treatment were included as covariates in the models for milk yield and composition, IGF-I, insulin, NEFA, cholesterol, DMI, BCS and calculated EBAL. Parity and calving date were included as adjustment variables in all repeated measures models; if non-significant, these variables were removed and the models were re-run. Follicle development, peak plasma E2 concentrations at oestrus, and day of luteolysis data were analysed using mixed models procedures with treatment as a fixed effect, block as a random effect, and calving date and parity included as adjustment variables.

Day of luteolysis was defined as the day before plasma P4 declined to less than 50% of the average for the four maximum P4 concentrations in the cycle. Additionally, plasma P4 must have declined again to less than 25% of the 4 maximum average P4 concentrations the following day [27]. Individual cows began undergoing luteolysis from day 14 after ovulation onwards; therefore P4 and CL volume data after day 14 were not included in the statistical analysis. Further specific analysis of plasma P4

concentration and CL volume was performed on data from days 5-7 after ovulation and from days 8-14 after ovulation.

All gene expression data was tested for normality using the UNIVARIATE procedure of SAS. A Box-Cox transformation analysis was performed using the TRANSREG procedure in SAS to obtain the appropriate lambda value for data that were not normally distributed. These data were then transformed by raising the variable to the power of lambda. Mixed models (PROC MIXED) were used to determine the effect of fat supplementation on the relative expression of each gene measured. The Tukey critical difference test was used to determine statistical differences between treatment means. In all statistical analyses, contrast statements were used to compare individual fat treatments against one another, and also to compare the two n-3 supplements combined (Flax and FO) against both Control and CLA supplements. Data were considered significant when $P < 0.05$, and a trend declared when $P < 0.1$.

3. RESULTS

3.1. Milk production, dry matter intake, body condition score and energy balance

Milk production, DMI, BCS and EBAL data are summarised in Table 5. Milk yield was greater for CLA-supplemented cows compared with all other treatments ($P = 0.007$); the remaining treatments did not differ from each other. There was a significant effect of treatment on milk fat concentration and yield (both $P < 0.001$). CLA supplementation reduced milk fat concentration and yield compared with all other treatments, and FO cows had reduced milk fat concentration and yield compared with those on the Control and Flax diets. Milk protein concentration was reduced for CLA-supplemented cows compared with cows receiving Control and Flax treatments ($P = 0.018$). Milk protein yield was reduced for FO-treated cows compared with both

Flax and CLA-supplemented cows, and was greater for Flax-treated cows compared with cows receiving the Control diet ($P = 0.03$). Milk lactose concentration was increased for cows receiving FO compared with those receiving the Control and CLA treatments, and cows receiving the Flax supplement had elevated milk lactose concentrations compared with cows on the CLA diet ($P = 0.008$). Overall, solids-corrected milk yield (SCM) was increased for cows on the Control and Flax diets compared with cows on the CLA and FO diets ($P < 0.001$). There was no effect of treatment on DMI, EBAL or BCS.

3.2. Ovarian follicular dynamics and peak oestradiol concentrations

Follicle development is summarised in Table 6. The type of fat supplement tended to affect both the length of the oestrous cycle and the number of follicles in the first wave of the cycle ($P = 0.09$ and 0.07 , respectively). The oestrous cycle of cows receiving the Flax supplement was 3.5 d shorter than for the Control group ($P = 0.04$), and 4.6 d shorter compared with cows offered the FO supplement ($P = 0.02$). The FO-supplemented cows had 2.2 fewer follicles in the first wave of the cycle compared with those fed the Control supplement ($P = 0.01$). Peak plasma E2 at the synchronised oestrus was 1.62 pg/mL greater ($P = 0.046$) in Flax-supplemented cows compared with FO-supplemented cows, and tended to be 1.32 pg/mL greater ($P = 0.08$) in Flax-supplemented cows compared with cows receiving the Control supplement. Peak plasma E2 at the spontaneous oestrus tended to be 0.85 pg/mL greater ($P = 0.09$) in Flax-supplemented cows compared with cows receiving the Control supplement. There was no overall treatment effect on the day of first wave peak follicle diameter, but peak follicle diameter in the first follicular wave occurred 1.5 days earlier after

ovulation in Control cows compared with cows receiving the FO diet ($P = 0.04$). No other follicle or E2 variables were affected by the type of fat supplement.

3.3. Plasma progesterone concentrations and corpus luteum volumes

The effect of fat supplementation on plasma P4 and CL volume is shown in Table 7. and Figure 1.

Days 2-14 after ovulation: There was a significant effect of treatment on both plasma P4 concentration and CL volume from days 2 – 14 of the oestrous cycle ($P = 0.013$ and 0.04 respectively). Mean plasma P4 concentrations were 0.62 ng/mL ($P = 0.04$) and 0.87 ng/mL ($P = 0.001$) less for cows receiving the Flax supplement compared with cows receiving the Control and CLA supplements, respectively. Mean plasma P4 concentrations in cows on the FO diet were 0.67 ng/mL ($P = 0.03$) and 0.92 ng/mL ($P = 0.01$) less compared with cows receiving the Control and CLA supplements, respectively. Corpus luteum volumes were 1464 mm^3 less ($P = 0.03$) and 2077 mm^3 less ($P = 0.005$) for FO-treated cows compared with cows receiving the Control and CLA supplement, respectively. The n-3-supplemented cows had lesser plasma P4 compared with Control (4.48 ng/mL vs. 5.13 ng/mL , $P = 0.01$) and CLA-supplemented cows (4.48 ng/mL vs. 5.38 ng/mL , $P = 0.004$). Corpus luteum volumes were lesser in n-3-supplemented cows compared with CLA-supplemented cows (6355 mm^3 vs. 7965 mm^3 , $P = 0.01$). There was a tendency for primiparous animals to have smaller CL volumes than multiparous cows (6380 mm^3 vs. 7632 mm^3 , $P = 0.06$).

There was no effect of treatment on the number of days from ovulation to luteolysis.

Days 5-7 after ovulation: There was no overall treatment effect on either plasma P4 or CL volumes; however, CLA-treated cows had 1.17 ng/mL greater plasma P4 concentrations ($P = 0.04$) and 2507 mm^3 larger CL volumes compared with cows

receiving the FO diet ($P = 0.03$). The n-3-supplemented cows had reduced plasma P4 concentrations (3.32 ng/mL vs. 4.24 ng/mL, $P = 0.04$) and CL volumes (6249.7 mm³ vs. 8125.7 mm³, $P = 0.04$) compared with the CLA-supplemented cows.

Days 8-14 after ovulation: There was a significant effect of treatment on plasma P4 from days 8-14 after ovulation. Mean plasma P4 concentrations in CLA treated cows were 1.20 ng/mL greater ($P = 0.006$) and 1.28 ng/mL greater ($P = 0.007$) compared with cows receiving the Flax and FO supplements, respectively. The n-3-treated cows had lesser mean plasma P4 concentrations compared with both the Control (6.50 ng/mL vs. 7.20 ng/mL, $P = 0.04$) and CLA-supplemented (6.50 ng/mL vs. 7.74 ng/mL, $P = 0.002$) cows. There was no overall treatment effect on CL volume on days 8-14 after ovulation, but both CLA-treated (9668.8 mm³ vs. 7353.7 mm³, $P = 0.04$) and Control (9490.2 mm³ vs. 7353.7 mm³, $P = 0.05$) cows had larger CL compared with cows receiving the FO diet.

3.4. Metabolites and metabolic hormones

Mean IGF-1, insulin, cholesterol and NEFA concentrations on days 15, 25 and 35 of dietary treatment are presented in Table 7. The type of fat supplement tended to affect plasma insulin ($P = 0.09$) and IGF-1 ($P = 0.07$) concentrations. Plasma insulin concentrations were 0.94 μ UI/mL greater ($P = 0.02$) in Control-treated cows compared with cows receiving the FO supplement. Plasma IGF-1 concentrations were 15.7 ng/mL greater ($P = 0.008$) for cows receiving the Flax diet compared with cows receiving the Control diet. The n-3-supplemented cows had greater plasma IGF-1 concentrations compared with cows receiving the Control diet (84.6 ng/mL vs. 73.4 ng/mL, $P = 0.03$)

There was a significant effect of treatment on plasma cholesterol concentrations ($P = 0.02$). Plasma cholesterol concentrations in CLA-supplemented cows were 0.97 mmol/L greater ($P = 0.002$) and 0.75 mmol/L greater ($P = 0.01$) compared with cows receiving the Control and FO supplements, respectively. The CLA-supplemented cows had greater plasma cholesterol concentrations compared with cows receiving the n-3 supplements (5.94 mmol/L vs. 5.32 mmol/L, $P = 0.02$).

There was a significant effect of treatment on plasma NEFA concentrations ($P = 0.01$). Cows receiving the Control supplement had 0.05 mmol/L less ($P = 0.03$), 0.07 mmol/L less ($P = 0.007$), and 0.08 mmol/L less ($P = 0.003$) plasma NEFA concentrations compared with cows receiving the Flax, CLA and FO supplements, respectively. Plasma NEFA concentrations were greater in n-3-treated cows compared with cows receiving the Control supplement (0.23 mmol/L vs. 0.16 mmol/L, $P = 0.003$).

3.5. Endometrial gene expression

Endometrial gene expression data are presented in Table 8. There was a significant effect of treatment on *PPAR γ* expression ($P = 0.015$). Endometrial *PPAR γ* expression was increased 1.9 fold ($P = 0.005$) and 1.5 fold ($P = 0.05$) in cows receiving the FO diet compared with cows offered the Control and CLA diets, respectively. Flax-treated cows had 1.7 fold greater *PPAR γ* expression compared with Control-treated cows ($P = 0.008$). The mRNA abundance of *PPAR γ* was increased 1.8 fold ($P = 0.002$) and 1.4 fold ($P = 0.03$) for n-3-treated cows compared with cows receiving the Control and CLA diets, respectively. Fat supplementation tended to affect both *PPAR δ* and *PTGS2* expression ($P = 0.09$ and 0.08 respectively). Endometrial *PPAR δ* expression was 2.0 fold greater for cows receiving the Flax treatment compared with

those receiving the Control supplement ($P = 0.02$). When the two n-3 treatments were combined, *PPAR δ* expression was 2.0 fold greater ($P = 0.02$) compared with cows receiving the Control diet. Endometrial *PTGS2* expression was 2.6 fold ($P = 0.01$) and 2.4 fold greater ($P = 0.04$) for Flax-treated cows compared with Control and FO-supplemented cows, respectively. Although there was no overall treatment effect on endometrial *PGFS2* expression, a 2.1 fold increase ($P = 0.04$) in *PGFS2* expression for n-3-supplemented cows compared with Control cows was observed. There was no overall treatment effect on endometrial *PLA₂* expression, but cows receiving the Flax treatment tended to have a 2.0 fold increase ($P = 0.07$) in endometrial *PLA₂* expression compared with Control-supplemented cows. There was no effect of treatment on endometrial expression of *PPAR α* , *OTR*, *PGFS1*, *PGES*, *CBR1* or *FADS2*.

3.6. Hepatic gene expression

Hepatic gene expression data are presented in Table 8. There was a significant effect of treatment on hepatic *ACSL1*, *FABP1* and *SREBF1* expression. Hepatic expression of *ACSL1* was increased for cows on Control (2.2 fold, $P = 0.002$), Flax (2.2 fold, $P = 0.002$) and CLA (2.3 fold, $P = 0.001$) treatments compared with cows receiving the FO treatment. Hepatic expression of *FABP1* was increased in cows on the Flax (3.7 fold, $P < 0.001$), Control (3.0 fold, $P = 0.005$) and CLA (3.0 fold, $P = 0.006$) treatments compared with cows on the FO treatment. Hepatic expression of *SREBF1* was increased in cows on the Control (3.5 fold, $P = 0.003$), Flax (2.8 fold, $P = 0.014$) and CLA (3.6 fold, $P = 0.002$) treatments compared with cows on the FO treatment. There was no effect of fat supplementation on hepatic expression of *ACADVL*, *ACOX1*, *CPT1A*, *DGAT1*, *GPAM*, *SCAP*, *SLC27A1*, *PPAR α* , *CYP3A*, or *CYP2C*.

4. DISCUSSION

The objective of the current study was to identify the effects of four fat supplements differing in fatty acid composition on follicle and CL development, steroid hormone concentrations and endometrial and hepatic gene expression in lactating dairy cows. The most important finding from this study was the differential effects of the fat supplements on plasma P4 concentration and CL volume.

The central role that P4 plays in optimising the uterine microenvironment to support embryo development in early pregnancy is widely accepted [28]. Cows that displayed both a delayed rise in plasma P4 concentrations after ovulation and lower luteal phase plateau concentrations of plasma P4 had retarded embryo development, with little or no interferon- τ (IFN- τ) production on day 16 after insemination compared with cows that had a normal P4 profile [29]. In the current study, mean plasma P4 concentrations were reduced for the n-3-supplemented cows throughout the oestrous cycle. Elevated P4 concentrations on days 5-7 and days 8-14 after ovulation were observed in cows on the CLA and Control treatments compared with cows on the n-3 supplements. The increase in plasma P4 at critical times in the oestrous cycle could alter uterine endometrial secretions to stimulate embryo development, leading to a larger embryo capable of signalling its presence to the dam. The differences in plasma P4 concentrations between the treatments were accompanied by similar differences in CL volume.

Dietary fat supplementation has consistently increased plasma cholesterol concentrations [2], the precursor for P4 synthesis. However, feeding whole flaxseed has been reported to decrease plasma cholesterol concentrations compared to fat

sources low in n-3 PUFA [30,31], and incubation of dispersed luteal cells with EPA and DHA reduced P4 concentrations [32]. In the current study, cows on the Control diet had the lowest concentrations of plasma cholesterol, yet greater plasma P4 concentrations compared with n-3-treated cows. This indicates that cholesterol availability was not the limiting factor in P4 synthesis, and that alternative mechanisms are responsible for the variations in plasma P4 concentrations observed. Transport of cholesterol from the cytoplasm to the inner cell membrane, mediated by the steroidogenic acute regulatory protein (StAR), is the rate limiting step in P4 biosynthesis [33]. Once inside the mitochondrion, Cytochrome P450 cholesterol side chain cleavage enzyme (P450scc) converts cholesterol to pregnenolone, which is then converted to P4 by 3 β -hydroxysteroid dehydrogenase/ Δ^5 , Δ^4 isomerase (3 β -HSD) [33]. It is possible that the dietary treatments utilised in the current study influenced the luteal expression and activity of one or more of the genes involved in P4 biosynthesis.

Following ovulation, the granulosa cells of the follicle differentiate into large luteal cells to form the CL, and these large luteal cells may be responsible for up to 80% of progesterone secretion in the mature CL [34]. It has been suggested that an increase in CL volume in cows supplemented with PUFA may be due to increased numbers of granulosa and theca cells from a larger dominant follicle [35]. The lack of treatment differences in ovulatory follicle diameter in the current study does not support this hypothesis. Treatment differences in CL volumes in the current study may be due to either greater numbers of granulosa cells undergoing differentiation at ovulation, or increased survival of these cells following formation of the CL. Further work is required to fully determine the mechanisms responsible for the larger CL volume

observed in the current study, and to determine if larger CL volume was associated with greater ovarian P4 secretion.

It has been suggested that dietary fat supplementation influences metabolic clearance rate (MCR) of progesterone in cattle [36]. The majority of P4 is catabolised by the cytochrome P450 2C (*CYP2C*) and cytochrome P450 3A (*CYP3A*) enzymes in the liver [37]. The lack of any differences between treatments in hepatic mRNA expression of the P4 catabolic enzymes *CYP2C* and *CYP3A* suggests that alterations in P4 synthesis rather than MCR was a more important source of the variation observed in plasma P4 in the current study.

Endometrial biopsies were collected on day 7 of the oestrous cycle in the current study. This time point was selected as it represents a critical stage in embryo development, when the embryo descends into the uterus [28]. A prerequisite for PG synthesis is the generation of the PUFA substrate within the cell (e.g., arachidonic acid for 2-series PGs). Arachidonic acid (AA) is liberated from the phospholipid membrane by the action of a phospholipase. Many phospholipases have been identified, of which intracellular Group IV cytosolic $A_{2\alpha}$ ($PLA_{2\alpha}$) appears most important in controlling the availability of free AA for PG synthesis [38]. The free AA is then metabolized by prostaglandin G/H synthase enzymes (PTGS2) to an intermediate, PGH₂, which is in turn converted to either PGF₂ or PGE₂ by the enzymes PGF synthase (PGFS1 and PGFS2), and PGE synthase (PGES). Additional PGF_{2 α} may be synthesised by the reduction of PGE₂, catalysed by the Carbonyl Reductase enzyme CBR 1 [39]. In the current study, a tendency towards increased endometrial expression of $PLA_{2\alpha}$ was observed for cows receiving the Flax supplement compared with Control animals. This may indicate increased availability of AA for PG synthesis, a theory supported by the concurrent increases in endometrial

expression of both *PTGS2* and *PGFS2* in cows receiving the Flax treatment compared with Control animals. To the authors' knowledge, the present study is the first to investigate the effect of Flax supplementation on endometrial expression of genes involved in PG synthesis.

Peroxisome proliferator-activated receptors ($PPAR\alpha$, $PPAR\delta$ and $PPAR\gamma$) are a family of nuclear receptors that are activated by binding of natural ligands, such as PUFA [40]. It has been suggested that $PPAR\delta$ is involved in the pregnancy recognition process of cattle and may mediate some of the proposed beneficial effects of n-3 PUFA supplementation on PG synthesis [13,41]. The findings of the current study do not support this hypothesis, as the increase in endometrial $PPAR\delta$ expression with n-3 PUFA supplementation was not accompanied by suppression of genes involved in the synthesis of $PGF_{2\alpha}$. It is important to note, however, that in the current study endometrial biopsies were collected on day 7 of the oestrous cycle, some 8-10 days prior to maternal recognition of pregnancy. This may provide some explanation for the contrast in results between the current study and the work of Coyne et al. [13], who used tissue collected after animals were slaughtered on day 17 of the oestrous cycle. The increase in endometrial $PPAR\gamma$ expression with n-3 PUFA supplementation in the current study are not consistent with the findings of MacLaren et al. [41] using cell culture, and Coyne et al. [13] using beef heifers, who found no effect of n-3 PUFA supplementation on endometrial $PPAR\gamma$ expression.

Polyunsaturated fatty acids are known to affect the expression of genes involved in diverse metabolic pathways [42]. Fatty acid regulation of hepatic gene transcription is controlled by the transcription factors, $PPAR\alpha$ and *SREBF-1* [43]. These transcription factors regulate the activity of a number of genes involved in fatty acid transport (*SLC27A1* and *FABP1*), triacylglycerol synthesis (*DGAT1*) and fatty acid oxidation

(*CPT1A*, *ACOX1* and *ASCL1*) [43]. Liver biopsies were taken on day 7 of the oestrous cycle in the current study. Day 7 was selected as the hormonal milieu (increasing progesterone, low oestradiol) would have been the same in all cows at this stage of the cycle. Any confounding effects of steroid hormones on hepatic metabolism [44] were therefore negated. Hepatic expression of *ASCL1*, *FABP1* and *SREBF1* were reduced in FO supplemented cows compared to all other treatments. The differences in fatty acid composition of the FO supplement are likely to be the cause of these differences in hepatic gene expression. In contrast to the other three supplements, which contain fatty acids with 18 carbon chains or less, FO contains significant amounts of the very long chain PUFA, EPA and DHA. Deckelbaum et al. [45] noted that EPA, DHA and arachidonic acid (AA) have a greater inhibitory effect on *SREBF1* expression than shorter chain PUFA. This inhibitory effect on *SREBF1* is in turn reflected in reduced *ACSL1* and *FABP1* expression, as *SREBF1* is a major modulator of these genes [45].

The development of ovarian follicles during the early postpartum period has been consistently demonstrated to be stimulated by fat supplementation [2]. Few differential effects of fat supplementation on ovarian follicular development were observed in the current study, in agreement with Childs et al. [46], who found no differences in follicle development when supplementing heifers with either whole soybean (n-6) or a fish oil (n-3) supplement. Similarly, Petit and Twagiramungu [35] were unable to determine any differences in follicle development in cows fed either Megalac, an n-3 flaxseed supplement or an n-6 soybean supplement.

Cows receiving the Flax treatment had shorter cycle lengths than cows fed either the Control or FO diets. As ovulatory follicle size was unaffected by treatment, it is plausible that a shorter oestrous cycle may have beneficial effects for subsequent likelihood of conception by reducing the likelihood of ovulation of follicles with

605 prolonged dominance [47,48]. Reduced E2 concentrations around the time of oestrus
606 have been demonstrated to be related to decreased duration and intensity of oestrus
607 behaviour in lactating dairy cows [49]. The differences in peak plasma E2 observed in
608 the current study are in agreement with the work of Robinson et al. [31], who
609 demonstrated increased peak plasma E2 concentrations for cows fed an n-3 flaxseed
610 supplement compared with cows fed an isoenergetic control supplement. The increase
611 in plasma IGF-1 concentrations in Flax supplemented cows in the current study may
612 provide some explanation for the increased E2 concentrations, as plasma E2
613 concentrations during pre-ovulatory follicle development have been shown to be
614 highly correlated with plasma IGF-1 concentrations [50]. Our observations of
615 increased plasma concentrations of IGF-1 in n-3 supplemented cows compared to
616 Control animals are consistent with the work of Childs et al. [15].

617 The treatment differences in plasma NEFA concentrations observed in the current
618 study are most likely due to the degree of saturation of the fatty acid supplement, as
619 the Control supplement was the only fully saturated fat source. The plasma NEFA
620 levels observed in the current study were generally low, and as such unlikely to have
621 influenced the reproductive mechanisms examined in the study.

622 *Trans*-10, *cis*-12 CLA is a potent inhibitor of milk fat synthesis and has been shown to
623 reduce milk energy output and improve energy balance in early lactation dairy cows
624 [7]. In the present study we observed reductions in milk fat concentration and yield
625 for CLA-supplemented cows compared with Control, Flax and FO-treated cows.
626 There also appears to have been an inhibitory effect of the FO treatment on milk fat
627 synthesis; both milk fat concentration and yield were reduced compared to Control
628 and Flax treatment, although to a lesser extent than observed for CLA-treated cows.

The reduction in milk fat synthesis with FO supplementation is consistent with other reports [51,52].

5. CONCLUSIONS

The fat supplements used in the current study had diverse effects on reproductive parameters. Plasma P4 concentrations were increased for Control and CLA-supplemented cows in comparison with cows receiving n-3 PUFA supplements. The increase in plasma P4 concentrations in Control and CLA-supplemented cows occurred without any concurrent deleterious effects on either follicle development or gene expression related to PG synthesis. The endometrial gene expression results indicate that n-3 PUFA supplementation does not suppress the expression of enzymes involved in PGF_{2α} synthesis on day 7 of the oestrous cycle. Compared to other fat sources, feeding n-3 PUFA to lactating dairy cows may have negative implications for embryo development, due to suppressive effects on plasma P4 concentrations.

6. ACKNOWLEDGEMENTS

The authors thank Mr J.P Murphy, Mr F. Coughlan and the Moorepark farm staff for the management and care of the animals. The technical assistance of Ms Norann Galvin (Teagasc Moorepark), Ms Penny Furney and Ms Niamh Hynes (UCD), Ms Assumpta Glynn (Teagasc Athenry) and Mr Matt McCabe (Teagasc Grange) is also much appreciated. Funding from the National Development Plan and the Dairy Levy Research Trust are gratefully acknowledged.

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Table 1: Ingredient and nutrient composition of the TMR and concentrate rations offeredTMR ingredients (% DM)

Grass Silage	34
Maize Silage	34
Straw	3
TMR premix	29

TMR premix ingredients (% DM)

Wheat	16
Citrus pulp	5
Barley	16
Maize gluten feed	30
Soybean meal	25
Molasses (cane)	5
Vitamins and minerals ¹	3

Nutrient Composition (DM basis)

DM (g/kg)	915.2
Net Energy (UFL/kg of DM) ²	0.85
Ash (g/kg of DM)	57.3
CP (g/kg of DM)	162.2
NDF (g/kg of DM)	410.0
Oil (acid hydrolysis; %)	2.5

Balancer Ration Composition (% as fed)

Wheat	20
Citrus Pulp	15
Barley	15
Maize Gluten Feed	35
Soybean meal	10
Molasses (cane)	5

<u>Total Concentrate Ration Composition (kg as fed)</u>	<u>Treatment</u>			
	<u>Control</u>	<u>Flax</u>	<u>CLA</u>	<u>FO</u>
Balancer Ration	1.5	1.5	1.5	1.5
Palmit 80	0.5	0.43	0.4	0.35
Flax 30	-	0.2	-	-
LE-CLA	-	-	0.1	-
Fish oil supplement	-	-	-	0.3
Total supplemental lipid fed per day	0.5	0.5	0.5	0.5
Total ration fed per day	2.0	2.13	2.0	2.15

Nutrient Composition (DM basis)

DM (g/kg)	941.1	942.9	944.6	945.4
CP (g/kg of DM)	125.75	136.04	125.73	116.21
CF (g/kg of DM)	63.76	63.63	62.11	62.06
Ash (g/kg of DM)	48.69	45.06	84.03	78.4
Oil (acid hydrolysis; %)	22.81	23.01	23.64	24.12

¹Vitamin and mineral mix: 15 g/kg of DiCa P, 8 g/kg of limestone flour, 5 g/kg of salt, 2.5 g/kg of Cal-Mag, 80 g of manganous oxide, 200 g of copper sulfate, 125 g of zinc oxide, 18 g of potassium iodate, 20 g of sodium selenite (4.6%), 10 g of cobalt sulfate, 8 MIU/t of vitamin A, 2 MIU/t of vitamin D3, 15,000 IU/t of vitamin E.

²UFL = unite fourragere lait; unit of net energy, equivalent to 1 kg of standard air-dried barley

Table 2: Fatty acid composition (g/100g) of the Palmit 80, Flax 30, LE-CLA and Fish Oil supplements

Fatty Acid	g/100g of total fatty acids			
	Palmit 80	Flax 30	LE-CLA	Fish Oil
12:0	0.20	0.09	1.52	0.13
13:0	0.00	0.14	0.03	0.05
14:0	1.72	0.31	1.85	1.85
15:0	0.32	0.10	0.16	0.29
16:0	86.78	9.79	20.03	11.74
<i>cis</i> 16:1	0.24	0.13	0.15	1.52
17:0	0.00	0.08	0.17	0.00
18:0	1.69	3.32	19.47	3.49
18:1 <i>cis</i> -9	7.03	17.79	16.93	6.56
18:1 <i>trans</i> -9	0.14	0.43	0.33	2.28
18:1 <i>trans</i> -11	0.00	0.00	0.05	0.00
18:2 <i>cis</i> -9, <i>cis</i> -12 (n-6)	1.27	13.48	1.26	2.79
18:2 <i>cis</i> -9, <i>trans</i> -11 CLA (n-6)	0.00	0.08	14.51	0.21
18:2 <i>trans</i> -10, <i>cis</i> -12 CLA (n-6)	0.00	0.00	15.52	1.20
All <i>trans</i> 18:2	0.05	0.13	3.13	0.64
18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 (n-6)	0.21	0.17	0.00	0.00
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 (n-3)	0.00	50.74	0.31	0.46
20:0	0.00	0.35	0.51	0.82
20:1 <i>cis</i> -11	0.00	0.32	0.08	2.54
20:2 <i>cis</i> -11, <i>cis</i> -14	0.00	0.03	0.00	0.33
20:3 <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 (n-6)	0.00	0.00	0.00	0.21
20:3 <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17 (n-3)	0.00	0.06	0.00	1.15
20:4 (n-6)	0.00	0.00	0.00	0.44
20:5 EPA (n-3)	0.00	0.00	0.00	19.35
22:0	0.00	0.58	0.64	0.93
22:1	0.00	0.00	0.00	0.00
22:4	0.00	0.35	0.00	0.63
22:5 DPA (n-3)	0.00	0.00	0.00	6.73
22:6 DHA (n-3)	0.00	0.00	0.00	20.05
24:0	0.00	0.00	0.00	1.77
24:1	0.00	0.00	0.00	3.63
All < C18	89.26	10.65	23.92	15.57
All C18	10.40	86.15	71.51	17.63
All > C18	0.00	1.68	1.22	58.58
Total	99.66	98.51	96.66	92.47

Table 3: Schedule of experimental procedures performed

Day of dietary treatment ¹	Experimental procedure
0	Blood sample collected
5	Synchronisation protocol started
7	Blood sample collected
13	CIDR Removed
14-19	Cows observed for oestrus
14-35	Blood samples collected daily for a complete oestrous cycle. Trans-rectal ultrasonography daily for a complete oestrous cycle
35-45	10 day rest period following first synchronised cycle
46	2 injections of PGF _{2α}
46-52	Cows observed for oestrus
53-59	Liver and endometrial biopsies taken 7 days after ovulation

¹Between cow variation occurred in the number of days to ovulation after synchrony treatment and in the length of the oestrous cycle, therefore exact day of dietary treatment varied for individual cows.

Table 4: Bovine oligonucleotide primers used to detect endometrial and hepatic gene transcripts

Gene Name ¹	Sequence	Accession Number	Product Size
<u>Endometrial tissue</u>			
<i>PGFS2</i>	F: 5'-TTCCCTTCAACCAGAGTTGG-3' R: 5'-TCCCTGGCTTCAGAGACACT-3'	M86544	113
<i>PGFS1</i>	F: 5'-TTTACAAGGAGCTGGGCTTC-3' R: 5'-GCAGCAGCACTTTATCACCA-3'	NM_001040598	186
<i>FADS2</i>	F: 5'-CAGCTCTGACTGGTGATGGA-3' R: 5'-TCCCTATGGATCCAGTCTGC-3'	NM_001083444	132
<i>PTGS2</i>	F: 5'-TTTGACCCAGAGCTGCTTTT-3' R: 5'-TCTGATCCTGGACCACTTCC-3'	NM_174445	106
<i>PPARγ</i>	F: 5'-AGGATGGGGTCCTCATATCC-3' R: 5'-GCGTTGAACTTCACAGCAAA-3'	BC116098	121
<i>PPARδ</i>	F: 5'-AGTACTGCCGCTTCCAGAAA-3' R: 5'-GTTGTGCTGACTCCCCTCAT-3'	NM_001083636	131
<i>OTR</i>	F: 5'-TGGTTCTTGGTGGCTGTGTA-3' R: 5'-GCTTGGTTTGTATGGTGGAGT-3'	NM_174134	137
<i>CBR1</i>	F: 5'-AAGAAATGCAGCCGTGAACT-3' R: 5'-CACCCCGTTCTTTGTGTCTT-3'	NM_001034513	108
<i>PGES</i>	F: 5'-GGAACGCTGCCTCAGAGCCCA-3' R: 5'-CGACGAAGGGGTTCGGTCCG-3'	NM_174443.2	101
<i>PLA2</i>	F: 5'-TTCGAGCCATGGTAGGATTC-3' R: 5'-GGCCCTTCTCTGGAAAATC-3'	NM_001075864.1	148
<i>UBQ</i>	F: 5'-AGATCCAGGATAAGGAAGGCA-3' R: 5'-GCTCCACCTCCAGGGTGAT-3'	NM_174133	198
<i>RSP9</i>	F: 5'-CCTCGACCAAGAGCTGAAG-3' R: 5'-CCTCCAGACCTCACGTTTGTTC-3'	NM_001101152.1	64
<u>Endometrial and hepatic tissue</u>			
<i>PPARα</i>	F: 5'-TTGTGGCTGCTATCATTTGC-3' R: 5'-AGAGGAAGACGTCGTCAGGA-3'	AF229356	135
<i>ACTB</i>	F: 5'-ACTTGCGCAGAAAACGAGAT-3' R: 5'-CACCTTCACCGTTCCAGTTT-3'	BT030480	123
<u>Hepatic tissue</u>			
<i>SLC27A1</i>	F: 5'-ACTGTCTGCCCCTGTACCAC-3' R: 5'-GGCTGGCTGAAAACCTTCTTG-3'	NM_001033625.2	102
<i>ACADVL</i>	F: 5'-CACCATGAAAGGCATCATTG-3' R: 5'-GTTGGCACTCACCATGTACG-3'	NM_174494	160
<i>ACOX1</i>	F: 5'-AGCAAGAGAAATGGCTGCAT-3' R: 5'-AGGGTCATAAGTGGCTGTGG-3'	NM_001035289	119
<i>CPT1A</i>	F: 5'-TCCTGGTGGGCTACCAATTA-3' R: 5'-TGCGTCTGTAAAGCAGGATG-3'	FJ415874	181
<i>SREBF1</i>	F: 5'-CCGAGGCCAAGTTGAATAAA-3' R: 5'-TTCAGCGATTTGCTTTTGTG-3'	NM_001113302	136
<i>SCAP</i>	F: 5'-GGCTGATCCATGGTCACTTT-3' R: 5'-AGTGGGTAGCAGCAGGCTAA-3'	NM_001101889	183

<i>DGAT1</i>	F: 5'-GCATCCTGAATTGGTGTGTG-3' R: 5'-CACAATGACCAGGCACAGAG-3'	NM_174693	158
<i>GPAM1</i>	F: 5'-ACGACGGAGGCTAGATGAGA-3' R: 5'-TTCCACTTCTTGAGCGTGTG-3'	NM_001012282.1	140
<i>FABP1</i>	F: 5'-GGAGTTCATGACTGGGGAGA-3' R: 5'-CCCTTCGTCATGGTACTGGT-3'	NM_175817	135
<i>ACSL1</i>	F: 5'-GGCATCTATCTCCACCCTGA-3' R: 5'-CTCCCTCGCGTTAGACTTTG-3'	NM_001076085	145
<i>CYP2C</i>	F: 5'-TATGGACTCCTGCTCCTGCT-3' R: 5'-CATACTGCTGGGGACAAGGT-3'	NM_001109792	177
<i>CYP3A</i>	F: 5'-GAAGCTGCAGGAGGAAATTG-3' R: 5'-CTCCCAGCAATTGGAAACAT-3'	Y10214	129
<i>CAP1</i>	F: 5'-AGGCGGTGACTTCAATGAGTTCCC-3' R: 5'-ACAAGGAACCCAGTGGCACTTCG-3'	NM_001035010.1	121
<i>PSMD2</i>	F: 5'-CTGTGGCTGGGCTGCTCACC-3' R: 5'-CCACATCCACTGCCTGGCCC-3'	NM_001101197.1	183

¹PGFS; prostaglandin F synthase, FADS; fatty acid desaturase, PTGS; prostaglandin G/H synthase, PPAR; peroxisome proliferator-activated receptor, OTR; oxytocin receptor, CBR; carbonyl reductase, PGES; prostaglandin E synthase, PLA; Phospholipase UBQ; ubiquitin, ACTB; Beta-actin, RSP9; ribosomal protein S9, SLC27A1; solute carrier family 27, ACADVL; Very long-chain specific acyl-CoA dehydrogenase, ACOX acyl-coenzyme A oxidase, CPT1A; carnitine palmitoyltransferase, SREBF; sterol regulatory element binding transcription factor, SCAP; SREBF chaperone, DGAT; diacylglycerol O-acyltransferase, GPAM; glycerol-3-phosphate acyltransferase, mitochondrial, FABP; fatty acid binding protein, ASCL; Long chain fatty acid CoA ligase, CYP; cytochrome P450, CAP1; adenylate cyclase-associated protein, PSMD; proteasome 26S subunit, non-ATPase.

Table 5: Milk production, dry matter intake, energy balance and body condition score of cows receiving Control, Flax, CLA and Fish Oil (FO) lipid supplements.

	Treatment				SEM	P-value	
	Control	Flax	CLA	FO		Trt	Trt*time
Milk Yield (kg/d)	20.98 ^a	21.64 ^a	23.23 ^b	21.11 ^a	0.548	0.007	0.2
Milk Fat							
%	4.33 ^a	4.37 ^a	2.94 ^b	3.92 ^c	0.098	<0.001	0.8
kg/d	0.93 ^a	0.94 ^a	0.67 ^b	0.80 ^c	0.027	<0.001	0.4
Milk Protein							
%	3.09 ^a	3.13 ^a	2.95 ^b	3.05 ^{ab}	0.059	0.018	0.6
kg/d	0.64 ^{ac}	0.69 ^b	0.68 ^{ab}	0.64 ^c	0.015	0.03	0.13
Milk Lactose							
%	4.65 ^{ab}	4.67 ^{ac}	4.60 ^b	4.72 ^c	0.026	0.008	0.6
kg/d	0.98 ^a	1.01 ^{ab}	1.05 ^b	1.00 ^{ab}	0.027	0.18	0.09
SCM yield (kg/d) ¹	20.72 ^a	21.10 ^a	17.88 ^b	18.94 ^b	0.492	<0.001	0.14
DMI (kg/d)	18.63	18.54	18.20	18.51	1.030	0.9	0.6
EBAL (UFL/d)	4.28	4.00	5.05	4.76	0.596	0.4	0.5
BCS	3.01	2.97	3.01	2.98	0.025	0.6	0.3

^{abc} Within row means not sharing a common superscript differ significantly (P < 0.05)

¹ Solids corrected milk yield = 12.3*milk fat yield + (6.56*(milk protein yield + milk lactose yield))- (0.0752*milk yield)

Table 6: Ovarian follicular dynamics and peak plasma oestradiol concentrations in cows receiving Control, Flax, CLA and Fish Oil (FO) lipid supplements.

	Treatment				SEM	P-value
	Control	Flax	CLA	FO		
Cycle Length	21.8 ^a	18.3 ^b	21.6 ^{ab}	22.9 ^a	1.21	0.09
Number of waves	2.30	2.40	2.17	2.43	0.202	0.8
Maximum diameter ovulatory follicle (mm)	16.60	16.57	17.46	15.93	0.609	0.4
Peak plasma oestradiol at synchronised oestrus (pg/ml)	4.01 ^{ab}	5.33 ^a	4.92 ^{ab}	3.71 ^b	0.543	0.15
Peak plasma oestradiol at spontaneous oestrus (pg/ml)	3.41	4.26	4.24	3.53	0.390	0.2
Day of 1st wave emergence	0.9	0.8	1.0	1.6	0.30	0.3
1st wave day of peak follicle diameter	7.5 ^a	8.2 ^{ab}	8.7 ^{ab}	9.0 ^b	0.63	0.2
Day of 2nd wave emergence	9.8	10.1	10.3	11.3	0.64	0.4
2nd wave day of peak follicle diameter	18.0	18.8	18.8	20.4	1.01	0.4
Number of follicles in 1st wave	6.30 ^a	5.40 ^{ab}	5.63 ^{ab}	4.13 ^b	0.557	0.07
Number of follicles in 2nd wave	6.50	4.97	6.67	5.01	0.752	0.2
Maximum diameter follicle 1st wave (mm)	16.17	16.82	15.75	14.83	0.796	0.4
Maximum diameter follicle 2nd wave (mm)	15.83	16.26	15.98	15.01	0.861	0.7
Wave 1-2 emergence interval (days)	8.9	9.3	9.3	10.0	0.58	0.6
Wave 1-2 peak interval (days)	10.4	10.3	10.2	10.9	1.25	1

^{abc} Within row means not sharing a common superscript differ significantly ($P < 0.05$)

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Table 7: Corpus luteum volume and plasma concentrations of progesterone, insulin, IGF-1, cholesterol and non-esterified fatty acids (NEFA) in cows receiving Control, Flax, CLA and Fish Oil (FO) lipid supplements.¹

	Treatment				P-value		
	Control	Flax	CLA	FO	SEM	Trt	Trt*time
Day 2 - 14							
Progesterone (ng/ml)	5.13 ^a	4.51 ^b	5.38 ^a	4.46 ^b	0.321	0.013	0.9
CL Volume (mm ³)	7352.3 ^a	6821.1 ^{ab}	7964.9 ^a	5888.2 ^b	509.29	0.04	0.8
Day 5-7							
Progesterone (ng/ml)	3.96 ^{ab}	3.47 ^{ab}	4.24 ^a	3.17 ^b	0.34	0.15	0.4
CL Volume (mm ³)	7287.2 ^{ab}	6880.8 ^{ab}	8125.7 ^a	5618.6 ^b	752.38	0.14	1
Day 8-14							
Progesterone (ng/ml)	7.20 ^{ab}	6.54 ^a	7.74 ^b	6.46 ^a	0.426	0.014	0.9
CL Volume (mm ³)	9490.2 ^a	8588.5 ^{ab}	9668.8 ^a	7353.7 ^b	723.16	0.14	0.4
Day of Luteolysis	17.3	16.7	17.3	16.9	0.56	0.75	-
Insulin (μUI/mL)							
	4.41 (3.84 - 5.07) ^a	4.18 (3.65 - 4.79) ^{ab}	3.71 (3.19 - 4.32) ^{ab}	3.47 (2.96 - 4.07) ^b	-	0.09	0.5
IGF-1 (ng/mL)							
	73.41 (64.29 - 83.83) ^a	89.13 (78.13 - 101.68) ^b	80.43 (70.50 - 91.76) ^{ab}	80.06 (69.92 - 91.68) ^{ab}	-	0.07	0.9
Cholesterol (mmol/L)							
	4.97 ^a	5.45 ^{ab}	5.94 ^b	5.19 ^a	0.212	0.02	0.3
NEFA (mmol/L)							
	0.16 (0.13 - 0.19) ^a	0.21 (0.18 - 0.25) ^b	0.23 (0.19 - 0.28) ^b	0.24 (0.20 - 0.29) ^b	-	0.01	0.8

^{a,b,c} Within row means not sharing the same superscript differ significantly (P < 0.05)

¹ Insulin, IGF-1 and NEFA values are back-transformed least-square means, followed by the 95 % confidence limits in parenthesis.

Table 8: Endometrial and hepatic gene expression in cows receiving Control, Flax, CLA and Fish Oil (FO) lipid supplements.^{1,2}

Gene ³	Treatment				P value
	Control	Flax	CLA	FO	
Endometrial tissue					
<i>PPARα</i>	0.78 (0.41 - 1.24)	0.93 (0.60 - 1.31)	0.67 (0.36 - 1.05)	0.87 (0.42 - 1.44)	0.8
<i>PPARδ</i>	0.55 (0.25 - 0.96) ^a	1.12 (0.74 - 1.59) ^b	0.74 (0.41 - 1.16) ^{ab}	1.07 (0.58 - 1.72) ^{ab}	0.085
<i>PPARγ</i>	1.33 (0.87 - 1.88) ^a	2.28 (1.71 - 2.94) ^{bc}	1.73 (1.24 - 2.30) ^{ac}	2.56 (1.81 - 3.43) ^b	0.015
<i>PTGS2</i>	0.97 (0.37 - 1.86) ^a	2.49 (1.62 - 3.54) ^b	1.62 (0.88 - 2.58) ^{ab}	1.03 (0.32 - 2.14) ^a	0.078
<i>OTR</i>	2.64 (1.57 - 3.91)	2.42 (1.57 - 3.40)	1.82 (1.02 - 2.79)	2.14 (1.02 - 3.56)	0.4
<i>PGFS1</i>	0.82 (0.41 - 1.33)	0.79 (0.46 - 1.19)	0.71 (0.37 - 1.14)	0.96 (0.45 - 1.62)	0.8
<i>PGFS2</i>	0.87 (0.35 - 1.71)	1.72 (0.98 - 2.72)	1.00 (0.47 - 1.80)	1.86 (0.86 - 3.36)	0.13
<i>PGES</i>	1.37 (0.64 - 2.38)	1.65 (0.94 - 2.56)	1.72 (0.95 - 2.71)	2.07 (1.03 - 3.47)	0.7
<i>CBR1</i>	0.68 (0.32 - 1.20)	0.93 (0.58 - 1.39)	0.61 (0.33 - 1.00)	1.12 (0.59 - 1.88)	0.4
<i>PLA₂</i>	0.92 (0.38 - 1.68)	1.86 (1.19 - 2.68)	1.41 (0.79 - 2.21)	1.33 (0.57 - 2.42)	0.3
<i>FADS2</i>	1.31 (0.74 - 2.05)	1.82 (1.19 - 2.58)	2.29 (1.59 - 3.13)	1.59 (0.85 - 2.57)	0.3
Hepatic tissue					
<i>ACADVL</i>	0.56 (0.36 - 0.78)	0.53 (0.36 - 0.71)	0.66 (0.46 - 0.87)	0.49 (0.27 - 0.73)	0.6
<i>ACOX1</i>	7.95 (7.50 - 8.37)	7.82 (7.46 - 8.16)	7.83 (7.44 - 8.21)	7.37 (6.85 - 7.86)	0.3
<i>ACSL1</i>	1.27 (0.98 - 1.56) ^a	1.25 (1.00 - 1.50) ^a	1.29 (1.02 - 0.89) ^a	0.57 (0.26 - 0.89) ^b	0.006
<i>CPT1A</i>	0.76 (0.46 - 1.11)	0.81 (0.55 - 1.11)	0.98 (0.67 - 1.32)	0.94 (0.56 - 1.35)	0.8
<i>DGAT</i>	1.81 (1.34 - 2.28)	1.74 (1.34 - 2.15)	2.00 (1.56 - 2.44)	1.74 (1.20 - 2.28)	0.6
<i>FABP1</i>	1.24 (0.85 - 1.67) ^a	1.57 (1.21 - 1.96) ^a	1.25 (0.88 - 1.66) ^a	0.42 (0.13 - 0.81) ^b	0.002
<i>GPAM</i>	1.91 (1.62 - 2.20)	1.75 (1.49 - 2.01)	1.98 (1.71 - 2.26)	1.72 (1.39 - 2.06)	0.5
<i>SCAP</i>	0.71 (0.39 - 1.08)	1.14 (0.82 - 1.49)	1.00 (0.66 - 1.37)	1.06 (0.64 - 1.52)	0.4
<i>SLC27A1</i>	1.19 (0.72 - 1.69)	1.55 (1.13 - 1.99)	1.59 (1.13 - 2.09)	1.73 (1.15 - 2.33)	0.4
<i>SREBF1</i>	1.46 (0.96 - 2.03) ^a	1.17 (0.77 - 1.63) ^a	1.50 (1.02 - 2.05) ^a	0.42 (0.10 - 0.88) ^b	0.01
<i>PPARα</i>	0.84 (0.57 - 1.13)	0.91 (0.67 - 1.16)	1.12 (0.84 - 1.41)	1.21 (0.87 - 1.56)	0.3
<i>CYP3A</i>	0.92 (0.58 - 1.30)	0.71 (0.44 - 1.02)	0.99 (0.66 - 1.35)	1.07 (0.66 - 1.53)	0.5
<i>CYP2C</i>	2.07 (1.48 - 2.68)	2.02 (1.51 - 2.54)	2.71 (2.14 - 3.30)	2.34 (1.65 - 3.05)	0.3

^{a,b,c} Within row means not sharing the same superscript differ significantly ($P < 0.05$)

¹ Gene expression values were normalized to the reference gene following adjustment for efficiencies and interplate variation and converted to values relative to the highest cycle threshold (Ct) within each data set.

² Real-time reverse transcription-PCR values are back-transformed least squares means, followed by the 95% confidence limits in parentheses

³ PPAR; peroxisome proliferator-activated receptor, PTGS; prostaglandin G/H synthase, OTR; oxytocin receptor, PGFS; prostaglandin F synthase, PGES; prostaglandin E synthase, CBR; carbonyl reductase, PLA; Phospholipase, FADS; fatty acid desaturase. SLC27A1; solute carrier family 27, ACADVL; Very long-chain specific acyl-CoA dehydrogenase, ACOX acyl-coenzyme A oxidase, ASCL; Long chain fatty acid CoA ligase, CPT1A; carnitine palmitoyltransferase, DGAT; diacylglycerol O-acyltransferase, FABP; fatty acid binding protein, GPAM; glycerol-3-phosphate acyltransferase, mitochondrial, SREBF; sterol regulatory element binding transcription factor, SCAP; SREBF chaperone, CYP; Cytochrome P450

Figure 1: Plasma progesterone (upper panel) and corpus luteum volume (lower panel) in cows receiving Control, Flax, CLA and Fish Oil (FO) lipid supplements. Statistical analysis was performed using data from day 2 to day 14 following ovulation. Data from day 15 to day 20 after ovulation are included for illustration purposes only. All values are LSM