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10 **Effect of supplementation with different fat sources on the mechanisms involved**

11 **in reproductive performance in lactating dairy cattle**

12

13 RUNNING HEAD: FATTY ACIDS AND REPRODUCTION

14

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26

27

28 **ABSTRACT**

29 Supplementary fat positively influences reproductive performance in dairy cattle,

30 although the mechanisms involved are not clearly defined. Our objective was to

31 determine the effects of four different fat supplements on follicle development,

32 plasma steroid hormone concentrations and prostaglandin (PG) synthesis in lactating

33 dairy cattle. Forty-eight early lactation Holstein-Friesian cows (21 primiparous, 27

34 multiparous) were used in a completely randomised block design. Cows were fed the

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

KEYWORDS: Fatty acids, reproduction, dairy cattle, progesterone.

60 **1. INTRODUCTION**

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

75 *Trans*-10, *cis*-12 CLA, an n-6 polyunsaturated fatty acid (PUFA), is a potent inhibitor
76 of milk fat synthesis, and has recently been demonstrated to decrease milk energy
77 output, with subsequent improvements in energy balance and body condition score
78 [7]. These improvements in energy balance have been shown to have a beneficial
79 effect on reproductive performance [8]. Other potential mechanisms by which CLA
80 might improve reproductive performance are yet to be fully elucidated.

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

85 potential to manipulate PG synthesis by adding n-3 PUFA supplements to the diet.
86 Increasing the proportion of n-3 PUFA in the diet can result in increased synthesis of
87 the less biologically active 3-series PGs at the expense of $PGF_{2\alpha}$ [10,11]. Studies
88 conducted *in vitro* [12], and *in vivo* using beef heifers [13] demonstrated that n-3
89 PUFA supplementation can alter endometrial expression of genes regulating $PGF_{2\alpha}$
90 synthesis, potentially leading to a reduction in uterine $PGF_{2\alpha}$ production. Inhibition of
91 uterine $PGF_{2\alpha}$ secretion may delay the regression of the corpus luteum (CL), and
92 hence improve embryo survival.
93 The present study was conducted to determine the effects of four different fat
94 supplements on follicle and CL development, steroid hormone concentrations and
95 endometrial and hepatic gene expression in lactating dairy cows.
96
97 **2. MATERIALS AND METHODS**
98 ***2.1. Animals and treatments***
99 All experimental procedures involving animals were licensed by the Department of
100 Health and Children, Ireland, in accordance with the Cruelty to Animals Act (1876)
101 and the European Community Directive 86/609/EEC. Twenty one primiparous and 27
102 multiparous Holstein-Friesian cows were blocked on the basis of parity, calving date,
103 average daily milk yield and body condition score (BCS), and randomly assigned to
104 one of four dietary fat supplements at 38 days in milk (DIM) (+/- 11 days (S.D)): 500
105 g palmitic acid (Palmit 80, Trouw Nutrition, Belfast, UK) (Control); 200 g pure
106 pressed flax seed (Flax 30, Bob Allen Feeds, Bandon, Ireland) containing 72 g α-
107 linolenic acid (Flax); 100 g lipid-encapsulated conjugated linoleic acid (CLA) (Lutrell
108 Pure, BASF, Ludwigshafen, Germany) containing 10 g each of *trans*-10, *cis*-12 CLA
109 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 $NaOCH_3$/methanol and BF_3/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

135 samples using a Milkoscan 605 (Foss Electric, Hillerod, Denmark). The following
136 equation was used to calculate solids corrected milk yield (SCM) [18]:
137 SCM = (12.3*milk fat yield + (6.56*(milk protein yield + milk lactose yield))-
138 (0.0752*milk yield)
139 Body condition score was recorded every two weeks using a 1 to 5 scale
140 (1 = emaciated, 5 = extremely fat) with 0.25 increments [19]. Energy balance (EBAL)
141 was estimated as the difference between energy intake and the sum of energy
142 requirements for maintenance and milk production, using the French net energy
143 system [20]. This system uses unité fourragère lait (UFL) as the unit of net energy,
144 which is equivalent to 1 kg of standard air-dried barley. The following equations were
145 used to determine the energy required for maintenance and output in milk [21]:
146 Energy required for maintenance (UFL/d) =1.4+0.6×BW/100;
147 Energy requirement for milk (UFL/kg of milk) = 0.0054 × FC + 0.0031 × PC +
148 0.0028 × LC-0.015; where FC = fat concentration (%), PC = protein concentration
149 (%), and LC = lactose concentration (%).
150 Of the 48 cows on the trial, 9 cows failed to respond to synchrony treatment, and a
151 further 5 cows did not ovulate at the subsequent spontaneous oestrus. In addition, two
152 cows were removed from the study due to illnesses unrelated to the experimental
153 treatments.
154
155 ***2.2. Synchronisation of the oestrous cycle***
156 A protocol to synchronise oestrus was initiated on day 5 of dietary treatment with
157 injection (i.m.) of GnRH (0.01 mg Buserelin (Receptal); Intervet, Dublin) and
158 insertion of an intravaginal progesterone releasing device (Eazi-breed CIDR
159 containing 1.38g P4, Pfizer Animal Health, Dublin, Ireland). On day 12 of dietary

160 treatment each cow received an injection (i.m.) of $PGF_{2\alpha}$ (Lutylase, Pfizer Animal
161 Health), and the following day the CIDR was removed. All cows had tail paint
162 applied at CIDR removal, and were observed for oestrus over the next 5 days. The
163 schedule of all experimental procedures performed is presented in Table 3.
164
165 ***2.3. Monitoring ovarian activity***
166 Follicles were counted and measured, and corpora lutea measured for each cow via
167 daily transrectal ovarian ultrasonography (Aloka SSD-900, Aloka Ltd, Tokyo, Japan,
168 7.5 MHz transducer) from the day after CIDR removal until ovulation following the
169 next spontaneous oestrus. In order to measure follicles and corpora lutea, images were
170 frozen on screen during ultrasonography, and dimensions were measured with internal
171 callipers on the Aloka SSD-990. The position of each large (≥10 mm diameter)
172 follicle on the ovary was mapped daily to facilitate identification of the dominant
173 follicle in each wave of follicular growth. Corpora lutea dimensions were recorded
174 and used to calculate the average diameter and radius. Corpus luteum volume (V) was
175 then calculated with the formula $V = 4/3x\ \pi\ x\ radius^3$. For CL with a fluid-filled
176 lacuna the volume of the lacuna was calculated and subtracted from the total volume
177 of the CL.
178
179 ***2.4. Blood sampling and hormone and metabolite analysis***
180 Blood samples were collected from each cow after the morning milking on the day of
181 initiation of dietary treatment, on day 7 of dietary treatment, on the day after CIDR
182 removal, and daily thereafter until the day of ovulation at the spontaneous oestrus
183 following the synchronised oestrus. Blood samples were collected from the coccygeal
184 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

210 were 12.7% (high), 9.8% (medium) and 25.7% (low), and intra-assay CV for E2 were
211 8.0 % (high), 8.9 % (medium) and 38.2 % (low). Plasma cholesterol and NEFA
212 concentrations were determined by enzymatic colorimetry using appropriate kits
213 (cholesterol kit supplied by Horiba ABX, Montpellier, France, NEFA kit supplied by
214 Wako Chemicals, GmbH, Nissanstrase, Germany).
215
216 ***2.5. Tissue biopsies***
217 Following the synchronised cycle when cows were scanned daily, the cows were
218 given a 10-day period of rest. The cows were then re-synchronised with two injections
219 (i.m.) of $PGF_{2\alpha}$ (Lutylase, Pfizer Animal Health) after consecutive morning and
220 evening milkings. Tail paint was applied to aid heat detection, and transrectal
221 ultrasonography was used following observed oestrus to confirm ovulation. Liver and
222 endometrial biopsies were collected 7 days after ovulation to examine gene expression
223 at this stage of the oestrous cycle.
224 For the liver biopsy, a site between the 11th and 12th ribs was shaved, sanitised with
225 Videne (Povidone-iodine, 7.5%; Ecolab, Leeds,UK) and methylated spirits, and
226 anaesthetised with Willcain (Dechra Ltd, Shrewsbury, UK). An incision of
227 approximately 1 cm was made through the skin and the biopsy instrument was used to
228 pierce the intercostal muscles and peritoneum. The liver was located and a 1 to 1.5 g
229 sample removed. The sample was immediately washed in saline, blotted dry, snap
230 frozen in liquid nitrogen and stored at -80 °C. The incision site was sutured and
231 treated topically with Duphacycline spray (Interchem, Naas, Ireland) and cows were
232 given 10 mL of a subcutaneous antibiotic as a prophylactic (Excenel RTU, Pfizer
233 Animal Health). For the endometrial biopsy, animals were given caudal epidural
234 anaesthesia with Willcain, the external genital area was sanitized, and a biopsy tool

235 passed through the cervix and into the uterine horn ipsilateral to the CL by using
236 transrectal manipulation. The open jaws of the biopsy basket (2 × 1 mm) were pressed
237 against the endometrium and samples (approximately 100 mg) of endometrium
238 removed. The samples were then washed in saline, blotted dry, snap frozen in liquid
239 nitrogen, and stored at −80 °C. Animals were given 500 mg of an intrauterine
240 prophylactic antibiotic (Metricure, Intervet, Bray, Co Wicklow, Ireland).
241
242 ***2.6. RNA extraction and cDNA synthesis***
243 Total RNA was isolated from frozen tissue using TRI® Reagent and chloroform
244 (Sigma-Aldrich Ireland Ltd. Dublin, Ireland). Homogenisation of the sample in TRI®
245 Reagent was performed using a tissue lyser (Qiagen Ltd., Crawley, UK) and the RNA
246 was subsequently extracted using chloroform and precipitated using isopropanol.
247 Quantity and quality of total RNA was assessed using the NanoDrop ND-1000
248 spectrophotometer (NanoDrop Technologies, DE, USA) and the Agilent Bioanalyser
249 2100 with the RNA 6000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd.
250 Dublin, Ireland), respectively. RNA quality was verified by ensuring all RNA samples
251 had an absorbance ($A_{260/280}$) of between 1.8 and 2 and RINs of between 7 and 10.
252 RNA samples were treated with RQ1 RNase-free DNase (Promega UK Ltd.,
253 Southampton, UK) and purified using the RNeasy1 mini kit (Qiagen Ltd.). DNase-
254 treated and purified total RNA was then reverse transcribed to cDNA, with random
255 hexamers, using the High Capacity cDNA Reverse Transcription kit (Applied
256 Biosciences, Ireland) according to the manufacturers instructions and stored at -20 °C
257 for subsequent analyses.
258
259 ***2.7. Primer design and reference gene selection***

260 All candidate and reference gene primers used to detect endometrial and hepatic gene
261 expression (Table 4) in this study were designed using the Primer3 web based
262 software program (http://frodo.wi.mit.edu/primer3/) [24], and obtained from a
263 commercial supplier (Sigma Aldrich Ltd., Dublin, Ireland). Primer specificity was
264 checked using the Basic Local Alignment Search Tool (BLAST) from the National
265 Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). All
266 the primers were validated using a pooled cDNA sample. A standard curve was
267 generated using serial dilutions of pooled cDNA. PCR products generated by
268 amplification were sequenced to verify their primer specific identity (Biochemistry
269 DNA Sequencing Facility, University of Cambridge). To determine the relative gene
270 expression levels, suitable highly stable reference genes were required. In the current
271 study, out of five tested, three reference genes for both endometrial (beta-actin
272 (*ACTB*), ubiquitin (*UBQ*) and ribosomal protein S9 (*RPS9*)) and hepatic tissue
273 (adenylate cyclase-associated protein (*CAP1*), proteasome 26S subunit, non-ATPase
274 (*PSMD*) and *ACTB* were used to normalise gene expression data. The principle behind
275 the selection of the reference gene is that the expression ratio of two perfect reference
276 genes should be constant across all samples. The expression stability of the reference
277 genes was validated with the software program, geNorm version 3.5, by calculating
278 the gene expression stability measure (M value). Using three reference genes, the M
279 values were 0.56 (*ACTB, UBQ, RPS9*) and 0.37 (*ACTB, PDSM2, CAP1*) for
280 endometrial and hepatic RNA samples, respectively, both of which were below the
281 default minimum coefficient of 1.5 as specified by the geNorm program [25].
282
283 ***2.8. Quantitative real time PCR (qPCR)***

284 Following reverse transcription, cDNA quantity was determined and standardised to
285 the required concentration for qPCR. Triplicate 20 μL reactions were carried out in
286 96-well optical reaction plates (Applied Biosystems, Warrington, UK), containing 1
287 μL cDNA (10-50 ng of RNA equivalents), 10 μL Power SYBR® Green PCR Master
288 Mix (Applied Biosystems), 8 μL nuclease-free H_2O, and 1 μL forward and reverse
289 primers (250-1000 nM per primer). Assays were performed using the ABI 7500 Fast
290 qPCR System (Applied Biosystems) with the following cycling parameters: 95 °C for
291 20 s and 40 cycles of 95 °C for 30 s, 60 °C for 30 s followed by amplicon dissociation
292 (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s). Gene expression
293 levels were recorded as Ct values, i.e. the number of PCR cycles at which the
294 fluorescence signal is detected above the threshold value and all samples were run in
295 triplicate. Amplification efficiencies were determined for all candidate and reference
296 genes using the formula $E=10\wedge^{(-1/\text{slope})}$, with the slope of the linear curve of cycle
297 threshold (Ct) values plotted against the log dilution [26]. Primer concentrations were
298 optimized for each gene and disassociation curves were examined for the presence of
299 a single PCR product. The efficiency of the reaction was calculated using a 5-fold
300 serial dilution of cDNA and generation of a standard curve. All PCR efficiency
301 coefficients were between 0.9 and 1.1 and therefore deemed acceptable. The software
302 package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden) was used for
303 efficiency correction of the raw Ct values, interplate calibration based on a calibrator
304 sample included on all plates, averaging of replicates, normalization to the reference
305 gene and the calculation of quantities relative to the highest Ct and $\log_2$
306 transformation of the expression values for all genes.
307

308 ***2.9. Statistical analysis***

309 All statistical analyses were carried out using SAS (SAS System Inc., Cary NC,
310 USA). Daily measurements of milk yield, DMI and EBAL were collapsed into weekly
311 means. A test for normality was performed on all the blood analyte data using the
312 UNIVARIATE procedure of SAS. Insulin, IGF-1 and NEFA variables were log-
313 transformed prior to analysis to generate a normal distribution. Milk production, milk
314 composition, DMI, BCS, EBAL, blood analyte and CL volume data were analysed
315 using mixed models with repeated measures, using the satterthwaite adjustment to
316 calculate denominator degrees of freedom. The appropriate covariance structure for
317 each repeated measures analysis was identified based on Akaike's Information
318 Criterion (AIC) model fit statistic. Where appropriate, measurements made during the
319 3 wk prior to the initiation of dietary treatment were included as covariates in the
320 models for milk yield and composition, IGF-I, insulin, NEFA, cholesterol, DMI, BCS
321 and calculated EBAL. Parity and calving date were included as adjustment variables
322 in all repeated measures models; if non-significant, these variables were removed and
323 the models were re-run. Follicle development, peak plasma E2 concentrations at
324 oestrus, and day of luteolysis data were analysed using mixed models procedures with
325 treatment as a fixed effect, block as a random effect, and calving date and parity
326 included as adjustment variables.

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

333 concentration and CL volume was performed on data from days 5-7 after ovulation
334 and from days 8-14 after ovulation.
335 All gene expression data was tested for normality using the UNIVARIATE procedure
336 of SAS. A Box-Cox transformation analysis was performed using the TRANSREG
337 procedure in SAS to obtain the appropriate lambda value for data that were not
338 normally distributed. These data were then transformed by raising the variable to the
339 power of lambda. Mixed models (PROC MIXED) were used to determine the effect
340 of fat supplementation on the relative expression of each gene measured. The Tukey
341 critical difference test was used to determine statistical differences between treatment
342 means. In all statistical analyses, contrast statements were used to compare individual
343 fat treatments against one another, and also to compare the two n-3 supplements
344 combined (Flax and FO) against both Control and CLA supplements. Data were
345 considered significant when $P<0.05$, and a trend declared when $P<0.1$.
346

347 **3. RESULTS**

348 ***3.1. Milk production, dry matter intake, body condition score and energy balance***

349 Milk production, DMI, BCS and EBAL data are summarised in Table 5. Milk yield
350 was greater for CLA-supplemented cows compared with all other treatments (P =
351 0.007); the remaining treatments did not differ from each other. There was a
352 significant effect of treatment on milk fat concentration and yield (both $P < 0.001$).
353 CLA supplementation reduced milk fat concentration and yield compared with all
354 other treatments, and FO cows had reduced milk fat concentration and yield compared
355 with those on the Control and Flax diets. Milk protein concentration was reduced for
356 CLA-supplemented cows compared with cows receiving Control and Flax treatments
357 (P = 0.018). Milk protein yield was reduced for FO-treated cows compared with both

358 Flax and CLA-supplemented cows, and was greater for Flax-treated cows compared
359 with cows receiving the Control diet (*P* = 0.03). Milk lactose concentration was
360 increased for cows receiving FO compared with those receiving the Control and CLA
361 treatments, and cows receiving the Flax supplement had elevated milk lactose
362 concentrations compared with cows on the CLA diet (*P* = 0.008). Overall, solids-
363 corrected milk yield (SCM) was increased for cows on the Control and Flax diets
364 compared with cows on the CLA and FO diets (*P* < 0.001). There was no effect of
365 treatment on DMI, EBAL or BCS.
366
367 ***3.2. Ovarian follicular dynamics and peak oestradiol concentrations***
368 Follicle development is summarised in Table 6. The type of fat supplement tended to
369 affect both the length of the oestrous cycle and the number of follicles in the first
370 wave of the cycle (*P* = 0.09 and 0.07, respectively). The oestrous cycle of cows
371 receiving the Flax supplement was 3.5 d shorter than for the Control group (*P* = 0.04),
372 and 4.6 d shorter compared with cows offered the FO supplement (*P* = 0.02). The FO-
373 supplemented cows had 2.2 fewer follicles in the first wave of the cycle compared
374 with those fed the Control supplement (*P* = 0.01). Peak plasma E2 at the synchronised
375 oestrus was 1.62 pg/mL greater (*P* = 0.046) in Flax-supplemented cows compared
376 with FO-supplemented cows, and tended to be 1.32 pg/mL greater (*P* = 0.08) in Flax-
377 supplemented cows compared with cows receiving the Control supplement. Peak
378 plasma E2 at the spontaneous oestrus tended to be 0.85 pg/mL greater (*P* = 0.09) in
379 Flax-supplemented cows compared with cows receiving the Control supplement.
380 There was no overall treatment effect on the day of first wave peak follicle diameter,
381 but peak follicle diameter in the first follicular wave occurred 1.5 days earlier after

382 ovulation in Control cows compared with cows receiving the FO diet (P = 0.04). No
383 other follicle or E2 variables were affected by the type of fat supplement.
384
385 ***3.3. Plasma progesterone concentrations and corpus luteum volumes***
386 The effect of fat supplementation on plasma P4 and CL volume is shown in Table 7.
387 and Figure 1.
388 **Days 2-14 after ovulation:** There was a significant effect of treatment on both plasma
389 P4 concentration and CL volume from days 2 – 14 of the oestrous cycle (P = 0.013
390 and 0.04 respectively). Mean plasma P4 concentrations were 0.62 ng/mL (P = 0.04)
391 and 0.87 ng/mL (P = 0.001) less for cows receiving the Flax supplement compared
392 with cows receiving the Control and CLA supplements, respectively. Mean plasma P4
393 concentrations in cows on the FO diet were 0.67 ng/mL (P = 0.03) and 0.92 ng/mL (P
394 = 0.01) less compared with cows receiving the Control and CLA supplements,
395 respectively. Corpus luteum volumes were 1464 mm^3 less (P = 0.03) and 2077 mm^3
396 less (P = 0.005) for FO-treated cows compared with cows receiving the Control and
397 CLA supplement, respectively. The n-3-supplemented cows had lesser plasma P4
398 compared with Control (4.48 ng/mL vs. 5.13 ng/mL, P = 0.01) and CLA-
399 supplemented cows (4.48 ng/mL vs. 5.38 ng/mL, P = 0.004). Corpus luteum volumes
400 were lesser in n-3-supplemented cows compared with CLA-supplemented cows (6355
401 mm^3 vs. 7965 mm^3, P = 0.01). There was a tendency for primiparous animals to have
402 smaller CL volumes than mulitparous cows (6380 mm^3 vs. 7632 mm^3, P = 0.06).
403 There was no effect of treatment on the number of days from ovulation to luteolysis.
404 **Days 5-7 after ovulation:** There was no overall treatment effect on either plasma P4
405 or CL volumes; however, CLA-treated cows had 1.17 ng/mL greater plasma P4
406 concentrations (P = 0.04) and 2507 mm^3 larger CL volumes compared with cows

407 receiving the FO diet (P = 0.03). The n-3-supplemented cows had reduced plasma P4
408 concentrations (3.32 ng/mL vs. 4.24 ng/mL, P = 0.04) and CL volumes (6249.7 mm^3
409 vs. 8125.7 mm^3, P = 0.04) compared with the CLA-supplemented cows.
410 **Days 8-14 after ovulation:** There was a significant effect of treatment on plasma P4
411 from days 8-14 after ovulation. Mean plasma P4 concentrations in CLA treated cows
412 were 1.20 ng/mL greater (P = 0.006) and 1.28 ng/mL greater (P = 0.007) compared
413 with cows receiving the Flax and FO supplements, respectively. The n-3-treated cows
414 had lesser mean plasma P4 concentrations compared with both the Control (6.50
415 ng/mL vs. 7.20 ng/mL, P = 0.04) and CLA-supplemented (6.50 ng/mL vs. 7.74
416 ng/mL, P = 0.002) cows. There was no overall treatment effect on CL volume on days
417 8-14 after ovulation, but both CLA-treated (9668.8 mm^3 vs. 7353.7 mm^3, P = 0.04)
418 and Control (9490.2 mm^3 vs. 7353.7 mm^3, P = 0.05) cows had larger CL compared
419 with cows receiving the FO diet.
420
421 ***3.4. Metabolites and metabolic hormones***
422 Mean IGF-1, insulin, cholesterol and NEFA concentrations on days 15, 25 and 35 of
423 dietary treatment are presented in Table 7. The type of fat supplement tended to affect
424 plasma insulin (P = 0.09) and IGF-1 (P = 0.07) concentrations. Plasma insulin
425 concentrations were 0.94 µUI/mL greater (P = 0.02) in Control-treated cows
426 compared with cows receiving the FO supplement. Plasma IGF-1 concentrations were
427 15.7 ng/mL greater (P = 0.008) for cows receiving the Flax diet compared with cows
428 receiving the Control diet. The n-3-supplemented cows had greater plasma IGF-1
429 concentrations compared with cows receiving the Control diet (84.6 ng/mL vs. 73.4
430 ng/mL, P = 0.03)

431 There was a significant effect of treatment on plasma cholesterol concentrations (*P* =
432 0.02). Plasma cholesterol concentrations in CLA-supplemented cows were 0.97
433 mmol/L greater (*P* = 0.002) and 0.75 mmol/L greater (*P* = 0.01) compared with cows
434 receiving the Control and FO supplements, respectively. The CLA-supplemented
435 cows had greater plasma cholesterol concentrations compared with cows receiving the
436 n-3 supplements (5.94 mmol/L vs. 5.32 mmol/L, *P* = 0.02).
437 There was a significant effect of treatment on plasma NEFA concentrations (*P* =
438 0.01). Cows receiving the Control supplement had 0.05 mmol/L less (*P* = 0.03), 0.07
439 mmol/L less (*P* = 0.007), and 0.08 mmol/L less (*P* = 0.003) plasma NEFA
440 concentrations compared with cows receiving the Flax, CLA and FO supplements,
441 respectively. Plasma NEFA concentrations were greater in n-3-treated cows compared
442 with cows receiving the Control supplement (0.23 mmol/L vs. 0.16 mmol/L, *P* =
443 0.003).
444

445 ***3.5. Endometrial gene expression***

446 Endometrial gene expression data are presented in Table 8. There was a significant
447 effect of treatment on *PPARγ* expression (*P* = 0.015). Endometrial *PPARγ* expression
448 was increased 1.9 fold (*P* = 0.005) and 1.5 fold (*P* = 0.05) in cows receivng the FO
449 diet compared with cows offered the Control and CLA diets, respectively. Flax-
450 treated cows had 1.7 fold greater *PPARγ* expression compared with Control-treated
451 cows (*P* = 0.008). The mRNA abundance of *PPARγ* was increased 1.8 fold (*P* =
452 0.002) and 1.4 fold (*P* = 0.03) for n-3-treated cows compared with cows receiving the
453 Control and CLA diets, respectively. Fat supplementation tended to affect both
454 *PPARδ* and *PTGS2* expression (*P* = 0.09 and 0.08 respectively). Endometrial *PPARδ*
455 expression was 2.0 fold greater for cows receiving the Flax treatment compared with

456 those receiving the Control supplement (P = 0.02). When the two n-3 treatments were
457 combined, *PPARδ* expression was 2.0 fold greater (P = 0.02) compared with cows
458 receiving the Control diet. Endometrial *PTGS2* expression was 2.6 fold (P = 0.01) and
459 2.4 fold greater (P = 0.04) for Flax-treated cows compared with Control and FO-
460 supplemented cows, respectively. Although there was no overall treatment effect on
461 endometrial *PGFS2* expression, a 2.1 fold increase (P = 0.04) in *PGFS2* expression
462 for n-3-supplemented cows compared with Control cows was observed. There was no
463 overall treatment effect on endometrial PLA_2 expression, but cows receiving the Flax
464 treatment tended to have a 2.0 fold increase (P = 0.07) in endometrial PLA_2
465 expression compared with Control-supplemented cows. There was no effect of
466 treatment on endometrial expression of *PPARα, OTR, PGFS1, PGES, CBR1 or*
467 *FADS2*.
468
469 ***3.6. Hepatic gene expression***
470 Hepatic gene expression data are presented in Table 8. There was a significant effect
471 of treatment on hepatic *ACSL1, FABP1* and *SREBF1* expression. Hepatic expression
472 of *ACSL1* was increased for cows on Control (2.2 fold, P = 0.002), Flax (2.2 fold, P =
473 0.002) and CLA (2.3 fold, P = 0.001) treatments compared with cows receiving the
474 FO treatment. Hepatic expression of *FABP1* was increased in cows on the Flax (3.7
475 fold, $P < 0.001$), Control (3.0 fold, P = 0.005) and CLA (3.0 fold, P = 0.006)
476 treatments compared with cows on the FO treatment. Hepatic expression of *SREBF1*
477 was increased in cows on the Control (3.5 fold, P = 0.003), Flax (2.8 fold, P = 0.014)
478 and CLA (3.6 fold, P = 0.002) treatments compared with cows on the FO treatment.
479 There was no effect of fat supplementation on hepatic expression of *ACADVL,*
480 *ACOX1, CPT1A, DGAT1, GPAM, SCAP, SLC27A1, PPARα, CYP3A, or CYP2C.*

481

482 **4. DISCUSSION**

483

484 The objective of the current study was to identify the effects of four fat supplements
485 differing in fatty acid composition on follicle and CL development, steroid hormone
486 concentrations and endometrial and hepatic gene expression in lactating dairy cows.
487 The most important finding from this study was the differential effects of the fat
488 supplements on plasma P4 concentration and CL volume.
489 The central role that P4 plays in optimising the uterine microenvironment to support
490 embryo development in early pregnancy is widely accepted [28]. Cows that displayed
491 both a delayed rise in plasma P4 concentrations after ovulation and lower luteal phase
492 plateau concentrations of plasma P4 had retarded embryo development, with little or
493 no interferon-τ (IFN-τ) production on day 16 after insemination compared with cows
494 that had a normal P4 profile [29]. In the current study, mean plasma P4 concentrations
495 were reduced for the n-3-supplemented cows throughout the oestrous cycle. Elevated
496 P4 concentrations on days 5-7 and days 8-14 after ovulation were observed in cows
497 on the CLA and Control treatments compared with cows on the n-3 supplements. The
498 increase in plasma P4 at critical times in the oestrous cycle could alter uterine
499 endometrial secretions to stimulate embryo development, leading to a larger embryo
500 capable of signalling its presence to the dam. The differences in plasma P4
501 concentrations between the treatments were accompanied by similar differences in CL
502 volume.
503 Dietary fat supplementation has consistently increased plasma cholesterol
504 concentrations [2], the precursor for P4 synthesis. However, feeding whole flaxseed
505 has been reported to decrease plasma cholesterol concentrations compared to fat

506 sources low in n-3 PUFA [30,31], and incubation of dispersed luteal cells with EPA
507 and DHA reduced P4 concentrations [32]. In the current study, cows on the Control
508 diet had the lowest concentrations of plasma cholesterol, yet greater plasma P4
509 concentrations compared with n-3-treated cows. This indicates that cholesterol
510 availability was not the limiting factor in P4 synthesis, and that alternative
511 mechanisms are responsible for the variations in plasma P4 concentrations observed.
512 Transport of cholesterol from the cytoplasm to the inner cell membrane, mediated by
513 the steroidogenic acute regulatory protein (StAR), is the rate limiting step in P4
514 biosynthesis [33]. Once inside the mitochondrion, Cytochrome P450 cholesterol side
515 chain cleavage enzyme (P450scc) converts cholesterol to pregnenolone, which is then
516 converted to P4 by 3β-hydroxysteroid dehydrogenase/ Δ^5, Δ^4 isomerase (3β-HSD)
517 [33]. It is possible that the dietary treatments utilised in the current study influenced
518 the luteal expression and activity of one or more of the genes involved in P4
519 biosynthesis.
520 Following ovulation, the granulosa cells of the follicle differentiate into large luteal
521 cells to form the CL, and these large luteal cells may be responsible for up to 80% of
522 progesterone secretion in the mature CL [34]. It has been suggested that an increase in
523 CL volume in cows supplemented with PUFA may be due to increased numbers of
524 granulosa and theca cells from a larger dominant follicle [35]. The lack of treatment
525 differences in ovulatory follicle diameter in the current study does not support this
526 hypothesis. Treatment differences in CL volumes in the current study may be due to
527 either greater numbers of granulosa cells undergoing differentiation at ovulation, or
528 increased survival of these cells following formation of the CL. Further work is
529 required to fully determine the mechanisms responsible for the larger CL volume

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

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

539 Endometrial biopsies were collected on day 7 of the oestrous cycle in the current
540 study. This time point was selected as it represents a critical stage in embryo
541 development, when the embryo descends into the uterus [28]. A prerequisite for PG
542 synthesis is the generation of the PUFA substrate within the cell (e.g., arachidonic
543 acid for 2-series PGs). Arachidonic acid (AA) is liberated from the phospholipid
544 membrane by the action of a phospholipase. Many phospholipases have been
545 identified, of which intracellular Group IV cytosolic $A_{2\alpha}$ ($PLA_{2\alpha}$) appears most
546 important in controlling the availability of free AA for PG synthesis [38]. The free
547 AA is then metabolized by prostaglandin G/H synthase enzymes (PTGS2) to an
548 intermediate, PGH2, which is in turn converted to either PGF2 or PGE2 by the
549 enzymes PGF synthase (PGFS1 and PGFS2), and PGE synthase (PGES). Additional
550 $PGF_{2\alpha}$ may be synthesised by the reduction of PGE2, catalysed by the Carbonyl
551 Reductase enzyme CBR 1 [39]. In the current study, a tendency towards increased
552 endometrial expression of $PLA_{2\alpha}$ was observed for cows receiving the Flax
553 supplement compared with Control animals. This may indicate increased availability
554 of AA for PG synthesis, a theory supported by the concurrent increases in endometrial

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

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

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

580 (*CPT1A, ACOX1* and *ASCL1*) [43]. Liver biopsies were taken on day 7 of the oestrous
581 cycle in the current study. Day 7 was selected as the hormonal milieu (increasing
582 progesterone, low oestradiol) would have been the same in all cows at this stage of the
583 cycle. Any confounding effects of steroid hormones on hepatic metabolism [44] were
584 therefore negated. Hepatic expression of *ASCL1*, *FABP1* and *SREBF1* were reduced
585 in FO supplemented cows compared to all other treatments. The differences in fatty
586 acid composition of the FO supplement are likely to be the cause of these differences
587 in hepatic gene expression. In contrast to the other three supplements, which contain
588 fatty acids with 18 carbon chains or less, FO contains significant amounts of the very
589 long chain PUFA, EPA and DHA. Deckelbaum et al. [45] noted that EPA, DHA and
590 arachidonic acid (AA) have a greater inhibitory effect on *SREBF1* expression than
591 shorter chain PUFA. This inhibitory effect on *SREBF1* is in turn reflected in reduced
592 *ACSL1* and *FABP1* expression, as *SREBF1* is a major modulator of these genes [45].
593 The development of ovarian follicles during the early postpartum period has been
594 consistently demonstrated to be stimulated by fat supplementation [2]. Few
595 differential effects of fat supplementation on ovarian follicular development were
596 observed in the current study, in agreement with Childs et al. [46], who found no
597 differences in follicle development when supplementing heifers with either whole
598 soybean (n-6) or a fish oil (n-3) supplement. Similarly, Petit and Twagiramungu [35]
599 were unable to determine any differences in follicle development in cows fed either
600 Megalac, an n-3 flaxseed supplement or an n-6 soybean supplement.
601 Cows receiving the Flax treatment had shorter cycle lengths than cows fed either the
602 Control or FO diets. As ovulatory follicle size was unaffected by treatment, it is
603 plausible that a shorter oestrous cycle may have beneficial effects for subsequent
604 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 $PGF_{2\alpha}$ 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.

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Table 1: Ingredient and nutrient composition of the TMR and concentrate rations offered

TMR 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			
	Treatment			
Total Concentrate Ration Composition (kg as fed)	Control	Flax	CLA	FO
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

[1]Vitamin 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 soduim 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.
[2]UFL = 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

	g/100g of total fatty acids			
Fatty Acid	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
cis 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 *cis*-9	7.03	17.79	16.93	6.56
18:1 *trans*-9	0.14	0.43	0.33	2.28
18:1 *trans*-11	0.00	0.00	0.05	0.00
18:2 *cis*-9, *cis*-12 (n-6)	1.27	13.48	1.26	2.79
18:2 *cis*-9, *trans*-11 CLA (n-6)	0.00	0.08	14.51	0.21
18:2 *trans*-10, *cis*-12 CLA (n-6)	0.00	0.00	15.52	1.20
All *trans* 18:2	0.05	0.13	3.13	0.64
18:3 *cis*-6, *cis*-9, *cis*-12 (n-6)	0.21	0.17	0.00	0.00
18:3 *cis*-9, *cis*-12, *cis*-15 (n-3)	0.00	50.74	0.31	0.46
20:0	0.00	0.35	0.51	0.82
20:1 *cis*-11	0.00	0.32	0.08	2.54
20:2 *cis*-11, *cis* -14	0.00	0.03	0.00	0.33
20:3 *cis*-8, *cis*-11, *cis*-14 (n-6)	0.00	0.00	0.00	0.21
20:3 *cis*-11, *cis*-14, *cis*-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 $PGF_{2\alpha}$
46-52	Cows observed for oestrus
53-59	Liver and endometrial biopsies taken 7 days after ovulation

[1]Between 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.

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

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

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

[1]PGFS; 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					P-value	
	Control	Flax	CLA	FO	SEM	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					
	Control	Flax	CLA	FO	SEM	P-value
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 ($P < 0.05$)

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-trasnformed 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]

	Treatment				
Gene[3]	Control	Flax	CLA	FO	P value
Endometrial tissue					
PPARα	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
PPARδ	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
PPARγ	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
PTGS2	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
OTR	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
PGFS1	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
PGFS2	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
PGES	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
CBR1	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
PLA_2	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
FADS2	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					
ACADVL	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
ACOX1	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
ACSL1	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
CPT1A	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
DGAT	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
FABP1	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
GPAM	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
SCAP	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
SLC27A1	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
SREBF1	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
PPARα	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
CYP3A	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
CYP2C	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

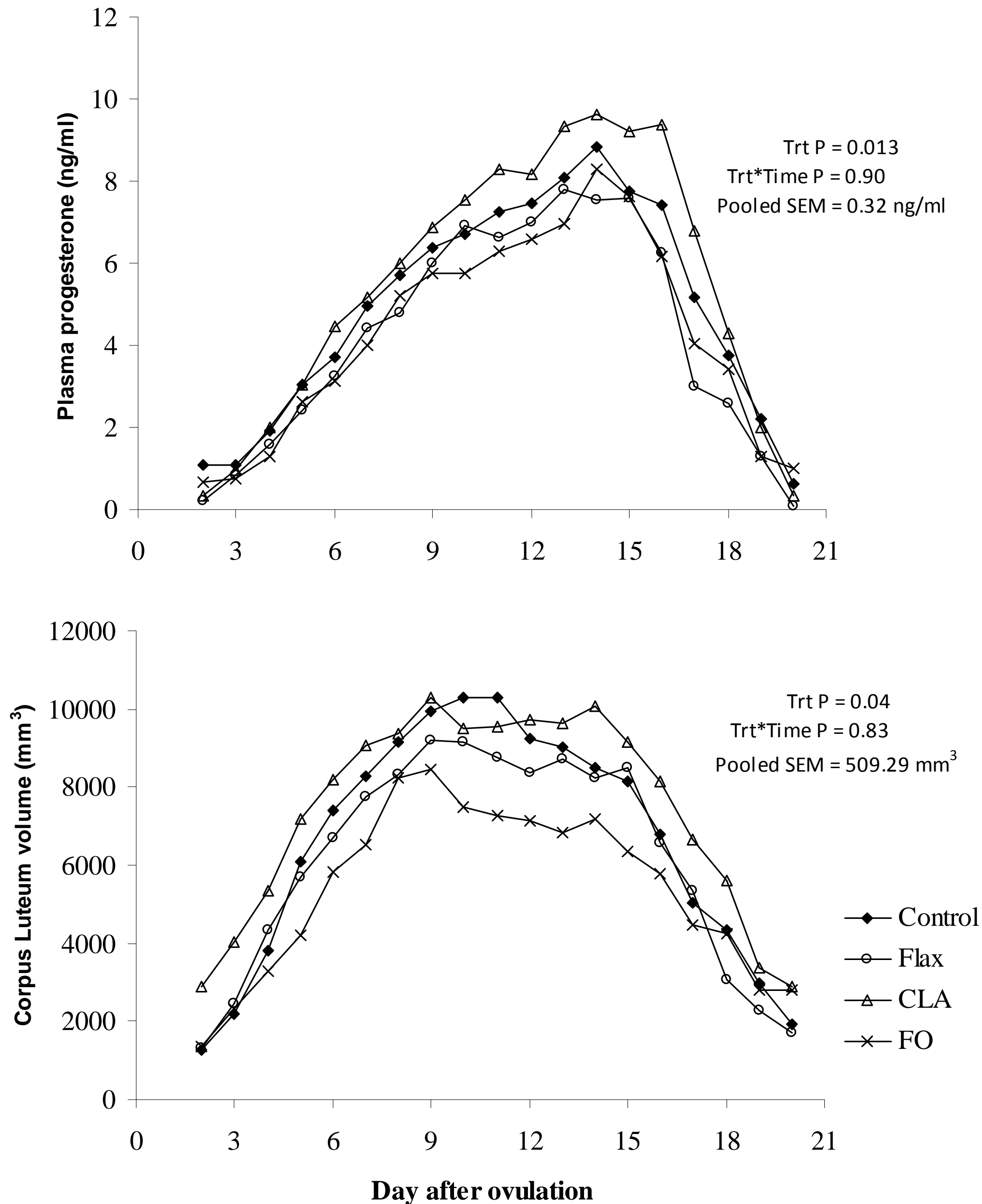

Figure 1: Plasma progesterone (upper panel) and corpus luteum volume (lower panel) in cows receiving Control, Flax, CLA and Fish Oil (FO) lipid supplements. Statistical analysis was performed using data from day 2 to day 14 following ovulation. Data from day 15 to day 20 after ovulation are included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