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Effect of dietary enrichment with either n-3 or n-6 fatty acids on systemic metabolite and hormone concentration and ovarian function in heifers

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*The objective of this experiment was to examine the effects of dietary n-3 or n-6 fatty acid (FA) supplementation on blood FA, metabolite and hormone concentrations, follicle size and dynamics and corpus luteum (CL) size. Reproductively normal heifers (*n *= 24) were individually fed diets of chopped straw and concentrate containing either (i) no added lipid (CON;* n *= 8); (ii) 2% added fat as whole raw soya beans (WSB, n-6;* n *= 8); or (iii) 2% added fat as fish oil (FO, n-3;* n *= 8). Following oestrous cycle synchronisation, blood samples were collected at appropriate times and intervals for the measurement of hormones, FAs and metabolites. On days 15 and 16 of the cycle, animals were subjected to an intravenous oxytocin challenge and prostaglandin* $F_{2\alpha}$ *($PGF_{2\alpha}$) response, measured as venous concentrations of 13,14-dihydro-15-keto* $PGF_{2\alpha}$ *(PGFM). Dry matter intake and average daily gain were similar among treatments (*$P > 0.05$*). Plasma concentration of linoleic acid was highest on WSB (*$P < 0.05$*), while eicosapentaenoic (EPA, n-3;* $P < 0.0001$*) and docosahexaenoic acid (DHA, n-3;* $P < 0.0001$*) were greatest in the FO group. Plasma concentrations of arachidonic acid were higher on FO (*$P < 0.05$*) compared with CON and WSB. Plasma triglyceride concentrations increased, while β-hydroxybutyrate (BHBA) decreased with time on all diets (*$P < 0.05$*). There was a diet × time interaction (*$P < 0.01$*) for non-esterified fatty acid (NEFA) concentrations. Plasma cholesterol was higher on WSB and FO (*$P < 0.01$*) compared with CON. Progesterone (P_4) and oestradiol (E_2) concentrations, as well as follicle growth rate and CL diameter were similar across diets (*$P > 0.05$*). There was a diet × day interaction for PGFM (*$P < 0.01$*). When corrected for systemic* $E_2 : P_4$ *ratio, day 15 concentrations of PGFM were higher in the WSB group at 15 and 30 min (*$P < 0.01$*) post oxytocin administration compared with CON and FO, which were similar (*$P > 0.05$*). Concentrations of PGFM on day 16 were similar for WSB and FO and were greater than CON at 15 (*$P < 0.01$*) and 45 min (*$P < 0.05$*) post oxytocin administration, and at 30 min for FO (*$P < 0.05$*). With the exception of PGFM, dietary lipid source did not affect the reproductive variables measured.*

Keywords: fish oil, cattle, prostaglandin metabolite, progesterone, linoleic acid

Introduction

Rapid genetic progress and better nutrition have led to significant improvements in dairy cow milk yield in recent decades; however, this has been accompanied by a dramatic decline in cow fertility. A decline in the first service conception rate of approximately 1% per annum has been reported worldwide (Butler *et al*., 1995; Royal *et al*., 2000; Evans *et al*., 2004). Reproductive inefficiency is a significant financial problem for the dairy industry and especially for seasonal pasture-based dairy production systems such as practised in Ireland (Horan *et al*., 2005).

Nutrition, particularly energy nutrition, fundamentally impacts reproduction. For example, in early *post partum* dairy cows because energy demand exceeds energy intake, cows typically enter a state of negative energy balance (NEB), which is associated with poor reproductive performance. Prolonged depletion of body reserves during early lactation (see review by Butler, 2001) or even short-term fluctuations in energy intake (Dunne *et al*., 1999) can impair both the resumption of ovarian activity *post partum* and/or conception rates.

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Supplemental fats are often included in the diet of dairy cows to reduce the energy deficit experienced in early lactation. Aside from the beneficial effects on the FA composition of milk (see review by Bauman and Griinari, 2003), fat supplementation, and in particular poly-unsaturated fatty acid (PUFA) supplementation, has been reported to positively influence cow reproductive performance (see reviews, Wathes *et al*., 2007; Staples *et al*., 1998); however, the specific mechanisms involved are ill defined (Wathes *et al*., 2007).

The inclusion of dietary fat has been reported to increase follicle number and diameter (Lucy *et al*., 1990; Lammoglia *et al*., 1997). Furthermore, fat supplementation may increase systemic concentrations of progesterone through either increased *de novo* synthesis or reduced hepatic metabolism (Hawkins *et al*., 1995). Additionally, Mattos *et al*. (2000) proposed that n-3 PUFA may reduce uterine $PGF_{2\alpha}$ secretion and/or decrease the sensitivity of the corpus luteum (CL) to $PGF_{2\alpha}$. This may improve fertility by reducing the degree of embryonic loss associated with the inadequate suppression of $PGF_{2\alpha}$ in early pregnancy.

In contrast to the effects of n-3 PUFA, the essential n-6 PUFA, linoleic acid, which is a precursor to arachidonic acid (AA) can increase dienoic PGs, including $PGF_{2\alpha}$ (Petit *et al*., 2002). While a reduction in $PGF_{2\alpha}$ would be beneficial in the context of inhibiting luteolysis during the breeding season, stimulation of early *post partum* $PGF_{2\alpha}$ has been associated with improved uterine health (Abayasekara and Wathes, 1999) and uterine involution (Guilbault *et al*., 1985).

The objective of the current study was to examine the effects of feeding diets rich in either n-3 (eicosapentaenoic (EPA), and docosahexaenoic acids (DHA)) or n-6 (linoleic acid) PUFA on concentrations of reproductive and metabolic hormones, metabolites, ovarian follicle dynamics and CL diameter in cattle. A nulliparous heifer model was used to avoid possible confounding effects of lactation and associated NEB.

Materials and methods

Animals and oestrous cycle synchronisation

Reproductively normal nulliparous crossbred beef heifers ($n = 24$) with a mean ± s.e.m. age of 20 ± 2 months and liveweight of 442 ± 10 kg were oestrus synchronised using two injections (PG1 & PG2) of a 500μg of prostaglandin F2 (PG) analogue (Cloprostanol, Estrumate®; Schering-Plough Ltd, Shire Park, Welwyn Garden City, Hertfordshire, UK), administered intramuscularly 11 days apart (Figure 1). Observation for oestrous activity was carried out using an electronic heat mount detection system (Heatwatch®; DDx Inc., Denver, CO, USA) combined with visual observations at 0700, 1100, 1500, 1900 and 2300 h beginning 24 h after the second prostaglandin administration; this heat check was repeated towards the end of the subsequent oestrous cycle.

Experimental diets and feeding regime

Animals were housed in slatted floor pens, blocked on liveweight and body condition score (BCS) and randomly assigned, within block, to one of three concentrate and straw-based diets ($n = 8$ per diet). The concentrates contained either (i) no added lipid (CON); (ii) 2% added fat as supplemental whole raw soya beans (WSB) or (iii) 2% added fat as supplemental fish oil (FO). The FO was derived from mixed fish species, predominantly herring and mackerel (United Fish Industries Grade 1 fish oil, United Fish Industries, Killybegs, Co. Donegal, Ireland). The ingredient composition and chemical analysis of the three concentrates as well as the chemical analysis of the straw is presented in Table 1. The FA composition of the WSB and fish oil is presented in Table 2. The WSB and FO diets were formulated to provide approximately 150 g/head per day of soya oil and fish oil, respectively. All diets were formulated to be isonitrogenous (14% crude protein (CP) in total diet dry matter (DM)). Animals were allocated to diets following response to PG1 and diets were offered for 32 days.

The animals were offered their respective daily lipid supplement in a 1 kg DM bolus feed at 0900 h each day. At 1200 h each day, animals were offered 4.5 kg DM of a balancer ration (Table 1) and 1.5 kg DM straw. All animals were individually fed using an electronic feeding system (Calan Inc., Northwood, NH, USA).

Liveweight and body condition scoring

Animals were weighed on days 0, 16, 27 and 32. At the start (days 0 and 1) and the end (days 31 and 32) of the

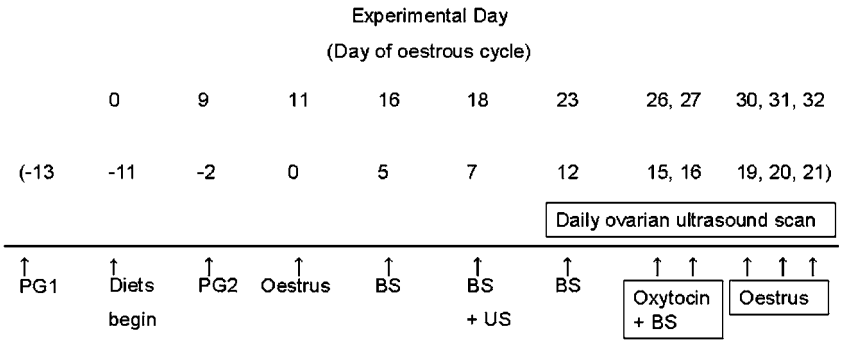

Figure 1 Sequence of injection and collection of samples. PG = prostaglandin (Estrumate®), BS = Blood sample, US = ovarian ultrasound scan.

Table 1 *Ingredient composition (g/kg as fed) and the chemical analysis (expressed as g/kg of DM unless otherwise stated) of the concentrates and forage*

	Control	WSB	FO	BAL	Straw
Barley	458	182	242	682	–
Soya bean meal (48%)	507	–	550	231	–
Whole Soya bean	–	768	–	–	–
Fish oil	–	–	153	–	–
Molasses (Cane)	–	20	20	50	–
*Vitamin mineral premix	20	20	20	20	–
Ground Limestone	6	7.5	4	2	–
Salt	3	2.5	3	3	–
Di-calcium phosphate	6	0	8	12	–
DM (g/kg)	866	870	886	848	861
CP	334	327	335	209	46.19
CF	54.76	61.97	43.60	54.29	535.70
ADF	76.34	84.67	63.18	71.22	581.10
ADL	9.45	7.93	6.69	10.94	87.11
NDF	145.82	144.89	112.31	151.64	881.29
Ash	44.50	50.27	42.42	31.17	35.03
Ether Extract	16.86	166.85	166.71	16.20	5.99
Gross Energy (MJ/kg DM) DM)	16.05	18.70	19.60	15.39	17.54

Abbreviations are: DM = dry matter; CP = crude protein; CF = crude fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; NDF = neutral detergent fibre.
*Vitamin mineral premix contained: vitamin A, 320 000 IU/kg; vitamin D_3 80 000 IU/kg; vitamin E (α-tocopherol), 100 mg/kg; cobalt as cobalt carbonate, 200 mg/kg; zinc as zinc oxide, 4000 mg/kg; iodine as potassium iodate, 320 mg/kg; calcium, 120 g/kg; phosphorus, 50 g/kg; and magnesium, 100 mg/kg.

Table 2 *Fatty acid composition of WSB and FO used (g/100 g FAME)*

	Supplementary Oil	
Fatty acid	WSB	FO
Myristic (C14:0)	0.30	7.36
Palmitic (C16:0)	12.18	16.36
Stearic (C18:0)	3.87	2.49
Vaccenic (t11C18:1)	0.39	0.12
Oleic (C18:1)	22.60	12.87
Linoleic (C18:2)	51.87	1.64
Linolenic (C18:3)	5.51	–
Eicosa-trienoic (C20:3)	–	0.03
Arachidonic (C20:4)	–	1.80
EPA (C20:5)	0.09	6.86
DPA (C22:5)	0.14	–
DHA (C22:)	–	10.41
Others and Unknowns[1]	3.05	40.06

Abbreviations are: EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.
[1]Denotes fatty acids identified but not reported and quantified but unidentified peaks.

experiment liveweight was taken as the mean of weights measured on each of two successive days. The weighing scales were calibrated at regular intervals using known weights.

Feed sampling and analysis

Weekly composite samples of straw and concentrates were stored at −20°C until analysed for DM, CP, crude fibre (CF), acid detergent fibre (ADF), acid detergent lignin (ADL), neutral detergent fibre (NDF), ether extract, ash and gross energy (GE). Samples were milled through a 1-mm screen using a Christy and Norris hammer mill (Christy and Norris Process Engineers Ltd, Chelmsford, England). DM was determined by oven drying at 104°C for a minimum of 16 h. Ash was determined on all materials after ignition of a known weight of ground material in a muffle furnace (Nabertherm, Bremen, Germany) at 550°C for 4 h. Crude fibre was determined on all samples using a Fibertec extraction unit (Tecator, Hoganas, Sweden) according to the method of Van Soest *et al.* (1991). CP (total nitrogen*6.25) was determined using the method of Sweeney (1989) using a Leco FP 528 nitrogen analyser (Leco Instruments, UK Ltd, Newby Road, Hazel Grove, Stockport, Cheshire, UK). Ether extract was determined using a Sortex instrument (Tecator, Hoganas, Sweden) while the gross energy of the samples was determined using a Parr 1201 oxygen bomb calorimeter (Parr, Moline IL, USA).

Blood sampling

Blood samples were collected for measurement of plasma concentrations of progesterone (P_4), oestradiol (E_2), 13,14 dihydro, 15-keto prostaglandin $F_{2\alpha}$ (PGFM), FAs, cholesterol, glucose, triglycerides, urea, non-esterified fatty acids (NEFA) and β-hydroxybuterate (BHBA). Blood was collected by jugular venipuncture under license in accordance with the European Community Directive, 86-609-EC. Blood sampling for FAs and metabolites was carried out in the morning immediately prior to feeding. Samples for determination of P_4 and E_2 were also collected in the morning

before feeding and again at 1500 h. Samples (10 ml) were collected into ethylene diamine tetraacetic acid (EDTA) heparinised vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) for the determination of plasma concentrations of P_4 and into lithium heparinised vacutainers (Becton Dickinson Vacutainer Systems) for plasma concentrations of E_2, FAs, metabolites and PGFM. On collection, samples were immediately stored in ice water and centrifuged at $1500 \times \mathbf{g}$ at 4°C for 15 min. Plasma was harvested, and stored at −20°C pending analysis. Samples for the measurement of progesterone in plasma were collected on the day of standing oestrus (day 0), and again on days 5, 6, 7, 10, 15, 16, 19 and 20. Blood samples for determination of plasma concentrations of oestradiol were collected on a daily basis from day 12 of the oestrus cycle through to day 17. Blood samples for FA and metabolite concentrations were collected on day 10 and day 27 of the experimental period.

Oxytocin challenge

To examine the effect of dietary treatment on plasma PGFM, the animals were administered an oxytocin challenge on days 15 and 16 of the oestrous cycle. Blood samples were taken at −60 and again at 0 min to measure basal PGFM concentrations. A 10 ml dose of oxytocin (50 IU; Oxytocin-TAD, AniMedica GmbH, Senden-Bosensell, Germany) was administered intravenously into a jugular vessel immediately after the 0 time point sample. Subsequent blood samples were collected again at 15, 30, 45, 60, 75 and 90 min after oxytocin administration.

Blood hormone and metabolite analysis

Progesterone was measured using the Coat-a-Count assay procedure (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation for samples containing low (0.26 ± 0.08 ng/ml), medium (2.43 ± 0.26 ng/ml) and high (6.59 ± 0.78 ng/ml) concentrations of P_4 were 13.1% and 27.0% (low), 6.8 and 8.6% (medium) and 9.7% and 8.3% (high). The minimum detectable concentration of the assay was 0.10 ± 0.04 ng/ml (mean ± s.e.m.).

Oestradiol was determined as described by Prendiville *et al.* (1995) using an oestradiol MAIA assay kit (Biostat Ltd, Stockport, UK). The mean intra-assay and inter-assay coefficients of variation for samples containing low (0.53 ± 0.02 pg/ml), medium (2.13 ± 0.08 pg/ml) and high (4.43 ± 0.30 pg/ml) estradiol concentrations were 10.7% and 4.2% (low), 7.0% and 3.9% (medium) and 11.3% and 6.9% (high), respectively. The sensitivity of the assay was 0.3 pg/ml.

Blood plasma was analysed for glucose, triglyceride, NEFA, BHBA, urea and cholesterol concentrations using appropriate kits and an ABX Mira auto analyser (ABX Mira, Cedex, France).

Plasma concentrations of PGFM were measured in 100 μl samples of whole plasma as described previously (Robinson *et al.*, 2002). The tracer (13,14-dihydro-15-keto[5, 6, 8, 9, 11, 12, 14(n)- ^{3}H]-prostaglandin $F_{2\alpha}$) was obtained from Amersham International PLC (Amersham, Bucks, UK), the standards were supplied by Sigma (Poole, UK) and the antiserum was a kind gift from Prof Rodney W Kelly (MRC Human Reproduction Sciences Unit, Centre for Reproductive Biology, University of Edinburgh, UK). The sensitivity of the assay was 14.0 pg/ml. Intra- and inter-assay coefficients of variation were 6.6% and 3.7%, respectively.

Fatty acid analysis of feed and plasma

Total lipids were extracted from 1 ml of plasma and from 6 g of feed sample using chloroform methanol (2 : 1 v/v) as described by Folch *et al.* (1957). Methylation was carried out for both plasma and feed samples by *in situ* transesterification with 0.5N methanolic NaOH followed by 14% boron trifluoride in methanol as described by Park and Goins (1994). The fatty acid methyl esters (FAME) were separated using a CP Sil 88 column (100 m × 0.25 mm i.d., 0.20 μm film thickness; Chrompack, Middleburg, The Netherlands) and quantified using a gas liquid chromatograph (3400; Varian, Harbor City, CA, USA). The internal standard used was heptadecanoic acid (C17:0; Sigma-Aldrich Ireland Ltd) and the GC was calibrated using a range of commercial FA standards (Sigma-Aldrich). The GC was fitted with a flame ionisation detector and helium (37 psi) was used as the carrier gas. The injector temperature was held isothermally at 225°C for 10 min and the detector temperature was 250°C. The column oven was held at an initial temperature of 140°C for 8 min and then programmed to increase at a rate of 8.5°C/min to a final temperature of 200°C, which was held for 41 min. Data were recorded and analysed on a Minichrom PC system (VG Data System, Manchester, UK).

Follicular dynamics and CL diameter

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

Statistical analyses

Data were first checked for adherence to a normal distribution (PROC UNIVARIATE, SAS v. 9.1, 2002). Continuous data were analysed using two-way ANOVA with terms included for diet and block. Variables having more than one observation per subject such as DM intake (DMI), average daily gain (ADG), plasma analytes and follicular dynamics were analysed using repeated measures ANOVA (PROC MIXED, SAS v. 9.1, 2002) with terms for diet, time period and their interaction included in the model and animal within diet set as the error term. The type of variance–covariance structure used was chosen depending on the

magnitude of the Akaike criterion (AIC) for models run under compound symmetry, unstructured, autoregressive or Toeplitz variance–covariance structures. The model with the lowest AIC was selected. The PDIFF (predicted difference) and CONTRAST (for orthogonal contrasts) statements of SAS (v. 9.1, 2002) were used, and the Tukey test was applied as appropriate to evaluate pairwise comparisons of treatment means. While individual concentrations of E_2 and P_4 did not effect PGFM concentrations, the $E_2 : P_4$ ratio was found to have an affect and therefore was used as a covariate in the analysis of treatment effects on PGFM.

Results

Dry matter intake and animal performance

There was no effect ($P > 0.05$) of diet on either daily DMI (7.19 ± 0.05 *v.* 7.17 ± 0.07 *v.* 7.07 ± 0.11 kg/day for CON, WSB and FO, respectively) or ADG (0.80 ± 0.17 *v.* 0.67 ± 0.07 *v.* 0.90 ± 0.11 kg/day for CON, WSB and FO, respectively).

Plasma concentration of fatty acids

The effect of diet and day of sampling on the FA profile of plasma is shown in Table 3. Amongst the shorter chain FAs, vaccenic acid increased on the FO diet, while palmitic and stearic acid remained similar. Several FAs showed a statistically significant treatment × day of sampling interaction: myristic acid increased with time on CON and WSB but not on FO whereas palmitic, stearic and oleic all decreased with time on WSB but not on CON or FO. As expected, heifers fed the WSB diet had higher concentrations of the n-6 PUFA linoleic acid in comparison with the other two diets. Concentrations of the n-3 PUFA linolenic acid decreased with time on treatment in CON and WSB but increased in FO. Heifers fed the FO diet had higher ($P < 0.0001$) concentrations of the n-3 PUFAs linolenic acid ($P < 0.001$), EPA and DHA in comparison with the control diet, whereas eicosatrienoic acid was reduced in FO. The n-6 PUFAs AA and t10, c12 CLA were both higher ($P < 0.0001$) on the FO diet than on either control or WSB diets. There were diet × day of sampling interactions for linolenic acid and EPA where both FA increased with time on FO but did not change ($P > 0.05$) on either WSB or CON.

Plasma metabolites

The effect of diet on plasma concentrations of metabolites is presented in Table 4. Plasma concentrations of triglycerides decreased with time on all diets whereas BHBA increased. Supplementation with PUFA increased cholesterol concentrations and also caused a time related change in NEFA: with increases with time on WSB ($P < 0.05$) but no change in NEFA with time in either CON or FO ($P > 0.05$). Heifers on WSB had the highest concentration of urea while plasma concentrations of glucose were not altered by diet ($P > 0.05$).

Plasma progesterone and oestradiol and the effect of progesterone, oestradiol and the oestradiol : progesterone ratio on concentrations of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$

There was no diet × sample day interaction or effect of diet on plasma concentrations of either progesterone or oestradiol ($P > 0.05$) on any day of the oestrous cycle (Figures 2 and 3).

Table 3 *Effect of diet on fatty acid concentrations (g/100 g FAME) of plasma collected on day 10 and 27 of the 32-day trial period (mean ± s.e.m.)*

	Diet								
	CON		WSB		FO				
	Day		Day		Day		Statistical significance		
Fatty acid	10	27	10	27	10	27	TRT	DAY	TRT × DAY
Myristic (C14:0)	$4.85 \pm 0.87^{a,x}$	$9.26 \pm 0.81^{a,y}$	$3.62 \pm 0.81^{a,x}$	$7.56 \pm 0.81^{a,y}$	$6.32 \pm 0.81^{a,x}$	$6.55 \pm 0.81^{a,x}$	NS	***	*
Palmitic (C16:0)	$11.77 \pm 0.54^{a,x}$	$10.51 \pm 0.51^{a,x}$	$12.46 \pm 0.51^{a,x}$	$9.29 \pm 0.51^{a,y}$	$12.55 \pm 0.51^{a,x}$	$11.89 \pm 0.51^{a,x}$	*	***	*
Stearic (C18:0)	$17.77 \pm 0.88^{a,x}$	$16.01 \pm 0.24^{a,x}$	$20.98 \pm 0.82^{a,x}$	$16.79 \pm 0.82^{a,y}$	$13.04 \pm 0.82^{b,x}$	$13.15 \pm 0.82^{a,x}$	***	*	*
Vaccenic (t11C18:1)	0.81 ± 0.22^{a}	0.86 ± 0.21^{a}	0.95 ± 0.21^{a}	0.70 ± 0.21^{a}	3.30 ± 0.21^{b}	3.02 ± 0.21^{b}	***	NS	NS
Oleic (C18:1)	6.73 ± 0.52^{ax}	$5.82 \pm 0.48^{a,x}$	$6.87 \pm 0.48^{a,x}$	$4.60 \pm 0.48^{b,y}$	$5.83 \pm 0.48^{a,x}$	$5.88 \pm 0.48^{a,x}$	NS	*	<0.10
Linoleic (C18:2)	16.22 ± 1.28^{a}	$16.11 \pm 1.20^{a,\ b}$	22.50 ± 1.20^{b}	20.33 ± 1.20^{a}	13.48 ± 1.20^{a}	15.23 ± 1.20^{b}	***	NS	NS
c9t11CLA (C18:2)	0.08 ± 0.02	0.11 ± 0.04	0.12 ± 0.03	0.05 ± 0.04	0.10 ± 0.02	0.13 ± 0.02	NS	NS	NS
t10c12CLA (C18:2)	$0.09 \pm 0.11^{a,b}$	0.05 ± 0.11^{a}	0.11 ± 0.05^{a}	0.09 ± 0.08^{a}	0.44 ± 0.04^{b}	0.53 ± 0.04^{b}	***	NS	NS
Linolenic (C18:3)	$1.40 \pm 0.12^{a,x}$	$1.16 \pm 0.11^{a,x}$	$1.87 \pm 0.11^{b,x}$	$1.32 \pm 0.11^{a,y}$	$1.65 \pm 0.11^{a,b,x}$	$2.05 \pm 0.11^{b,x}$	***	NS	***
Eicosa-trienoic (C20:3)	0.91 ± 0.09^{a}	1.07 ± 0.08^{a}	0.89 ± 0.08^{a}	1.00 ± 0.08^{a}	0.55 ± 0.08^{b}	0.46 ± 0.08^{a}	***	NS	NS
Arachidonic (C20:4)	1.22 ± 0.11^{a}	$1.35 \pm 0.10^{a,b}$	1.48 ± 0.10^{a}	1.28 ± 0.10^{a}	1.92 ± 0.10^{b}	1.71 ± 0.10^{b}	***	NS	NS
EPA (C20:5)	$0.81 \pm 0.27^{a,x}$	$0.59 \pm 0.25^{a,x}$	$0.85 \pm 0.25^{a,x}$	$0.52 \pm 0.25^{a,x}$	$2.83 \pm 0.25^{b,x}$	$3.92 \pm 0.25^{b,y}$	***	NS	*
DHA (C22:6)	0.25 ± 0.07^{a}	0.23 ± 0.07^{a}	0.23 ± 0.07^{a}	0.25 ± 0.07^{a}	0.90 ± 0.07^{b}	0.815 ± 0.07^{b}	***	NS	NS
Others[1]	6.67 ± 0.53	7.46 ± 0.49	6.43 ± 0.49	6.56 ± 0.49	8.41 ± 0.49	6.99 ± 0.49	<0.10	NS	<0.10
Unknowns[2]	30.52 ± 2.53	29.54 ± 2.36	20.77 ± 2.36	29.75 ± 2.36	28.70 ± 2.36	27.70 ± 2.36	NS	NS	<0.10

[a,b]Within diet, concentrations with different superscripts are different ($P<0.05$). [x,y]Within day, concentrations with different superscripts are different ($P < 0.05$). $P < 0.10$ is accepted as approaching statistical significance.
[1]Denotes fatty acids quantified but not reported.
[2]Denotes fatty acids quantified but not identified.

Table 4 *The effect of diet on plasma metabolite concentrations (mmol/l; mean ± s.e.m.)*

	Diet								
	CON		WSB		FO				
	Day		Day		Day		Statistical significance		
Metabolite	10	27	10	27	10	27	TRT	DAY	TRT × DAY
Cholesterol	2.05 ± 0.224	1.99 ± 0.215	2.90 ± 0.224	3.23 ± 0.215	2.54 ± 0.224[a]	3.41 ± 0.215[b]	***	*	<.10
Triglycerides	0.31 ± 0.030	0.21 ± 0.024	0.31 ± 0.030	0.21 ± 0.024	0.23 ± 0.030	0.17 ± 0.024	NS	***	NS
Glucose	4.44 ± 0.150	4.33 ± 0.166	4.37 ± 0.150	4.23 ± 0.166	4.45 ± 0.150	4.36 ± 0.166	NS	NS	NS
Urea	4.96 ± 0.324	4.64 ± 0.335	5.99 ± 0.324	5.70 ± 0.335	4.65 ± 0.324	5.48 ± 0.335	*	NS	NS
BHBA	0.18 ± 0.017[a]	0.36 ± 0.026[b]	0.18 ± 0.017[a]	0.32 ± 0.026[b]	0.22 ± 0.017[a]	0.35 ± 0.026[b]	NS	***	NS
NEFA	0.35 ± 0.055	0.39 ± 0.041	0.31 ± 0.055[a]	0.45 ± 0.041[b]	0.49 ± 0.055	0.40 ± 0.041	NS	NS	*

Within diet, concentrations with different superscripts are different ($P < 0.05$).
$P < 0.10$ is accepted as approaching statistical significance.

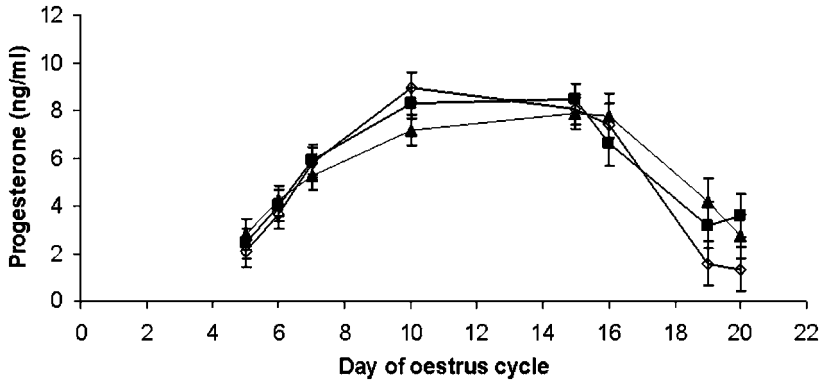

Figure 2 Plasma concentrations of progesterone (ng/ml) of heifers fed CON (■, $n = 8$), WSB (◇, $n = 8$) or FO (▲, $n = 8$) diets.

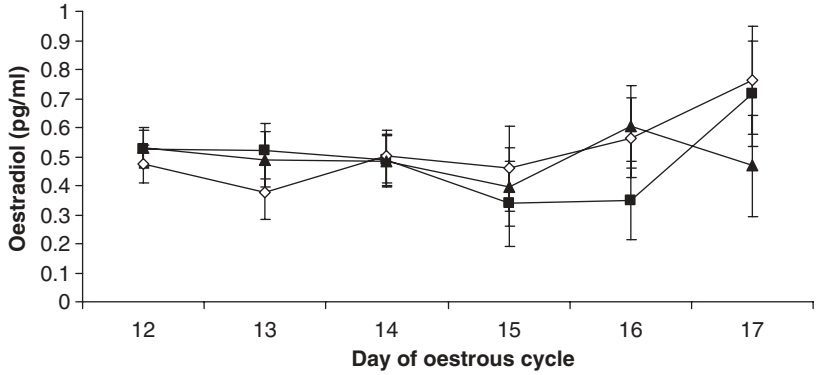

Figure 3 Plasma concentrations of oestradiol (pg/ml) of heifers fed CON (■, $n = 8$), WSB (◇, $n = 8$) or FO (▲, $n = 8$) diets.

There was no effect ($P > 0.05$) of concentrations of either progesterone or oestradiol on plasma PGFM on either day 15 or 16 of the oestrous cycle. However, there was an effect ($P < 0.001$) of the oestradiol : progesterone ratio on PGFM on both days. Consequently, the oestradiol : progesterone ratio was included as a covariate in the analysis of diet effects on PGFM concentrations outlined in the next section.

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

The effect of diet on plasma concentrations of 13, 14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) produced in response to an oxytocin challenge on days 15 and 16 of the oestrous cycle is shown in Figures 4 and 5. Although there were no ($P > 0.05$) diet × day × time or diet × time interactions for concentrations of PGFM, there was a diet × day of oestrous cycle interaction ($P < 0.01$). On day 15, PGFM

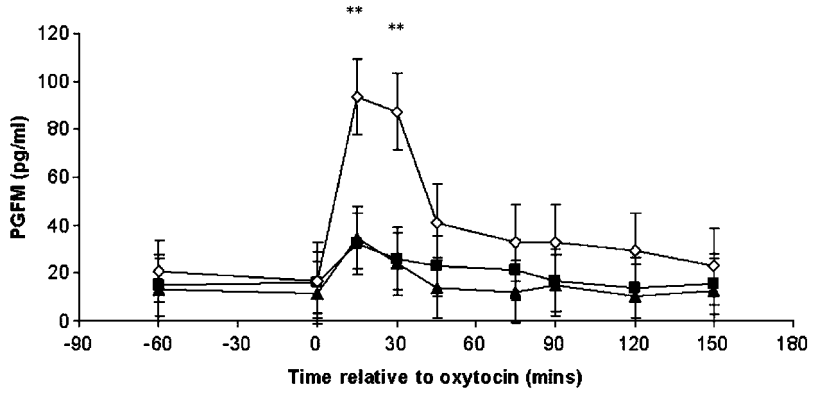

Figure 4 Plasma concentrations of prostaglandin F2α metabolite (PGFM) on day 15 of the oestrus cycle of heifers fed CON (■, $n = 8$), WSB (◇, $n = 8$) or FO (▲, $n = 8$) diets following correction for $P_4 : E_2$ (**$P < 0.01$).

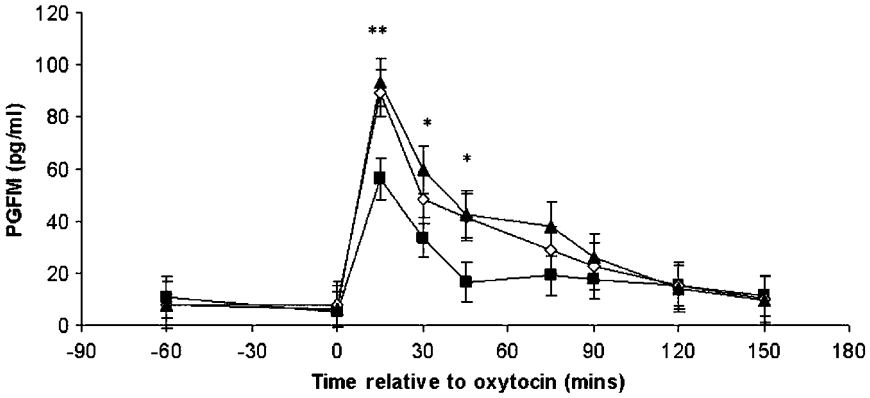

Figure 5 Plasma concentrations of prostaglandin F2α metabolite (PGFM) on day 16 of the oestrus cycle of heifers fed CON (■, $n = 8$), WSB (◇, $n = 8$) or FO (▲, $n = 8$) diets following correction for $P_4 : E_2$ ratio (**$P < 0.01$; *$P < 0.05$).

Table 5 *The effect of treatment on cycle length, dominant follicle size and growth rate and corpus luteum diameter on day 7 post oestrous (mean ± s.e.m.)*

	Diet		
	Control	WSB	FO
Cycle length (days)	22.13 ± 0.950	20.00 ± 0.500	21.50 ± 0.730
Follicles			
DF growth rate (mm/day)	1.31 ± 0.130	1.53 ± 0.570	1.23 ± 0.240
DF diameter (mm)	14.55 ± 1.404	14.18 ± 0.640	14.21 ± 0.403
Corpus Luteum			
CL diameter (mm)	23.05 ± 0.990	21.43 ± 1.400	21.88 ± 1.530

concentrations were higher ($P < 0.01$) on WSB at 15 and 30 min post oxytocin compared with either CON or FO, which were similar ($P > 0.05$). On day 16, however, concentrations of PGFM at 15 min post oxytocin were similar ($P > 0.05$) for FO and WSB, which were both higher ($P < 0.01$) than CON while at 30 min post oxytocin concentrations for FO and WSB groups were again similar ($P > 0.05$) but only FO was higher than CON ($P < 0.05$). At 45 min post oxytocin on day 16, PGFM concentrations on FO and WSB were similar and both higher ($P < 0.05$) than CON.

Oestrous cycle length, ovulatory follicle and corpus luteum diameter

There was no effect of diet on the length of the oestrous cycle, or on the maximum diameter or growth rate of the pre-ovulatory follicle ($P > 0.05$; Table 5). The diameter of the CL (Table 5) measured on day 7 post oestrus was not affected by diet ($P > 0.05$). There was no relationship between CL diameter measured on day 7 of the oestrous cycle and P_4 concentrations on any day of the oestrous cycle ($P > 0.05$).

Discussion

In this study there was no effect of diet on DMI. Whitlock *et al.* (2006) reported no adverse effects on DMI when feeding fish oil up to 1% of dietary DM to lactating dairy cows; however, inclusion rates of 2% resulted in an 11% decrease in DMI (Whitlock *et al.*, 2002). Jordan *et al.* (2006), using the same WSB source as used in the current study, attributed the 20% depression in DMI recorded in young bulls fed a 30% WSB ration *ad libitum* to a reduction in the palatability of the diet. However, the relatively low level of WSB (12.5%) fed in the current study was unlikely to have had a negative impact on DMI. The similarity in daily DMI across diets was mirrored in animal performance with no effect on ADG, consistent with the report of Wistuba *et al.* (2006).

Despite evidence of a diet $\times$ day interaction for plasma concentrations of some FA measured, concentrations of most FA were generally consistent with other published reports (Filley *et al.*, 2000; Burns *et al.*, 2003) involving similar dietary approaches. Consequently, rather than discussing the effect of diet on each FA measured, we focus on a select number of FAs in the context of their potential biological roles in metabolism and reproduction. The addition of the whole soya beans increased the concentrations of linoleic acid in plasma consistent with other studies involving dietary supplementation of cattle with a source of linoleic acid (Filley *et al.*, 2000; Burns *et al.*, 2003). The linoleic acid content of cattle follicular fluid and of oocytes is apparently important for oocyte developmental competence (Homa and Brown, 1992) and has been shown to be important for blastulation in *in vitro* fertilisation studies (Zeron *et al.*, 2001). A recent study by Scholljegerdes *et al.* (2007) has shown the oviduct to be more sensitive in terms of reflecting the effects of additional dietary linoleic acid compared to either the uterus or hypothalamus, further suggesting a potential role for this PUFA in fertilisation and early embryo development.

Despite low available dietary concentrations, the relatively higher plasma concentrations of the essential n-3 PUFA linolenic acid in the FO-fed animals, compared with either WSB or CON, suggest that FO may have interrupted the ruminal biohydrogenation of the linolenic acid coming from the other ingredients in the ration. Linolenic acid is the parent n-3 PUFA, with some limited elongation to the longer chain n-3 PUFA, EPA and DHA occurring. An *in vitro* study by Mattos *et al.* (2003) showed linolenic acid to be effective in suppressing $PGF_{2\alpha}$ from phorbol ester-stimulated bovine endometrial (BEND) cells. Furthermore, cows supplemented with linolenic acid were shown to have had higher concentrations of oestradiol during the follicular phase of the oestrous cycle than unsupplemented cows (Robinson *et al.*, 2002).

In the current study, FO supplementation increased plasma concentrations of EPA and DHA in agreement with previous studies involving dietary enrichment with either fish oil (Mattos *et al.*, 2004) or fish meal (Burns *et al.*, 2003; Wamsley *et al.*, 2005). However, despite a greater dietary concentration, the transfer of DHA from diet to plasma was less than for EPA in the current study. This may have been a consequence of greater ruminal biohydrogenation and/or a poorer efficiency of absorption. While the majority of studies have shown a greater rate of transfer from diet to milk for DHA compared with EPA (Cant *et al.*, 1997; Mattos *et al.*, 2004), a recent study from our laboratory has shown EPA to be transferred more efficiently to a number of key reproductive tissues including uterine endometrium and follicular fluid (Childs *et al.*, 2007).

There was little effect of diet on the plasma metabolites measured in the current study. While glucose is the main energy source of the ovary (Rabiee *et al.*, 1997) and of the post-blastulation bovine embryo (see review, Boland *et al.*, 2001), there was no effect of dietary lipid on systemic glucose. The literature on the effects of fat supplementation on systemic glucose is inconsistent and this may be a consequence of variable dietary effects on DMI and milk production, neither of which were factors in the present study. Triglycerides, the storage form of excess fat, were unaffected by diet, consistent with previous studies (Hightshoe *et al.*, 1991; Lammoglia *et al.*, 1996), but in contrast to others that have shown increases in triglyceride concentrations in animals on a high lipid diet (Wehrman *et al.*, 1991) or abomasally infused with fats (Oldick *et al.*, 1997). These differences can be attributed to varying energy and physiological states of the animals employed across the different studies. Dietary lipid source did not affect plasma concentrations of either NEFA or BHBA in the present study. There is some disagreement in the literature regarding the effect of fat supplementation on systemic concentrations of both NEFA and BHBA and these differences may again be attributed to variance in the metabolic status of the animals employed in the different studies.

Circulating cholesterol is the primary precursor for mammalian synthesis of luteal progesterone (Staples *et al.*, 1998). In the present study, fat supplementation increased plasma cholesterol consistent with previous reports (Grummer and Carroll, 1991; Hawkins *et al.*, 1995). However, despite the increase, we failed to establish any effect of either fat source on systemic concentrations of progesterone, in agreement with the findings of some studies (Lammoglia *et al.*, 1997; Mattos *et al.*, 2002; Wamsley *et al.*, 2005) but in contrast to others who reported increases (Hawkins *et al.*, 1995; Burke *et al.*, 1996) and decreases (Robinson *et al.*, 2002). The literature on the effect of fat supplementation on systemic oestradiol is similarly confused with studies reporting increases (Lammoglia *et al.*, 1997; Robinson *et al.*, 2002), decreases (Hightshoe *et al.*, 1991) or no change (Beam and Butler, 1997; Sartori *et al.*, 2004). As in these latter reports, the systemic concentrations of oestradiol were not affected by diet in the current study, despite the fact that variation due to stage of cycle was eliminated through the use of oestrous cycle synchronisation.

The size of the pre-ovulatory follicle has been positively related to improved pregnancy rate in cattle (Lopes *et al*., 2007). However, we found no effect of diet on follicle size or dynamics, in agreement with Petit *et al*. (2001). Other studies, however, have reported increases in the size of follicles in both dairy (Robinson *et al*., 2002) and beef cows (Lammoglia *et al*., 1996) supplemented with fat.

AA is an essential pre-cursor to the synthesis of $PGF_{2\alpha}$. In the current study, AA was highest in the plasma of animals supplemented with FO and this is consistent with other studies on plasma (Burns *et al*., 2003) and studies on milk (Mattos *et al*., 2002). AA is converted to PGH2, the pre-cursor of the other prostaglandins, through the actions of the enzyme PGH synthase (PGHS; Mattos *et al*., 2003). Despite FO supplementation increasing systemic AA, there is evidence from this laboratory (Childs *et al*., 2007) and others (Burns *et al*., 2003) that a high n-3 PUFA diet can reduce uterine endometrial tissue concentrations of AA.

In vitro work by Mattos *et al*. (2003) demonstrated that the addition of AA increased, while n-3 PUFA decreased the $PGF_{2\alpha}$ secretion of immortalised bovine endometrial cells. The ability to reduce the endometrial synthesis and secretion of the luteolysin, $PGF_{2\alpha}$, during early pregnancy would be beneficial in the context of the successful establishment of pregnancy (Mattos *et al*., 2000). Conversely, promotion of $PGF_{2\alpha}$ secretion during the early *post partum* period is conducive to more rapid uterine involution (Garcia-Bojalil *et al*., 1998). We hypothesised that FO (n-3 PUFA) supplementation would reduce and WSB (n-6 PUFA) would increase PGFM concentrations in response to an oxytocin challenge administered on days 15 and 16 of the oestrous cycle. We found a diet $\times$ day of oestrous cycle interaction on PGFM, with concentrations in the animals on the FO diet similar to CON on day 15 but increasing similar to WSB, on day 16. On day 15, PGFM concentrations were higher on WSB than CON or FO; however, FO failed to attenuate PGFM relative to the control diet. Furthermore, there was no direct relationship between plasma P_4, E_2 and PGFM, but a higher $E_2 : P_4$ ratio was related to an increased concentration of PGFM on both sampling days. An increase in PGFM has been reported previously for dietary (Fahey *et al*., 2002; Petit *et al*., 2004) and intravenously infused (Filley *et al*., 1999) sources of linoleic acid. However, results from trials involving fish meal and fish oil supplementation have been inconsistent in their effects on PGFM, with some reporting reductions (Mattos *et al*., 2002 and 2004), and others, reporting no effect (Wamsley *et al*., 2005; Heravi Moussavi *et al*., 2007). In the study of Heravi Moussavi *et al*. (2007), it is suggested that the use of oestradiol priming prior to administration of an oxytocin challenge may be responsible for the inconsistency in the results reported, as studies reporting attenuation of PGFM concentrations following either fish meal or fish oil supplementation have used oestradiol prior to oxytocin (Mattos *et al*., 2002 and 2004) while studies yielding no response involved administration of oxytocin but without oestradiol (Wamsley *et al*., 2005; Heravi Moussavi *et al*., 2007). The results of these latter studies and those of the current study, therefore, may represent a more physiologically normal response. While, as previously stated, there was no effect of diet on P_4 or E_2 in the current study, the inclusion of the $E_2 : P_4$ ratio in the statistical model was highly statistically significant in terms of explaining variation in PGFM concentrations. This may be related to the natural decline in P_4 and rise in E_2 prior to luteolysis. As E_2 upregulates the uterine oxytocin receptor and consequently $PGF_{2\alpha}$ synthesis (Goff, 2004), heifers with greater $E_2 : P_4$ ratios would be more likely to produce more $PGF_{2\alpha}$ in response to oxytocin.

In conclusion, this study showed little difference in the effect of dietary n-3 or n-6 PUFA supplementation on a range of reproductive variables. We found, however, that n-6 PUFA supplementation increased concentrations of circulating PGFM, the metabolite of $PGF_{2\alpha}$. As $PGF_{2\alpha}$ plays an important role in early *post partum* uterine function, this information may be of benefit to the formulation of post-calving diets for dairy cows.

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