Chapter 6

Assessing the Impact of a Diet Enriched in PUFA on Systemic and Endometrial Tissue Fatty Acid Concentrations in Cattle

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Abstract

The objective of the first trial was to determine the effects of a diet enriched in ω-6 fatty acids (whole soybean) or ω-3 fatty acids (fish oil) on the concentration of conjugated linoleic acid (CLA) found in the plasma of Holstein Friesian heifers (n = 24). Significant increases in the concentration of the *t*10, *c*12 CLA isomer were observed in the plasma of animals fed fish oil ($P < 0.05$), relative to animals on the whole soybean or control diets, however, the concentration of the *c*9, *t*11 CLA isomer remained unchanged. Plasma triglyceride concentrations increased, while β-hydroxybutyrate (BHBA) decreased with time on all diets ($P < 0.05$). There was a diet x time interaction ($P < 0.01$) for non-esterified fatty acid (NEFA) concentrations. Plasma cholesterol was higher on whole soybean and fish oil ($P < 0.01$) compared with control. The objective of the second trial was to determine if a ruminally protected fish oil enriched in EPA and DHA could be used to significantly increase the concentration of ω-3 fatty acids in the plasma and key sites associated with reproduction in Holstein Friesian heifers (n = 40). Animals receiving the MED or HIGH concentrations of the fish oil supplement had significantly higher concentrations of ω-3 PUFA in their plasma than animals on the control diet ($P < 0.05$). Similar trends were also evident in the endometrial tissue ($P < 0.05$) and follicular fluid ($P < 0.05$) of animals on the MED and HIGH diet relative to the control. There was a strong positive relationship between plasma and uterine endometrial concentrations of both EPA (R2 = 0.86; $P < 0.0001$) and total ω-3 PUFA (R2 = 0.77; $P < 0.0001$). There was a linear increase in cholesterol in response to increasing fish oil supplementation. Urea increased ($P < 0.0005$) while NEFA decreased ($P < 0.01$) linearly in response to increasing dietary fish oil intake.

6.1 Introduction

The inclusion of lipids in the diet of ruminants has been successfully employed to positively modulate the fatty acid composition of both the milk and meat of these animals but has also been associated with improving the health of these animals. Indeed, fatty acids such as α-linolenic acid, eicosapentanoic (EPA), docosahexaenoic acid (DHA) and the CLA isomers have been successfully used to improve both the health of the animal and the fatty acid composition of the milk and meat subsequently derived from these animals (Hennessy *et al.*, 2007; Ponnampalam *et al.*, 2006; Scislowski *et al.*, 2005). Furthermore, a range of other unsaturated fatty acids such as linoleic acid have also been employed to improve the endogenous production of fatty acids such as the *c*9, *t*11 CLA isomer, which has displayed potent health promoting properties both in animals and humans (Dhiman *et al.*, 2005; Hennessy *et al.*, 2007; Lawless *et al.*, 1998).

In recent years the high proportion of embryonic loss which occurs in cattle during the first three weeks of pregnancy has become a major issue for the dairy industry with a reported 1% decline in the first service conception rate per annum (Childs *et al.*, 2008c). Although several factors contribute to this problem, critical areas include the metabolic effects of lactation on reproduction, mechanisms linking disease to reproduction, and early embryonic mortality (Lucy, 2001). Recent studies have suggested that supplementation of the diet of Holstein Friesian cows with health promoting fatty acids such as α-linolenic acid, EPA, DHA or the CLA isomers may be capable of offsetting these losses (Ambrose *et al.*, 2006; Castaneda-Gutierrez *et al.*, 2005; Hawkins *et al.*, 1995; Mattos *et al.*, 2000; Rodriguez-Sallaberry *et al.*, 2006). Potential explanations for these reductions in embryonic loss include reductions in the production of the pro-inflammatory prostanoid, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which plays an important role in luteolysis

(destruction of the corpus luteum), and increases in plasma progesterone which suppresses the maternal immune response to fetal antigens (Achard *et al.*, 1997; Mattos *et al.*, 2003; McCracken *et al.*, 1999).

The first objective of the present study was to evaluate the effect of the provision of soy meal rich in linoleic acid or fish oil rich in the ω-3 polyunsaturated fatty acids (PUFA) EPA and DHA, on serum CLA concentrations. We hypothesised that through the supplementation of the bovine diet with these oils, plasma CLA concentrations could be increased to concentrations where an improvement in bovine fertility has previously been reported (Castaneda-Gutierrez *et al.*, 2007a; Rodriguez-Sallaberry *et al.*, 2006). A nulliparous heifer model was chosen for this study in order to avoid the possible confounding effects of milk production and associated negative energy balance. In the second study we investigated the effects of feeding a partially rumen protected ω-3 PUFA (EPA/DHA rich) supplement on the fatty acid compositions of rumen fluid and plasma along with the fatty acid composition of the endometrial tissue and follicular fluid which is thought to play a crucial role in bovine fertility. We hypothesised that increasing dietary ω-3 PUFA intake would increase systemic and tissue concentrations of the ω-3 PUFA, EPA and DHA.

6.2 Materials and methods

6.2.1 Animals and diets (Trial 1)

Reproductively normal nulliparous crossbred beef heifers (*n* = 24) with a mean ± standard error of mean (S.E.M.) age of 20 ± 2 months and liveweight of 442 ± 10 kg were selected for Trial 1. These animals were oestrus synchronised using two injections (PG1 & PG2) of a 500 mg of the $PGF_{2\alpha}$ analogue (Cloprostanol, Estrumate®; Schering-Plough Ltd, Shire Park, Welwyn Garden City, Hertfordshire, UK), administered intramuscularly 11 days apart. Observations for oestrous activity were carried out using an electronic heat mount detection system (Heatwatch®; DDx Inc., Denver, CO, USA) combined with visual observations at 07:00, 11:00, 15:00, 19:00 and 23:00 h, beginning 24 h after the second prostaglandin administration. This heat check was repeated towards the end of the subsequent oestrous cycle. 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 (control); (ii) 2% added fat as supplemental whole soybeans (WSB) (linoleic acid rich) or (iii) 2% added fat as supplemental fish oil (Fish oil). The fish oil 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 6.1.** The fatty acid composition of the WSB and fish oil are presented in **Table 6.2.** The WSB and fish oil diets were formulated to provide approximately 150 g/head per day of soy oil and fish oil, respectively. All diets were formulated to be isonitrogenous (14% crude protein) in total dietary dry matter (DM) and were

Table 6.1 Ingredient composition (g/kg as fed) and chemical analysis (expressed as g/kg of DM unless otherwise stated) of concentrates and forage, Trial 1.

	Control	Linoleic acid	Fish oil	Bal	Straw
Barley	458	182	242	682	–
Soybean meal (48%)	507	–	550	231	–
Whole Soybean	–	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
Crude protein	334	327	335	209	46.19
Crude fibre	54.76	61.97	43.6	54.29	535.7
Acid detergent fibre	76.34	84.67	63.18	71.22	581.1
Acid detergent lignin	9.45	7.93	6.69	10.94	87.11
Neutral detergent fibre	145.82	144.89	112.31	151.64	881.29
Ash	44.5	50.27	42.42	31.17	35.03
Ether Extract	16.86	166.85	166.71	16.2	5.99
Gross Energy (MJ/kg DM)	16.05	18.7	19.6	15.39	17.54

*Vitamin mineral premix contained: Vitamin A = 320,000 IU/kg; Vitamin D_3 = 80,000 IU/kg; Vitamin E = 100 mg/kg; Cobalt carbonate = 200 mg/kg; Zinc oxide = 4,000 mg/kg; Potassium iodate = 320 mg/kg; Calcium = 120 mg/kg; Phosphorus = 50 g/kg; and Magnesium = 100 mg/kg.

offered for 32 days. The animals were offered their respective daily lipid supplement in a 1 kg DM bolus feed at 09:00 h each day. At 12:00 h each day, animals were offered 4.5 kg DM of a balancer ration **(Table 6.1)** and 1.5 kg DM straw. All animals were individually fed using an electronic feeding system (Calan Inc., Northwood, NH, USA).

6.2.2 Liveweight and body condition scoring (Trial 1)

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 experiment liveweight was taken as the mean of weights measured on each of two successive days. Body condition score (BCS) was estimated on a linear scale of 1–5 (1 = emaciated, 5 = extremely fat) (Lowman *et al.*, 1976). The weighing scales were calibrated at regular intervals using known weights.

6.2.3 Animals and diets (Trial 2)

Reproductively normal nulliparous crossbred heifers (n = 40) with a mean ± S.E.M. liveweight of 479.4 ± 8.4 kg and BCS of 3.3 ± 0.1 were selected for Trial 2. These animals were oestrus synchronised using two injections (PG1 & PG2) of a 500 mg of the $PGF_{2\alpha}$ analogue (Cloprostanol, Estrumate®; Schering-Plough Ltd, Shire Park, Welwyn Garden City, Hertfordshire, UK), administered intramuscularly 11 days apart. Observation for oestrous activity was carried out as previously described. Animals were blocked by liveweight and BCS and randomly assigned, within block, to one of four concentrate and straw-based diets (n = 10 per diet). All animals were individually fed a barley straw (1.40 kg DM), molasses (0.28 kg DM) and concentrate (5.50 kg DM)-based ration, containing one of four levels of a partially rumen protected EPA and DHA enriched fish oil (ω-3 PUFA) supplement

Table 6.2 Fatty acid composition of whole soybean and fish oil (g/100g FAME), Trial 1.

	Whole soybean	Fish oil
Myristic (C14:0)	0.30	7.36
Palmitic (C16:0)	12.18	16.36
Stearic (C18:0)	3.87	2.49
Vaccenic (*t* 11-C18:1)	0.39	0.12
Oleic (C18:1)	22.60	12.87
Linoleic (C18:2) (ω-6)	51.87	1.64
α-linolenic (C18:3) (ω-3)	5.51	–
Eicosatrienoic (C20:3) (ω-6)	–	0.03
Arachidonic (C20:4) (ω-6)	–	1.80
EPA (C20:5) (ω-3)	0.09	6.86
DPA (C22:5) (ω-3)	0.14	–
DHA (C22:) (ω-3)	–	10.41
Others and Unknowns[1]	3.05	40.06

[1] Denotes fatty acids identified but not reported and quantified but not identified peaks.

(Trouw Nutrition, 36 Ship Street, Belfast BT15 1JL; EPA:DHA, 1.5:1) to provide (1) 0 g (Control); (2) 65 g (LOW; 39 g EPA, 26 g DHA); (3) 140 g (MED; 84 g EPA, 56 g DHA) or (4) 275 g (HIGH; 165 g EPA, 110 g DHA) of EPA and DHA combined on a DM basis. Concentrations of fish oil in the total DM were 0, 1.04, 2.08 and 4.15% for the control, LOW, MED and HIGH diets, respectively. Ruminal protection was achieved via encapsulation in a pH sensitive matrix, which remains intact at ruminal pH but breaks down at the lower pH in the abomasum releasing the constituents for absorption. The fish oil was derived from anchovy, sardine and salmon oil, which were distilled in order to concentrate the EPA and DHA content. The ingredient composition and chemical analysis of the lipid supplemented experimental concentrates, balancer rations, and straw are presented in **Table 6.3**. The fatty acid composition of the experimental concentrates and balancer rations offered is presented in **Table 6.4**. All diets were formulated to be isonitrogenous (14% crude protein in total diet) and isolipogenous and were fed for a total of 45 days. Each day at 09:00 h, the animals received their entire daily allocation of supplementary lipid in the form of 1.40 kg DM bolus feed mixed with 0.28 kg DM heated molasses to enhance palatability and a further 0.90 kg of a 26% crude protein ration (Balancer 1; **Table 6.3**) to balance the low crude protein concentration of the bolus diet. At 12:00 h, animals were offered 3.20 kg DM of a second balancer ration (Balancer 2; 14.6% crude protein; **Table 6.3**) and 1.40 kg DM straw **(Table 6.3)**.

6.2.4 Liveweight and body condition scoring (Trial 2)

Animals were weighed and BCS was scored on days 0, 1, 16, 27, 37 and on the day prior to (day 45) and on the day of slaughter (day 46). In the interest of accuracy in the determination of average daily gain (ADG), animals were weighed on each of

Table 6.3. Ingredient composition and chemical analysis (expressed as g/kg of DM unless otherwise stated) of the ω-3 PUFA supplemented rations, balancer rations and straw, Trial 2.

	Control	LOW	MED	HIGH	Bal 1	Bal 2	Straw
Barley	340	278	220	110	225	420	–
Soybean meal (48%)	5	29	55	105	430	110	–
Molassed sugar beet pulp	345	296	236	118	230	421	–
Palmit 80 prills*	310	230	155	–	–	–	–
EPA/DHA supplement	–	167	334	667	–	–	–
Molasses (Cane)	–	–	–	–	45	20	–
Vitamin mineral premix[a,b]	–	–	–	–	50	20	–
Ground limestone	–	–	–	–	15	7	–
Salt	–	–	–	–	1	1	–
Di-calcium phosphate	–	–	–	–	4	1	–
DM (g/kg)	908	918	925	928	890	918	917
Crude protein	67.5	69.28	65.75	67.91	256.19	142.03	43.37
Crude fibre	62.84	53.10	55.20	44.22	48.45	67.31	404.80
Ash	36.80	105.70	171.90	325.70	123.40	78.70	38.60
Ether extract	27.07	33.34	30.46	31.91	1.40	1.21	0.79
Gross energy (MJ/kg DM)	20.76	20.79	20.36	18.24	14.77	15.57	16.02

*Minimum content of palmitic acid (C16:0) = 80%; Myristic acid (C14:0) < 2%; Stearic acid (C18:0) = 4-7%; Oleic acid (C18:1) =8-12%; Linoleic acid (C18:2) < 3%, product of Trouw Nutrition, 36 Ship Street, Belfast, BT15 1JL, Northern Ireland.

[a]Premix supplied per kilogram of supplement: 22400 IU of Vitamin A; 5600 IU of vitamin D3; and 700 mg of vitamin E.

[b]Premix supplied per kilogram of supplement: 2.1 mg selenium as sodium selenite; 22.4 mg iodine as calcium iodate; 87.5 mg copper as cupric sulphate; 280 mg zinc as zinc oxide; 175 mg manganese as manganese oxide and 7 mg cobalt as cobalt carbonate.

Table 6.4 Fatty acid concentration of concentrates fed (g/100g FAME; mean ± S.E.M.), Trial 2.

	Control	LOW	MED	HIGH	Bal 1	Bal 2
Myristic (C14:0)	2.66	2.29	1.63	0.26	0.39	0.31
Palmitic (C16:0)	82.20	70.69	47.94	5.39	18.70	22.16
Stearic (C18:0)	5.40	5.34	4.70	3.85	3.69	2.34
Oleic (C18:1)	5.13	5.55	6.51	7.85	14.22	13.13
ω-6 PUFA						
Linoleic (C18:2)	1.51	1.13	1.61	1.78	50.00	45.59
Eicosatrienoic (C20:3)	–	0.04	0.14	0.31	–	0.02
Arachidonic (C20:4)	0.01	0.27	0.80	2.16	–	0.11
ω-3 PUFA						
α-linolenic (C18:3)	0.19	0.28	0.54	0.99	6.95	5.44
EPA (C20:5)	0.01	4.25	13.01	29.26	0.11	1.94
DPA (C22:5)	–	0.37	0.70	1.68	–	0.32
DHA (C22:)	<0.01	2.97	9.10	19.03	0.01	1.39
ω-3 family	0.21	7.50	22.65	49.28	7.08	8.77
ω-6 family	1.52	1.40	2.41	3.93	50.00	45.71
Ratio ω-6 to ω-3	7.27	0.19	0.11	0.08	7.06	5.21

N.D: Not detected
C18:3 + C20:5 + C22:6
C18:2 + C20:4

two consecutive days at both the start (days 0 and 1) and the end (days 45 and 46) of the experimental period and the mean of these weights was taken as the start and end weights, respectively. BCS was estimated as previously described.

6.2.5 Feed sampling and analysis

Weekly composite samples of straw and concentrates were stored at -22^{o}C until analysed for DM, crude protein, crude fibre, acid detergent fibre, acid detergent lignin, neutral detergent fibre, ether extract, ash and gross energy. 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^{o}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^{o}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). Crude protein (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).

6.2.6 Blood sampling

During Trial 1, blood samples for fatty acid and metabolite analysis were collected on day 10 and day 27 of the 35 day experimental period, whilst for Trial 2 blood samples for fatty acid and metabolite analysis were collected on day 20 and 44 of the 45 day experimental period. Blood was collected by jugular venipuncture under

license in accordance with the European Community Directive, 86-609-EC. Samples (10 ml) were collected into lithium heparinised vacutainers (Becton Dickinson, Vacutainer Systems). On collection, samples were immediately stored in ice and centrifuged at 1500 x g at 4^{o}C for 15 min. Plasma was harvested and stored at -20^{o}C until analysed.

6.2.7 Tissue sampling

Following Trial 2, Heifers were slaughtered in an EU licensed abattoir (Martin Jennings Wholesale Ltd., Neale Road, Ballinrobe, Co., Mayo, Ireland) on either day 45 or 46 or the experimental period which corresponded to either day 17 or 18 of the synchronised oestrous cycle. Endometrial tissue from the anti-mesometrial border of the uterine horn ipsilateral to the corpus luteum was dissected from the myometrium as described previously by Bilby *et al.* (2006). Follicular fluid was collected from the dominant follicle using a half inch 20 gauge needle (Becton Dickinson, Plymouth, UK) and a 2 ml syringe, while rumen fluid samples were collected by filtering the fluid from a sample of the rumen contents. All samples were collected into sterile containers within 40 min of slaughter, flash frozen in liquid nitrogen and stored at -80^{o}C pending analysis for fatty acid concentrations. Rumen samples were collected approximately 20 h after the animals had received their final feed allocation.

6.2.8 Fatty acid analysis of feeds, plasma, rumen and follicular fluid, and endometrial tissue

Total lipids were extracted from 1 ml of plasma, rumen fluid and follicular fluid, and from 6 g of the feed sample, using chloroform methanol (2:1, v/v) as described previously (Folch *et al.*, 1957). Endometrial tissue lipids were extracted from 1 g of

tissue, by homogenising the tissue in chloroform methanol (2:1) using the Tissue Tearor™ (Biospec Products Inc., Bartlesville, OK, USA; model no. 985370). Following overnight storage at 4^{o}C, extraction was carried out as described by Folch *et al.* (1957). Methylation was carried out for all samples by *in situ* transesterification with 0.5 N methanolic NaOH followed by 14% boron trifluoride in methanol as described by Park & Goins (1994). The fatty acid methyl esters (FAME) were separated using a CP Sil 88 column (100 m x 0.25 mm i.d., 0.20 mm film thickness; Chrompack, Middleburg, The Netherlands) and quantified using a gas liquid chromatograph (GLC) (3400; Varian, Harbor City, CA, USA). The internal standards used were heptadecanoic acid (C17:0; Sigma Aldrich Ireland Ltd.) for plasma, endometrium, rumen and follicular fluid samples and tridecanoic acid (C13:0; Sigma Aldrich) for feed samples. The GLC was calibrated using a range of commercial fatty acid standards (Sigma Aldrich). The GLC was fitted with a flame ionization detector (FID) and helium (37 psi) was used as the carrier gas. The injector temperature was held isothermally at 225^{o}C for 10 min and the detector temperature was 250^{o}C. The column oven was held at an initial temperature of 140^{o}C for 8 min and then programmed to increase at a rate of 8.5^{o}C/min to a final temperature of 200^{o}C, which was held for 41 min. Data were recorded and analysed on a Minichrom PC system (VG Data System, Manchester, UK).

6.2.9 Metabolites

Plasma glucose, triglycerides, non-esterfied fatty acids (NEFA), β-hydroxybuterate (BHBA), urea and cholesterol concentrations were analysed using commercial biochemical assay kits (Boehringer Mannheim, Mannheim, Germany, and Randox

Private Ltd., Crumlin, UK) on an automated biochemical analyzer (ABX Mira, Cedex 4, France).

6.2.10 Statistical analyses

All data were checked for adherence to a normal distribution (PROC UNIVARIATE, SAS v9.1, 2002). Data were analysed using two-way ANOVA with terms included for treatment and block. Variables having more than one observation per subject such as dry matter intake (DMI), ADG and plasma analytes were analysed using repeated measures ANOVA (PROC MIXED (*Trial 1*), PROC UNIVARIATE (*Trial 2*), SAS v9.1, 2002) with terms for treatment, time period and their interaction included in the statistical model. 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 chosen. The PDIFF (predicted difference) and CONTRAST (for orthogonal contrasts) statements of SAS (v9.1, 2002) were used to test for linear, quadratic and cubic effects of incremental fish oil inclusion on continuous variable of interest. The Tukey test was applied to evaluate pairwise comparisons of treatment means. Linear and stepwise multiple regression procedures (PROC REG and PROC STEPWISE, SAS) were also used as appropriate. P values < 0.05 were accepted as being statistically significant while those < 0.10 were considered to indicate tendency towards statistical significance.

6.3 Results

6.3.1 Dry matter intake and animal performance (Trial 1)

There was no effect ($P > 0.05$) of diet on either daily DMI (7.19 ± 0.05 vs. 7.17 ± 0.07 vs. 7.07 ± 0.11 kg/day for the control, WSB and fish oil diets, respectively) or ADG (0.80 ± 0.17 vs. 0.67 ± 0.07 v. 0.90 ± 0.11 kg/day for the control, linoleic acid and ω-3 PUFA diets, respectively).

6.3.2 Plasma concentration of fatty acids (Trial 1)

The effect of diet and day of sampling on the fatty acid profile of plasma is shown in **Table 6.5**. Amongst the medium chain fatty acids, vaccenic acid, the immediate precursor to the *c*9, *t*11 CLA isomer, was observed to be significantly higher in animals fed the fish oil diet, while palmitic and stearic acid remained similar to the control. Several fatty acids showed a statistically significant treatment x day of sampling interaction: myristic acid increased with time on the control and WSB diets but not in animals fed the fish oil diet, whereas palmitic, stearic and oleic all decreased with time on the WSB diet but not on the control or fish oil diets. Comparatively, heifers fed the linoleic acid rich WSB diet had higher concentrations of the ω-6 PUFA, linoleic acid, relative to animals fed the control or fish oil diets. Concentrations of the ω-3 fatty acid, α-linolenic acid, decreased with time on treatment in both the control and WSB diets but increased in animals fed the fish oil diet. Heifers fed the fish oil diet had significantly higher concentrations of α-linolenic acid ($P < 0.0001$), EPA and DHA ($P < 0.001$) relative to animals fed the control diet, whereas eicosatrienoic acid was reduced in the fish oil diet. Arachidonic acid concentrations were significantly higher ($P < 0.0001$) in heifers on the fish oil diet than in animals on either the control or WSB diets. There were

Table 6.5. Effect of diet on fatty acid concentrations (g/100g FAME) of plasma collected on day 10 and 27 of the 32 day experimental period (mean ± S.E.M.), Trial 1.

Diet	CON		WSB		FO				
	Day		Day		Day		Statistical significance		
Fatty acid	10	27	10	27	10	27	TRT	DAY	TRTx DAY
Myristic (C14:0)	4.85 ± 0.87^{a,x}	9.26 ± 0.81^{a,y}	3.62 ± 0.81^{a,x}	7.56 ± 0.81^{a,y}	6.32 ± 0.81^{a,x}	6.55 ± 0.81^{a,x}	NS	***	*
Palmitic (C16:0)	11.77 ± 0.54^{a,x}	10.51 ± 0.51^{a,x}	12.46 ± 0.51^{a,x}	9.29 ± 0.51^{a,y}	12.55 ± 0.51^{a,x}	11.89 ± 0.51^{a,x}	*	***	*
Stearic (C18:0)	17.77 ± 0.88^{a,x}	16.01 ± 0.24^{a,x}	20.98 ± 0.82^{a,x}	16.79 ± 0.82^{a,y}	13.04 ± 0.82^{b,x}	13.15 ± 0.82^{a,x}	***	*	*
Vaccenic (*t* 11-C18:1)	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^{a,x}	5.82 ± 0.48^{a,x}	6.87 ± 0.48^{a,x}	4.60 ± 0.48^{b,y}	5.83 ± 0.48^{a,x}	5.88 ± 0.48^{a,x}	NS	*	0.1
Linoleic (C18:2)	16.22 ± 1.28^{a}	16.11 ± 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
c 9, *t* 11CLA (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
t 10, *c* 12CLA (C18:2)	0.09 ± 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
α-linolenic (C18:3)	1.40 ± 0.12^{a,x}	1.16 ± 0.11^{a,x}	1.87 ± 0.11^{b,x}	1.32 ± 0.11^{a,y}	1.65 ± 0.11^{a,b,}	2.05 ± 0.11^{b,x}	***	NS	***
Eicosatrienoic (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 ± 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 ± 0.27^{a,x}	0.59 ± 0.25^{a,x}	0.85 ± 0.25^{a,x}	0.52 ± 0.25^{a,x}	2.83 ± 0.25^{b,x}	3.92 ± 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}	0.82 ± 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	0.1	NS	0.1
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	0.1

^{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. $P < 0.001$ *, $P < 0.01$ **, $P < 0.05$ ***
[1]Denotes fatty acids quantified but not reported.
[2]Denotes fatty acids quantified but not identified.

diet x day of sampling interactions for α-linolenic acid and EPA, where both fatty acids increased with time on fish oil diet but did not change ($P > 0.05$) on either the linoleic acid or control diets. Supplementation of the bovine diet with WSB or fish oil did not result in a significant increase in the concentration of *c*9, *t*11 CLA isomer relative to animals on the control diet. The concentration of the *t*10, *c*12 CLA isomer present in the plasma of heifers fed the fish oil diet was found to be significantly greater than that of animals on the control or WSB diet ($P < 0.001$). A significant interaction between treatment and day of sampling was not observed for the *t*10, *c*12 CLA isomer.

6.3.3 Plasma metabolites (Trial 1)

The effect of diet on plasma concentrations of metabolites is presented in **Table 6.6**. 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 linoleic acid rich WSB diet ($P < 0.05$), but there was no change in NEFA with time in either the control or fish oil diets ($P > 0.05$). Heifers on WSB diet had the highest concentration of urea, while plasma concentrations of glucose were not altered by diet ($P > 0.05$).

6.3.4 Dry matter intake and animal performance (Trial 2)

Mean daily DMI was lower ($P < 0.001$) for animals on HIGH compared with any of the other three treatment groups, which did not differ (7.16, 7.16, 7.13 and 6.88 kg/day; S.E.M. = 0.034 for the control, LOW, MED and HIGH, respectively) and this provided approximately 0, 65, 139 and 264 g of the ω-3 PUFA, EPA and DHA combined. Similarly, ADG was lower on HIGH ($p < 0.001$) compared with any

Table 6.6 The effect of diet on plasma metabolite concentration (mmol/l; mean ± S.E.M.), Trial 1.

Diet	Control		WSB		Fish oil				
	Day		Day		Day		Statistical significance		
Metabolite	10	27	10	27	10	27	TRT	DAY	TRTx 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]	***	*	<0.10
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. $P < 0.001$ *, $P < 0.01$ **, $P < 0.05$ ***

of the other treatments, which again did not differ (0.76, 0.50, 0.58 and 0.00 kg/day; S.E.M. = 0.075, for the control, LOW, MED and HIGH, respectively). The mean change in BCS was 0.00 ± 0.12 units and was not affected by diet ($P > 0.05$).

6.3.5 Rumen fluid fatty acids (Trial 2)

The fatty acid profile of rumen fluid collected at slaughter is presented in **Table 6.7**. There was no effect of diet on the concentrations of *c*9, *t*11 CLA ($P > 0.05$). There were quadratic responses to fish oil supplementation for both linoleic ($P < 0.001$) and to a lesser degree, α-linolenic acid ($P = 0.01$). With the exception of the linear decline in stearic acid ($P < 0.001$) and the quadratic response in palmitic acid ($P < 0.001$), the concentrations of the other fatty acids generally increased with increasing intake of fish oil. There were linear increases in the concentrations of vaccenic acid, *t*10, *c*12 CLA isomer, DPA and DHA ($P < 0.0001$) and also myristic, oleic, eicosatrienoic and arachidonic acid, and EPA ($P < 0.001$).

6.3.6 Plasma concentration of fatty acids (Trial 2)

The effect of diet on the fatty acid profile of plasma is shown in **Table 6.8**. As no diet x day of sampling interaction was detected, the mean value of the samples collected on days 20 and 44 of the 45-day trial period is shown. The linear increase ($P < 0.001$) in total ω-3 fatty acids and decrease ($P < 0.0001$) in total ω-6 PUFA were in keeping with the formulation objectives of the experiment. Furthermore, the ω-3 PUFA, EPA, DPA and DHA concentration increased ($P < 0.0001$) with increasing dietary fish oil intake, resulting in a linear reduction in the ω-6:ω-3 ratio ($P < 0.0001$). There was no effect of diet ($P > 0.05$) on the concentrations of myristic acid, *c*9, *t*11 CLA isomer or α-linolenic acid (C18:3). Palmitic, vaccenic and linoleic acid concentrations decreased ($P < 0.0001$) as the concentration of ω-3

Table 6.7. Effect of increasing intake of dietary ω-3 PUFA supplementation on the fatty acid concentration (g/100g FAME) of rumen fluid collected at slaughter (mean ± S.E.M.), Trial 2.

Fatty acid	Diet				*P* -value		
	Control	LOW	MED	HIGH	Pooled SEM	Linear	Quadratic
Myristic (C14:0)	4.01^{a}	4.73^{a}	6.73^{ab}	9.08^{b}	1.021	<0.001	NS
Palmitic (C16:0)	47.71^{a}	42.22^{ab}	36.55^{ab}	17.00^{c}	1.587	<0.0001	<0.0001
Stearic (C18:0)	20.19^{a}	16.40^{b}	11.25^{c}	9.07^{c}	0.871	<0.0001	NS
Oleic (C18:1)	2.75^{a}	2.80^{a}	2.73^{a}	4.24^{b}	0.262	<0.001	<0.01
Vaccenic (C18:1-*t* 11)	2.23^{a}	4.27^{a}	8.27^{b}	10.55^{b}	0.892	<0.0001	NS
c 9*t* 11 CLA (C18:2)	0.17	0.24	0.10	0.34	0.089	NS	NS
t 10*c* 12 CLA (C18:2)	0.10^{a}	0.37^{a}	0.71^{b}	1.17^{c}	0.079	<0.0001	NS
ω-6 PUFA							
Linoleic (C18:2)	1.30^{a}	1.10^{ab}	0.63^{b}	1.60^{a}	0.158	NS	<0.001
Eicosatrienoic (C20:3)	0.00^{a}	0.02^{a}	0.17^{ab}	0.27^{b}	0.055	<0.001	NS
Arachidonic (C20:4)	0.15^{a}	0.27^{a}	0.59^{ab}	1.39^{b}	0.239	<0.001	NS
ω-3 PUFA							
α-linolenic (C18:3)	0.57^{a}	1.37^{b}	0.75^{a}	0.94^{a}	0.104	NS	<0.01
EPA (C20:5)	0.01^{a}	0.07^{a}	0.23^{ab}	0.51^{b}	0.091	<0.001	NS
DPA (C22:5)	0.44^{ab}	0.32^{a}	0.62^{b}	0.88^{c}	0.066	<0.0001	<0.01
DHA (C22:6)	0.00^{a}	0.20^{a}	0.77^{a}	2.10^{b}	0.241	<0.0001	<0.05
ω-3 family[1]	0.58^{a}	1.63^{a}	1.75^{a}	3.55^{b}	0.332	<0.0001	NS
ω-6 family[2]	1.45^{a}	1.37^{a}	1.21^{a}	2.99^{b}	0.349	<0.01	<0.05
Ratio ω-6 to ω-3	0.00^{a}	0.20^{a}	0.77^{a}	2.10^{b}	0.241	<0.0001	<0..05

$^{a, b, c}$ Within row, concentrations with different superscripts are different (P <0.05)

[1]C18:3 + C20:5 + C22:6

[2]C18:2 + C20:4

PUFA supplementation increased. There was a linear decline in concentrations of stearic acid ($P < 0.001$), while concentrations of oleic, *t*10, *c*12 CLA isomer and arachidonic acid increased ($P < 0.0001$) with increasing dietary fish oil supplementation.

6.3.7 Uterine endometrial fatty acids (Trial 2)

The effect of diet on the endometrial tissue concentration of fatty acids is shown in **Table 6.9**. The increases in the concentration of EPA, DPA and DHA led to a linear increase in the concentration of total ω-3 PUFA present in the tissue ($P < 0.0001$). Equally, the decline in the concentrations of linoleic acid and arachidonic acid led to a linear decline in total ω-6 PUFA ($P < 0.0001$) with a consequent decline in the ratio of ω-6:ω-3 PUFA ($P < 0.0001$). There was no effect of diet on the endometrial concentrations of palmitic or stearic acid ($P > 0.05$). The concentrations of myristic acid, vaccenic acid and *c*9, *t*11 CLA increased with increasing fish oil supplementation ($P < 0.0001$). Concentrations of oleic acid ($P < 0.05$) declined with increasing ω-3 PUFA supplementation while the response of eicosatrienoic acid was quadratic ($P < 0.01$).

6.3.8 Follicular fluid fatty acids (Trial 2)

The effect of diet on the fatty acid profile of follicular fluid is shown in **Table 6.10**. Increased EPA ($P < 0.0001$) and decreased linoleic acid ($P < 0.0001$) concentrations with increasing dietary fish oil led to an overall decrease in the ω-6:ω-3 PUFA ratio of follicular fluid. There was no clear effect of treatment on follicular fluid concentrations of myristic acid, palmitic acid, DPA or DHA. Similarly there was no effect of diet on the concentrations of stearic, eicosatrienoic or arachidonic acid or *t*10, *c*12 CLA ($P > 0.05$). There was a linear increase in the concentrations of

Table 6.8 Effect of level of dietary ω-3 PUFA supplementation on fatty acid concentration (g/100g FAME) of plasma[†] (mean ± S.E.M.), Trial 2.

Fatty acid	Diet				*p*-value		
	CON	LOW	MED	HIGH	Pooled SEM	Linear	Quadratic
Myristic (C14:0)	1.10[a]	1.06[a]	0.99[a]	0.87[a]	0.104	NS	NS
Palmitic (C16:0)	16.79[a]	17.97[b]	18.82[b]	13.47[c]	0.322	<0.0001	<0.0001
Stearic (C18:0)	12.80[a]	11.37[ab]	10.28[b]	10.11[b]	0.501	<0.001	NS
Oleic (C18:1)	0.42[a]	0.43[a]	0.72[ab]	0.86[b]	0.081	<0.0001	NS
Vaccenic (C18:1-*t*11)	6.78[a]	5.27[b]	3.92[c]	3.70[c]	0.226	<0.0001	<0.01
*c*9*t*11 CLA	0.10[a]	0.08[a]	0.08[a]	0.08[a]	0.011	NS	NS
*t*10*c*12 CLA (C18:2)	0.07[a]	0.10[a]	0.18[b]	0.28[c]	0.015	<0.0001	<0.05
ω-6 PUFA							
Linoleic (C18:2)	25.19[a]	20.16[b]	15.11[c]	9.27[d]	0.707	<0.0001	NS
Eicosatrienoic (C20:3)	0.92[a]	0.27[b]	0.47[bc]	0.61[c]	0.072	<0.05	<0.0001
Arachidonic (C20:4)	2.13[a]	1.94[a]	2.67[b]	3.34[c]	0.111	<0.0001	<0.001
ω-3 PUFA							
α-linolenic (C18:3)	1.90[a]	1.89[a]	1.84[a]	1.69[a]	0.094	NS	NS
EPA (C20:5)	3.90[a]	9.05[b]	15.88[c]	23.30[d]	0.76	<0.0001	NS
DPA (C22:5)	0.93[a]	1.19[ab]	1.40[b]	1.35[b]	0.069	<0.0001	<0.05
DHA (C22:6)	1.09[a]	1.79[b]	1.96[b]	2.03[b]	0.124	<0.0001	<0.05
ω-3 family[1]	6.91[a]	12.82[b]	19.80[c]	27.05[d]	0.757	<0.0001	NS
ω-6 family[2]	27.34[a]	22.18[b]	17.81[c]	12.62[d]	0.812	<0.0001	NS
Ratio ω-6 to ω-3	4.69[a]	1.60[b]	0.89[c]	0.44[c]	0.324	<0.0001	<0.001

[a, b, c] Within row, concentrations with different superscripts are different ($P < 0.05$)

[†]As no treatment x day of sampling interaction was detected data represent the mean value of samples collected on day 20 and 44 of the 45 day trial period.

[1]C18:3 + C20:5 + C22:6

[2]C18:2 + C20:4

Table 6.9 Effect of level of dietary ω-3 PUFA supplementation on the fatty acid concentration (g/100g FAME) of uterine endometrial tissue collected at slaughter (mean ± S.E.M.), Trial 2.

Fatty acid	Diet				*P*-value		
	Control	LOW	MED	HIGH	Pooled SEM	Linear	Quadratic
Myristic (C14:0)	0.99^{ab}	0.84^{a}	1.23^{bc}	1.36^{c}	0.075	<0.0001	NS
Palmitic (C16:0)	14.50	15.20	14.12	14.35	0.336	NS	NS
Stearic (C18:0)	16.02	16.02	15.45	16.01	0.301	NS	NS
Oleic (C18:1)	15.42^{a}	15.04^{a}	12.51^{b}	13.93^{ab}	0.514	<0.01	NS
Vaccenic (C18:1-*t* 11)	0.88^{a}	1.18^{ab}	1.52^{b}	2.40^{c}	0.085	<0.0001	<0.01
c 9*t* 11 CLA	0.16^{a}	0.20^{a}	0.20^{a}	0.32^{b}	0.022	<0.0001	NS
ω-6 PUFA							
Linoleic (C18:2)	7.42^{a}	7.25^{a}	5.74^{b}	4.51^{c}	0.273	<0.0001	0.06
Eicosatrienoic (C20:3)	1.66^{a}	1.11^{b}	1.12^{b}	1.36^{ab}	0.110	0.08	<0.01
Arachidonic (C20:4)	7.39^{a}	5.37^{b}	4.58^{b}	5.09^{b}	0.303	<0.0001	<0.001
ω-3 PUFA							
α-linolenic (C18:3)	0.60^{a}	0.54^{a}	0.33^{b}	0.39^{b}	0.032	<0.0001	NS
EPA (C20:5)	0.92^{a}	2.42^{b}	3.39^{c}	4.47^{d}	0.239	<0.0001	NS
DPA (C22:5)	2.23^{a}	3.17^{b}	3.43^{bc}	3.77^{c}	0.128	<0.0001	<0.05
DHA (C22:6)	2.98^{a}	3.71^{b}	3.49^{ab}	3.75^{b}	0.183	<0.05	NS
ω-3 family[1]	4.50^{a}	6.68^{b}	7.21^{b}	8.61^{c}	0.309	<0.001	NS
ω-6 family[2]	14.81^{a}	12.61^{b}	10.32^{c}	9.60^{c}	0.379	<0.0001	NS
Ratio ω-6 to ω-3	3.52^{a}	1.90^{b}	1.44^{bc}	1.13^{c}	0.175	<0.0001	<0.001

$^{a, b, c}$ Within row, concentrations with different superscripts are different ($P < 0.05$)

[1]C18:3 + C20:5 + C22:6

[2]C18:2 + C20:4

Table 6.10 Effect of level of dietary ω-3 PUFA on the fatty acid concentration (g/100g FAME) of follicular fluid collected at slaughter (mean ± S.E.M.), Trial 2.

Fatty acid	Diet				*P*- value		
	Control	LOW	MED	HIGH	Pooled SEM	Linear	Quadratic
Myristic (C14:0)	8.69	6.25	5.28	9.29	1.458	NS	<0.05
Palmitic (C16:0)	13.88	15.78	16.37	14.85	0.793	NS	<0.05
Stearic (C18:0)	13.28	13.41	13.43	13.76	0.785	NS	NS
Oleic (C18:1)	7.55	7.31	5.98	6.18	0.560	<0.05	NS
Vaccenic (C18:1-*t* 11)	0.37^{a}	0.74^{a}	1.33^{b}	1.73^{b}	0.150	<0.0001	NS
*c*9*t*11 CLA	0.09^{ab}	0.16^{a}	0.11^{a}	0.19^{b}	0.020	<0.05	NS
t 10*c* 12 CLA (C18:2)	0.14	0.17	0.28	0.19	0.051	NS	NS
ω-6 PUFA							
Linoleic (C18:2)	17.37^{a}	13.87^{ab}	9.45^{b}	3.37^{c}	1.217	<0.0001	NS
Eicosatrienoic (C20:3)	0.54	0.36	0.3	0.45	0.115	NS	NS
Arachidonic (C20:4)	1.57	1.33	1.63	1.39	0.160	NS	NS
ω-3 PUFA							
α-linolenic (C18:3)	1.44^{ab}	1.48^{a}	1.39^{ab}	1.03^{b}	0.118	<0.05	NS
EPA (C20:5)	3.03^{a}	6.34^{ab}	9.99^{b}	8.78^{b}	1.065	<0.0001	<0.05
DPA (C22:5)	0.72	0.99	1.12	0.83	0.108	NS	<0.05
DHA (C22:6)	0.58	0.95	0.94	0.74	0.106	NS	<0.05
ω-3 family[1]	4.99^{a}	8.77^{ab}	12.32^{b}	10.55^{b}	1.213	<0.001	<0.05
ω-6 family[2]	18.95^{a}	15.20^{ab}	11.09^{b}	4.77^{c}	1.322	<0.0001	NS
Ratio ω-6 to ω-3	5.07^{a}	1.72^{bc}	0.92^{c}	0.49^{c}	0..374	<0.0001	<0.001

$^{a, b, c}$ Within row, concentrations with different superscripts are different ($P < 0.05$)

[1]C18:3 + C20:5 + C22:6

[2]C18:2 + C20:4

vaccenic acid ($P < 0.0001$) and *c*9, *t*11 CLA ($P < 0.05$) while oleic acid and α-linolenic acid ($P < 0.05$) decreased with increasing fish oil supplementation.

6.3.9 Relationship between plasma concentrations of PUFA and concentrations in endometrial tissue and follicular fluid (Trial 2)

Regression analysis was used to establish the relationship between concentrations of selected saturated fatty acids (SFA) and PUFA in plasma and their concentrations in endometrial tissue **(Table 6.11a)** and follicular fluid **(Table 6.11b)**. The relationship between SFA concentrations in plasma and their concentration in either endometrial tissue or follicular fluid were weak. For most PUFA, the relationship between their concentrations in plasma and in both the endometrial tissue and follicular fluid was linear in direction. There were moderate to strong positive relationships between plasma concentrations of linoleic acid, EPA, total ω-3 PUFA, total ω-6 PUFA and the ω-6:ω-3 ratio and their concentrations in both endometrial tissue and follicular fluid.

6.3.10 Plasma metabolites (Trial 2)

The effect of diet on plasma metabolite concentrations is shown in **Table 6.12**. As there were no diet x day of sampling interactions detected, mean values of samples collected on days 20 and 44 of the experimental period are presented. There was a linear increase in cholesterol in response to increasing fish oil supplementation. There was no effect of the supplementation regime on the concentrations of triglycerides, glucose or BHBA ($P > 0.05$). Urea increased ($P < 0.0005$) while NEFA decreased ($P < 0.01$) linearly in response to increasing dietary fish oil intake.

Table 6.11a Regression co-efficients for the relationship between plasma and endometrial concentrations of selected saturated fatty acids (SFA), along with ω-3 and ω-6 PUFA, Trial 2.

Fatty acid	β_0	β_1	β_2 †	R^2
SFA				
Myristic (C14:0)	1.96***	-1.32	0.70 $^{p=0.07}$	0.13
Palmitic (C16:0)	13.18***	0.08		0.04
Stearic (C18:0)	16.14***	-0.32		0.004
ω-6 PUFA				
Linoleic (C18:2)	0.87	0.44**	-0.01*	0.66
Eicosatrienoic (C20:3)	0.80***	0.97***		0.69
Arachidonic (C20:4)	7.67*	-0.75*		0.12
ω-3 PUFA				
α-linolenic (C18:3)	0.48	-0.002		0.00
EPA (C20:5)	-0.05	0.27***	-0.004 $^{p=0.05}$	0.79
DPA (C22:5)	1.20**	1.57***		0.39
DHA (C22:6)	2.24	0.76		0.44
ω-3 family[1]	3.41***	0.19***		0.77
ω-6 family[2]	5.70***	0.29***		0.62
Ratio ω-6 to ω-3	0.65***	0.90***	-0.06***	0.93

Table 6.11b Regression co-efficients for the relationship between plasma and follicular fluid concentrations of selected saturated fatty acids (SFA), along with ω-3 and ω-6 PUFA, Trial 2.

Fatty acid	β_0	β_1	β_2 †	R^2
SFA				
Myristic (C14:0)	7.12*	0.22		0.0003
Palmitic (C16:0)	48.12**	-4.55*	0.15*	0.25
Stearic (C18:0)	11.64***	0.17		0.04
ω-6 PUFA				
Linoleic (C18:2)	-14.04*	2.15**	-0.04*	0.65
Eicosatrienoic (C20:3)	0.24*	0.29 $^{p=0.06}$		0.14
Arachidonic (C20:4)	1.37	0.04		0.003
ω-3 PUFA				
α-linolenic (C18:3)	0.98*	0.18		0.02
EPA (C20:5)	-1.18	1.04***	-0.025**	0.52
DPA (C22:5)	0.64*	0.23		0.04
DHA (C22:6)	0.59*	0.14		0.05
ω-3 family[1]	-3.10	1.30***	-0.03**	0.48
ω-6 family[2]	-23.02*	2.76**	-0.05*	0.59
Ratio ω-6 to ω-3	0.12	0.84***	0.04**	0.99

β_0, β_1, β_2 are the regression co-efficients for the intercept, linear and quadratic components respectively.

†Where a quadratic term was not found to be statistically significant ($P < 0.05$) it was omitted from the regression model.

The probability of a co-efficient being not statistically significantly different from zero is denoted as follows *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

[1]C18:3 + C20:5 + C22:6, [2]C18:2 + C20:4

Table 6.12 Effect of level of dietary ω-3 PUFA supplementation on plasma[†] metabolite (mmol/l; mean ± S.E.M.) concentrations, Trial 2.

	Diet				*p* value		
Metabolite	Control	LOW	MED	HIGH	Pooled SEM	Linear	Quadratic
Cholesterol	4.18[a]	3.93[a]	4.90[a,b]	5.97[b]	0.232	<0.001	<0.05
Triglycerides	0.26	0.22	0.22	0.24	0.025	NS	NS
Glucose	3.91	3.85	3.94	3.81	0.086	NS	NS
Urea	3.79[a]	3.81[a]	4.31[a,b]	4.63[b]	0.174	<0.001	NS
BHBA	0.27[a]	0.35[b]	0.28[a,b]	0.26[a]	0.020	NS	<0.05
NEFA	0.45[a]	0.34[a,b]	0.35[a,b]	0.30[b]	0.032	<0.01	NS

6.4 Discussion

In the first study we observed no effect of diet on dry matter intake. Whitlock *et al.* (2006) reported no adverse effects on dry matter intake when feeding fish oil up to 1% of dietary dry matter to lactating dairy cows; however, inclusion rates of 2% resulted in an 11% decrease in dry matter intake (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 x day interaction for plasma concentrations of some fatty acids measured, concentrations of most fatty acids were generally consistent with other published reports involving similar dietary approaches (Burns *et al.*, 2003; Filley *et al.*, 2000). Consequently, rather than discussing the effect of diet on each fatty acid measured, we focus on a select number of fatty acids in the context of their potential biological roles in metabolism and reproduction.

The WSB diet (linoleic acid rich) increased the concentrations of linoleic acid in plasma and is consistent with similar studies (Burns *et al.*, 2003; Filley *et al.*, 2000). Interestingly, the increased supply of linoleic acid did not significantly increase the concentration of *c*9, *t*11 CLA isomer, *t*10, *c*12 CLA isomer or the CLA precursor vaccenic acid found in the plasma. This observation is contrary to results of similar studies in cows where the increased supply of linoleic acid from soybeans was associated with increased concentrations of both *c*9, *t*11 CLA isomer and vaccenic acid in the plasma and milkfat of ruminants (Dhiman *et al.*, 2000; Dhiman *et al.*, 2005; Loor & Herbein, 2003).

Despite low available dietary concentrations, increases in the concentration of the ω-3 PUFA, α-linolenic acid were observed in the plasma of animals fed the fish oil diet relative to animals fed with either the WSB or control diets. This may be a result of the fish oil diet interrupting the ruminal biohydrogenation of α-linolenic acid coming from the other ingredients in the ration. Interestingly, α-linolenic acid the parent fatty acid from which all other ω-3 PUFA are formed, has itself been shown to be an effective suppressor of $PGF_{2\alpha}$ during both *in vitro* and *in vivo* studies (Ambrose *et al.*, 2006; Mattos *et al.*, 2003).

In the current study, the fish oil diet was found to increase 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 being present in greater dietary concentrations, 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 poorer efficiency of absorption. The inclusion of the fish oil supplement in the animal's diet did not significantly increase the concentration of the *c*9, *t*11 CLA isomer found in the plasma. It was however found, that the concentration of *t*10, *c*12 CLA isomer in the plasma was significantly higher in the fish oil group than either the control or WSB fed groups. Despite an overall increase in plasma CLA concentrations as a result of the provision of the fish oil diet (10.5 μg/ml CLA), increases of the magnitude of those observed in cows on similar diets were not observed (34.3 μg/ml CLA) (Loor *et al.*, 2005). Statistically significant increases in the concentration of vaccenic acid, the CLA precursor, were observed in the plasma of animals on the fish oil diet compared to those on the control or linoleic acid rich WSB diet. Vaccenic acid has the potential to be converted to CLA endogenously via the enzyme Δ^9-desaturase found in the adipose tissue and

mammary gland, thus the increases in this fatty acid may suggest the potential for increased CLA production (Hennessy *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 (Boland *et al.*, 2001), there was no effect of dietary lipid on systemic glucose. The literature detailing 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. In the present study, fat supplementation increased plasma cholesterol consistent with previous reports (Grummer & Carroll, 1991; Hawkins *et al.*, 1995).

Overall, the provision of linoleic acid in the form of WSB was not observed to be an effective strategy in increasing the production of CLA or its immediate precursor vaccenic acid. This may be a result of the unavailability of the linoleic acid found in the soybean for ruminal biohydrogenation as a result of being

presented in the form of an intact oilseed. Indeed, a number of studies have found that when oil seeds are presented in rolled, cracked, extruded, or roasted forms, significantly greater concentrations of endogenously produced CLA can be achieved (Chouinard *et al.*, 2001; Dhiman *et al.*, 2000). In a similar observation to that achieved with WSB, supplementation of the diet of Holstein Friesian heifers with EPA and DHA in the form of fish oil was not found to be an effective strategy for increasing the concentration of CLA found in the plasma. However, significant increases in the concentration of vaccenic acid, an immediate precursor of the *c*9, *t*11 CLA isomer were observed. The reasons for the ineffectiveness of dietary oils, such as those used in part one of this study, to mediate an increase in the concentration of CLA detected in the plasma of the heifers used in this study may lie in the relative immaturity of the animal. Previous studies investigating the factors which influence CLA production by Holstein Friesian cows determined that animals with higher lactation numbers produce significantly more CLA than animals with lower lactation numbers (Lal & Narayanan, 1984; Stanton *et al.*, 1997). While the factors responsible for these differences were not elucidated, a potential reason may be the relative immaturity of the mammary gland in animals with a lower lactation number. Indeed, it is estimated that approximately 75-90% of the CLA found in milkfat is present as a result of the endogenous conversion of ruminally produced vaccenic acid to the *c*9, *t*11 CLA isomer via the action of the enzyme Δ^9-desaturase in the mammary gland (Hennessy *et al.*, 2007). As a result of the poor bioconversion of both linoleic acid from WSB and EPA/DHA from fish oil to CLA in heifers, we concluded that the inclusion of dietary oils in the bovine diet in an attempt to increase endogenous CLA by the animal did not merit further investigation.

While increased production of endogenous CLA could not be achieved through the supplementation of the diet of Holstein Friesian heifers with WSB or fish oil, it was observed that significant increases in the concentration of the health promoting fatty acids EPA and DHA could be achieved in the plasma of animals through the provision of a fish oil supplemented diet. Thus, in our second study it was decided to pursue the effect of supplying the animals with increasing concentrations of fish oil with the aim of assessing the diets impact on the fatty acid composition of key targets associated with bovine reproduction. To further increase the concentration of EPA and DHA delivered to the plasma it was additionally decided to ruminally protect the fish oil. Furthermore, given the higher adsorption of EPA than DHA which was observed in our first study it was decided to tailor the fish oil used in this study to contain higher EPA concentrations, thus, maximising the ω-3 PUFA uptake. In this second study, supplementation of the basal diet with the highest level of the fish oil supplement (HIGH, 4.15% fish oil) led to a significant reduction in DMI comparable to that reported in previous studies (Cant *et al.*, 1997; Donovan *et al.*, 2000; Wistuba *et al.*, 2006). The observations of such reductions when feeding fish oil in excess of 1% and may have been due to poorer palatability as a consequence of a strong fishy odour from the supplement offered and/or modification of the ruminal environment of the animals. Indeed, poor palatability due to odour has previously been reported as a potential reason for reduced DMI in dairy cows fed fish oil (Cant *et al.*, 1997). The lack of an effect of supplementation on DMI in animals on the LOW and MED diets is similar to previous reports, despite the percentages of oil in the diets being in excess of the 1% outlined previously (Mattos *et al.*, 2002). Consistent with the DMI, the ADG of the heifers was greater in animals on the control, LOW and MED diets compared to the HIGH fish oil diet. This is in contrast to a study by Wistuba *et al.* (2006) in which feeding

fish oil to steers at a rate of 3% dietary DM, reduced average daily feed intake but did not affect ADG. The literature relating to ruminal hydrogenation of the long chain ω-3 PUFA is inconsistent. Extensive hydrogenation of EPA and DHA has been reported *in vivo* (Castaneda-Gutierrez *et al.*, 2007b; Scollan *et al.*, 2001), while *in vitro* work has suggested that the effects of hydrogenation on the long-chain PUFA are negligible (Ashes *et al.*, 1992). It is generally believed that in order to appreciably alter tissue concentrations of the long-chain PUFA, some form of ruminal protection is necessary. In this study we fed a fish oil supplement that was partially ruminally protected. As evidenced by the linear increases in the ω-3 PUFA concentration in plasma with increasing dietary level, it would appear that a significant amount of the ω-3 PUFA did not undergo biohydrogenation. We found the affect of dietary fish oil supplementation on the plasma and tissue concentrations of the saturated and monounsaturated fatty acids measured to be consistent with other published studies (Burns *et al.*, 2003; Filley *et al.*, 2000). Consequently, discussion of the results of the current study will focus on a number of long-chain PUFA reported to have putative roles in the reproductive process. The linoleic acid concentration of both plasma and endometrial tissue was reduced by increasing fish oil supplementation in a linear fashion in the current study. This is consistent with previous reports in beef heifers (Childs *et al.*, 2008c), non-lactating beef (Burns *et al.*, 2003) and lactating dairy cows (Mattos *et al.*, 2004). As linoleic acid is the precursor to arachidonic acid, which is the precursor of luteolytic $PGF_{2\alpha}$, reductions in availability of the fatty acid both at plasma and tissue level may affect the biosynthesis of this luteolysin. However, the linoleic acid content of follicular fluid and oocytes is also believed to be important for the developmental competence of the oocyte (Homa & Brown, 1992) and for blastulation following *in vitro* maturation (IVM)/*in vitro* fertilisation (IVF) (Zeron *et al.*, 2001). In the current

study, there were no effects of fish oil supplementation on plasma concentrations of α-linolenic acid, however, the concentrations in endometrial tissue declined with increasing fish oil supplementation. A recent study at this laboratory reported an increase in plasma α-linolenic acid concentrations in response to supplementation with fish oil (Childs *et al.*, 2008c). Spain *et al.* (1995) has also reported increases in plasma concentrations of α-linolenic acid with increasing fish meal supplementation. In contrast, Burns *et al.* (2003) recorded a decline following fishmeal supplementation in plasma concentrations of α-linolenic acid but found no effect on endometrial concentrations. Mattos *et al.* (2004) found no effect of fish oil supplementation on the α-linolenic acid concentrations of uterine caruncular tissue. Consistent with this report, Moussavi *et al.* (2007) have also reported no effect of both calcium salts of fish oil fatty acids and incremental levels of fishmeal on the concentrations of α-linolenic acid in the endometrial tissue of lactating dairy cows. The plasma EPA concentrations of the fish oil supplemented animals increased in a dose responsive manner as did the EPA concentrations of endometrial tissue, which is consistent with previous studies (Burns *et al.*, 2003; Wamsley *et al.*, 2005). Spain *et al.* (1995) also reported linear increases in plasma EPA in dairy cows fed increasing concentrations of fishmeal.

Increases in the concentration of DHA in both plasma and endometrial tissue found here are consistent with some previous reports (Burns *et al.*, 2003; Wamsley *et al.*, 2005). Although there was a positive relationship between both dietary and plasma concentrations of this long chain ω-3 PUFA and its concentration in the uterine endometrium, the relationship was not as strong as that of EPA. Similarly, other studies have found that increasing tissue DHA concentrations is not readily achievable, through dietary manipulation (Burns *et al.*, 2003; Wamsley *et al.*, 2005). It is important to emphasise that while the endometrial

DHA concentrations in the current study were similar to those reported for uterine caruncular (Mattos *et al.*, 2004) and endometrial tissue (Moussavi *et al.*, 2007), the EPA and total ω-3 PUFA concentrations reported here are several multiples of the concentrations reported in the aforementioned studies. Therefore it would appear that the provision of a ruminally protected fish oil in the diet of Holstein Friesian heifers is an excellent strategy for increasing the concentration of PUFA found in targets associated with the reproductive process.

In the present study, plasma concentrations of arachidonic acid increased with increasing fish oil supplementation. This, again is similar to the report of Burns *et al.* (2003) and with more recent work from our laboratory (Childs *et al.*, 2008c). However, despite the increase in plasma concentrations of arachidonic acid, the endometrial concentrations of this fatty acid were reduced with increasing level of dietary fish oil. Similar results have previously been reported by Burns *et al.* (2003) in beef cows and also in the caruncular tissue in dairy cows (Mattos *et al.*, 2004). The higher plasma concentrations may be a function of either higher concentrations available in the diet, or due to the ability of both EPA and DHA to displace arachidonic acid in membrane phospholipids (Mattos *et al.*, 2003), resulting in a reduction in tissue uptake and thus causing a relative 'accumulation' of arachidonic acid in plasma. While Moallem *et al.* (1999) have previously reported fatty acid concentrations in follicular fluid, this is the first study, to our knowledge to report concentrations of the long chain ω-3 PUFA, EPA and DHA in cattle. Significantly greater concentrations of DHA in the follicular fluid of ewes have previously been reported when feeding calcium soaps of fish oil fatty acids (Zeron *et al.*, 2002). While the majority of fatty acids in follicular fluid were unaffected by the ω-3 PUFA supplementation, EPA concentrations in the fluid increased in a linear and quadratic fashion, while linoleic and oleic acid

concentrations both decreased linearly. Furthermore, we found strong positive relationships between the concentrations of a number of ω-3 and ω-6 PUFA in follicular fluid and their concentrations in plasma. In a previous study by Zeron *et al.* (2001), follicular fluid of Holstein cows in summer contained a higher proportion of saturated fatty acids compared to winter when PUFA were the predominant form. The summer period also coincided with reduced fertility in that study. Not withstanding the fact that changes in seasonal fertility are probably a result of multifactorial processes (Zeron *et al.*, 2001), the relationship between PUFA content, biophysical behaviour of oocytes and low fertility of the cows outlined by Zeron *et al.* (2001) suggest that PUFA modification can influence ovarian dynamics and biophysical behaviour of oocytes (Zeron *et al.*, 2002), and increasing the concentration of PUFA in follicular fluid may be of benefit for increasing conception rates.

The use of the ruminally protected fish oil supplement was also found to have an effect on systematic and tissue CLA concentrations. This result corresponds well with similar studies where ruminally protected marine algae supplements rich in EPA and DHA have been associated with increases in milkfat CLA (Franklin *et al.*, 1999). In our study, linear increases in the concentration of the *c*9, *t*11 CLA isomer were observed in both the endometrial tissue and follicular fluid of the animal's fed the dietary fish oil. Similar increases in the *c*9, *t*11 CLA isomer concentration of endometrial tissue were observed by Moussavi *et al.* (2007) when the bovine diet was supplemented with increasing concentrations of fishmeal. The presence of dose related amounts of this isomer in both the endometrial tissue and follicular fluid confirms the relationship between increasing dietary fish oil concentrations and the production and utilisation of CLA by the cow. Linear increases in the concentration of the *t*10, *c*12 CLA isomer were observed in the

rumen fluid, plasma and endometrial tissue of the animal, however, this trend was not replicated in the follicular fluid. This suggests a preference by the follicular fluid for the uptake of the *c*9, *t*11 CLA isomer over the *t*10, *c*12 isomer. The presence of increased CLA in bovine endometrial cells (BEND) has been associated with a decrease in $PGF_{2\alpha}$ production in response to stimuli while a recent *in vivo* study has associated CLA with increased plasma progesterone levels (Castaneda-Gutierrez *et al.*, 2007a; Rodriguez-Sallaberry *et al.*, 2006). Given these observations it is may be possible to associate the reproductive benefits of a ruminally protected fish oil dietary supplement in cows not just with increased systemic and tissue concentrations of EPA and DHA but also to an extent with increased CLA concentrations.

The importance of glucose as an energy source for the bovine ovary and the post-blastulation embryo has previously been documented (Boland *et al.*, 2001). In agreement with previous work at this laboratory there was no effect of ω-3 PUFA supplementation on systemic glucose concentrations, however, the literature on this issue is inconsistent. For example, reductions (Mattos *et al.*, 2004), increases (Moussavi *et al.*, 2007) and no response (Petit *et al.*, 2002) in systemic glucose in postpartum cows fed fish oil supplements have all been reported. Reviewing the literature, Grummer and Carroll (1991) concluded that fat supplementation does not routinely increase blood glucose and stable systemic glucose concentrations during fat supplementation may indicate a reduction in hepatic gluconeogenesis. The lack of a dietary effect on systemic triglyceride concentrations is consistent with previous work from this laboratory (Childs *et al.*, 2008c) and others (Hightshoe *et al.*, 1991; Lammoglia *et al.*, 1996). Similarly, consistent with some reports (Moussavi *et al.*, 2007) but not others (Mattos *et al.*, 2004) we found no effect on BHBA concentrations. NEFA concentrations decreased with increasing dietary fish

oil intake despite the lower overall feed intake and performance on the HIGH diet. This was surprising and is in contrast to the study of Robinson *et al.* (2002) who showed no effect of providing lactating dairy cows with either linoleic or α-linolenic acid-based supplements on systemic NEFA concentrations of lactating dairy cows. It is suggested that lower levels of NEFA may reflect a greater insulin-induced suppression of lipolysis during ω-3 PUFA enrichment (Lovegrove *et al.*, 1997). Although insulin has not been previously shown to be affected by ω-3 PUFA supplementation in cattle (Bilby *et al.*, 2006a; Childs *et al.*, 2008a), this observation may reflect an increase in insulin sensitivity, due to incorporation of EPA and DHA into membrane phospholipids, resulting in a reduced NEFA release (Lovegrove *et al.*, 1997). The mechanisms involved in this apparent ω-3 PUFA mediated reduction in NEFA requires further investigation. Despite this, overall, our results are consistent with the positive energy balance status and absence of lipolysis in the animals employed. Plasma urea concentrations in the present study increased as the level of fish oil increased in the diet, which conflicts with previous reports for cattle supplemented with either fish oil (Mattos *et al.*, 2004) or fishmeal (Spain *et al.*, 1995), and the given the fact that the diets were formulated to be isonitrogenous. Despite the fact that the diets were formulated to be isolipoid, concentrations of total cholesterol were increased by ω-3 PUFA supplementation, suggesting a possible effect on cholesterol synthesis. Furthermore, as most of the cholesterol found in bovine blood is in the form of high-density lipoproteins (HDL) (Staples *et al.*, 1998), the increase in cholesterol recorded is likely to be at the expense of low-density lipoprotein (LDL) as fish oil supplementation is thought to reduce activity of cholesterol ester transfer protein responsible for transfer of cholesterol esters from HDL to LDL (Nestel, 2000). Moreover, circulating cholesterol is the primary substrate for the synthesis of progesterone (Williams & Stanko, 1999), and

therefore increases in cholesterol could be expected to have a positive effect on the concentrations of progesterone, as has been documented elsewhere (Childs *et al.*, 2008b; Grummer & Carroll, 1991; Hawkins *et al.*, 1995). An increase in systemic progesterone could be important in the context of cow conception rate (Hawkins *et al.*, 1995).

6.5 Conclusions

While the inclusion of fish oil in the ruminant diet can be successfully used to elevate the concentration of *t*10, *c*12 CLA in the plasma of Holstein Friesian heifers and the CLA precursor vaccenic acid it cannot be used to significantly increase *c*9, *t*11 CLA production. Furthermore, the increases in plasma CLA observed using this strategy are not sufficient to have an impact on bovine reproduction. Supplementation of the diet of Holstein Friesian heifers with whole soybean was not observed to be an effective strategy for increasing the concentration of CLA found in plasma.

A more effective use of fish oil is to include it in the diet as a ruminally protected feed supplement with the aim of increasing the concentration of ω-3 PUFA which have been successfully utilised to improve bovine fertility. In our second study we confirmed that the inclusion of a ruminally protected fish oil in the diet of Holstein Friesian heifers can be successfully utilised to significantly increase the ω-3 PUFA content of the plasma, endometrial tissue and follicular fluid. Furthermore, we have observed that analysis of the plasma for concentrations of EPA and to a lesser extent DHA can be successfully used to predict their concentrations in key reproductive tissues. Furthermore, the study provides some evidence that improvements in reproductive performance following fish oil supplementation may be, in some part, mediated through increased systemic cholesterol leading to increases in progesterone.

6.6 References

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