RMIS: 5541

An examination of the molecular mechanisms controlling the tissue accumulation of conjugated linoleic acid (CLA) in cattle

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SUMMARY

Long chain n-3 polyunsaturated fatty acids (n-3 PUFA) and conjugated linoleic acid (CLA) have demonstrable and potential human health benefits in terms of preventing cancer, diabetes, chronic inflammation, obesity and coronary heart disease. Supplementation of cattle diets with a blend of oils rich in n-3 PUFA and linoleic acid have a synergistic effect on the accumulation of ruminal and tissue concentrations of trans vaccenic acid (TVA), the main substrate for Δ-9 desaturase which is responsible for *de novo* tissue synthesis of the cis 9, trans 11 isomer of CLA. This dietary strategy translates into increases in milk concentrations of CLA in dairy cows; however, concentrations in the muscle of beef animals have not always been increased. There is an apparent paradox in that n-3 PUFA supplementation enhances ruminal synthesis of trans-vaccenic acid (TVA), but then inhibits its conversion to CLA possibly through altering the activity of Δ-9 desaturase. Recently, the promoter regions of the bovine Δ-9 desaturase gene has been isolated and analysed and has been shown to contain a conserved PUFA response region.

The first objective of this study was to examine the effect of level and duration of feeding of a n-3 PUFA enriched fishoil (FO) supplement, in combination with soyoil (SO), on the transcriptional regulation of Δ-9 desaturase gene expression in bovine muscle. In this study, beef bulls (n=40) were assigned to one of four isolipid and isonitrogenous concentrate diets, fed *ad libitum*, for a 100 day finishing period. Concentrates were supplemented with one of the following: (i) 6% SO (CON); (ii) 6% SO + 1% FO (FO1); (iii) 6% SO + 2% FO (FO2) or (iv) 8% palmitic acid for the first 50 days and 6% SO + 2% FO for the latter 50 days (FO2(50)). Samples of *M. longissimus dorsi* were harvested and concentrations of fatty acids were measured.

Total RNA was isolated and the gene expression of Δ-9 desaturase was determined. mRNA expression of putative regulators of Δ-9 desaturase gene expression, sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator activated receptor-α (PPAR-α) were also measured in the CON and FO2 groups. Expression of mRNA for Δ-9 desaturase was decreased 2.6, 4.4 and 4.9 fold in FO1, FO2(50) and FO2 compared with CON, respectively ($P<0.05$). Expression of Δ-9 desaturase mRNA tended to be reduced ($P=0.09$) by increasing FO from 1% to 2%, but was not affected by duration of supplementation ($P>0.05$). Expression of mRNA for SREBP-1c was decreased 2 fold on FO2 compared with CON ($P<0.05$) whereas PPAR-α was not affected ($P>0.05$). There was a positive relationship between Δ-9 desaturase and SREBP-1c gene expression ($P<0.01$) but the expression of both genes was negatively related to tissue concentrations of n-3 PUFA ($P<0.05$) and positively related to n-6 PUFA concentration ($P<0.01$). Simultaneous enhancement of tissue concentrations of CLA and n-3 PUFA concentrations in bovine muscle may be hindered by negative interactions between n-3 PUFA and Δ-9 desaturase gene expression, possibly mediated through reduced expression of SREBP-1c.

As a negative relationship was exhibited between the gene expression of both Δ-9 desaturase and SREBP-1c; and tissue n-3 PUFA concentrations, the second objective of this project was to examine the effect of eicosapentaenoic acid (EPA), specifically on the transcriptional regulation of Δ-9 desaturase *in vitro*. Firstly, a novel primary bovine intramuscular adipocyte cell line was developed and validated. Intramuscular adipose tissue was obtained from the *M. longissimus* thoracis of a beef heifer. Mature adipocytes were isolated and cultured; and subsequently harvested and evaluated for lipid accumulation and the expression of genes regulating key functional adipocyte

protein markers at passage 10, 20 and 30. Isolated cells were shown to accumulate lipid in culture over time. Fatty acid analysis by gas chromatography was carried out at passage 30. Thirteen fatty acids ranging from tetradecanoic acid (C14:0) to the polyunsaturated fatty acid, docosohexanoic acid (C22:6) were easily detected and measured. High quality total RNA was isolated from adipocytes and the expression of peroxisome proliferator activated receptor (PPAR)-γ, fatty acid synthase (FAS), fatty acid binding protein (FABP)-4, adipocyte lipid binding protein (aP2), CD36, Δ-9 desaturase, sterol regulatory element binding protein (SREBP), microsomal triglyceride transfer protein. (MTP) and leptin genes were identified by reverse transcriptase-PCR and sequence analysis. Expression of the negative control, liver-specific hepatocyte nuclear factor (HNF)-1alpha was not detected. Adipocytes were subsequently incubated in medium containing 0, 50 or 100 μM EPA for 24h. Increasing the EPA concentration of the culture media led a linear increase in adipocyte EPA concentration ($P < 0.01$). Expression of Δ-9 desaturase mRNA was decreased 5 and 7 fold, respectively following 50 and 100 μM EPA incubation compared to the control. Gene expression of SREBP-1c was decreased by 6 and 18 fold in cells supplemented with 50 and 100 μM EPA, respectively compared to the control. Regression analysis showed a negative linear relationship between EPA concentration and the gene expression of both Δ-9 desaturase ($P<0.001$) and SREBP-1c ($P<0.001$), while there was a significant positive relationship observed between Δ-9 desaturase and SREBP-1c gene expression ($P<0.001$). This is the first report demonstrating that EPA treatment of bovine intramuscular adipocytes cells decreased gene expression of both Δ-9 desaturase and SREBP-1c *in vitro.* The bovine adipocyte cell line is an important resource for future studies facilitating less expensive, rapid screening of research hypotheses and circumventing the limitations associated with

the use of experimental animals including cost, inter animal variation, pre-experimental management and ethics.

This RMIS project is comprised of the following two studies:

1. Effect of level and duration of dietary n-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of Δ-9 desaturase in muscle of beef cattle.

2. Effect of level of eicosapentaenoic acid on the transcriptional regulation of Δ-9 desaturase using a novel *in vitro* bovine intramuscular adipocyte cell culture model.

Study 1. Effect of level and duration of dietary n-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of Δ-9 desaturase in muscle of beef cattle

INTRODUCTION

Long chain n-3 polyunsaturated fatty acids (n-3 PUFA) and the ruminant derived conjugated linoleic acid (CLA) have potential human health benefits (Ip et al., 1999; Wahle et al., 2004). The cis9, trans11 isomer of CLA is most prevalent comprising 80-90% of total CLA in food products from ruminants (Parodi, 2003). Thus, increasing the concentration of both n-3 PUFA and cis9, trans11 CLA in milk and meat is beneficial to public health. Supplementation of cattle diets with a blend of oils rich in n-3 PUFA and linoleic acid results in a synergistic accumulation of ruminal and tissue concentrations of vaccenic acid (VA) (AbuGhazaleh et al., 2002, Kenny et al., 2007), the substrate for Δ-9 desaturase catalyzed *de novo* tissue synthesis of the cis9, trans11 isomer of CLA. However, despite increases in its substrate, muscle tissue concentrations of cis9, trans11 CLA have not increased using this strategy (Kenny et al., 2007).

Studies have reported that the expression of the Δ-9 desaturase gene is regulated by the transcription factors sterol regulatory element binding protein (SREBP)-1c and peroxisome proliferator activator receptor (PPAR)-α (Sampath and Ntambi, 2006; Renaville et al., 2006). The promoter region of the bovine Δ-9 desaturase gene has been isolated and characterized (Keating et al., 2005) and apparently contains a conserved PUFA response region including a critical binding site for a SREBP

transcription factor. Indeed, studies both with mice (Tabor et al., 1999) and human intestinal cells (Renaville et al., 2006) have suggested that PUFAs down-regulate activity of the Δ-9 desaturase gene by interfering with the SREBP-1c function. There is no published information, however, on the effect of n-3 PUFA on Δ-9 desaturase gene expression or indeed its transcriptional regulators, in bovine muscle. Thus, the objective of the current study was to investigate the effect of dietary level and duration of supplementation with n-3 PUFA on the expression of Δ-9 desaturase, SREBP-1c and PPARα genes in the muscle of beef cattle.

MATERIALS AND METHODS

Animals, Experimental Design and Animal Measurements

Animals were maintained at University College Dublin Lyons research farm, Newcastle, Co. Dublin, Ireland. Forty continental cross young beef bulls were blocked on age, bodyweight, and breed (Charolais and Limousin) and assigned to one of four isolipid and isonitrogenous (15% CP) dietary treatments over a 100-day finishing period as described by Kenny et al. (2007). Briefly, animals were individually offered straw (10% of DMI) and barley-based concentrate rations (90% of DMI) *ad libitum*. The concentrates contained either (i) 6% soyoil (Redmills Animal Feeds, Goresbridge, Co. Kilkenny) (control; CON); (ii) 6% soyoil + 1% fishoil (Nutreco, Boxmeer, The Netherlands) (FO1) for 100 days; (iii) 6% soyoil + 2% fishoil (FO2) for 100 days or (iv) 8% palmitic acid (Palmit 80®, Trouw Nutrition, Belfast, Northern Ireland) for first 50 days and 6% soyoil + 2% fishoil (FO2) for latter 50 days (FO2(50)). Palmit 80 was added to CON and FO1 as appropriate to give 8% added lipid on a DM basis. The soyoil had a linoleic acid concentration of 53% while the fishoil (FO) had concentrations of eicosopentanoic acid (EPA) and docosohexanoic acid (DHA) of 39 and 24 % respectively. The ingredient and chemical composition of the experimental concentrates and straw is outlined in Table 1. All rations were prepared on the farm and were fed within 30 days of manufacture. Feed intake data has been described by Kenny et al. (2007). Briefly, the average daily

DMI for CON, FO1, FO2 and FO2(50) were 8.78, 7.31, 6.50 and 6.84 kg, respectively. There was a linear decrease in DMI with increasing dietary inclusion of fishoil ($P<0.05$). However there was no effect of duration of supplementation (FO2 v FO2(50); $P>0.05$).

Table 1. Ingredients (g/kg) and chemical composition (g/kg DM unless otherwise stated) of the experimental concentrates and straw

Ingredient	Control	FO1	FO2	FO2(50)*	Straw
Barley	747	747	747	747	
Soyabean meal	141	141	141	141	
Soya oil	60	60	60	0	
Palmit 80	18	9	0	78	
Fishoil	0	9	18	0	
Ground limestone	11.00	11.00	11.00	11.00	
Di-calcium phosphate	1.00	1.00	1.00	1.00	
Salt	2.00	2.00	2.00	2.00	
Vitamins & minerals†	20	20	20	20	
DM %	94.94	95.06	95.18	94.35	93.66
Protein	149.77	153.87	153.9	160.32	34.56
Ash	52.95	53.35	54.58	50.59	70.84
NDF	153.01	159.53	151.05	163	846.09
ADF	54.53	50.92	51.53	57.22	548.14
Oil	76.8	73.6	78.0	90.6	0
GE (MJ/kg DM)	19.38	19.22	19.25	19.22	17.31

*Animals on the FO2(50) treatment were offered this diet for the first 50 days and then switched over to FO2 for the latter 50 days prior to slaughter

†The mineral and vitamin mix contained Ca (30%), P (5%), Vitamin A (320,000 iu/kg), Vitamin D_3 (80,000 iu/kg), Vitamin E (20,000 iu/kg), cobalt carbonate (200 mg/kg), cupric sulphate (2,500 mg/kg), calcium iodate (320 mg/kg) Manganous oxide (2,500 mg/kg), sodium selenite (30 mg/kg) and zinc oxide (4,000 mg/kg) on an as fed basis.

Tissue Collection, RNA Extraction and Purification

All procedures were carried out under license in accordance with the European Community Directive, 86-609-EC. Animals were slaughtered in a licensed abattoir (Meadow Meats, Rathdowney, Co. Laois, Ireland). All surgical instruments used for tissue collection were sterilised and treated with RNA Zap (Ambion, Applera Ireland, Dublin, Ireland). Samples of *M. longissimus dorsi* were harvested, washed with sterile PBS, snap frozen in liquid nitrogen and stored immediately at -80°C. Muscle fatty acid concentrations were determined using gas chromatography as described by Kenny et al., (2007).

A Δ-9 desaturase index to estimate the activity of the Δ-9 desaturase enzyme at tissue level was calculated as described by Kelsey et al. (2003): [cis-9, trans-11 CLA]/[cis-9, trans-11 CLA + trans-11 18:1]. Muscle lipid concentrations were measured and analysed as described by Kenny et al. (2007).

Total RNA was isolated from fragmented frozen muscle tissue using TRIzol reagent and chloroform and subsequently precipitated using isopropanol. Samples were treated with RQ1 RNase-free DNase (Promega UK Ltd., Southampton, UK) and purified using the RNeasy® mini kit (Qiagen Ltd, Crawley, UK). RNA quantity was determined by absorbance at 260 nm using a Nanodrop spectrophometer. RNA quality was also assessed using 18S/28S ratio and RNA integrity number (RIN) on an Agilent Bioanalyser with the RNA 6000 Nano LabChip kit (Agilent Technologies Ireland Ltd., Dublin, Ireland).

cDNA synthesis

First-strand cDNA was synthesized using the Reverse Transcription system according to manufacturer's instructions (Promega UK Ltd., Southampton, UK). One μg of total RNA from each sample was reverse transcribed into cDNA using random hexamers. The converted cDNA was quantified by absorbance at 260 nm, diluted to 50 ng μL^{-1} working stocks and stored at -20°C for subsequent analyses.

Real time quantitative RT PCR (qRT-PCR)

Primers for real time RT-PCR were commercially synthesized (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Primers were first tested using end point PCR to optimize amplification conditions. All amplified PCR products from this study were also sequenced to verify their identity. In the case of all genes examined in this study, DNA sequences were 100% identical to published sequences.

As the effect of various experimental treatments on the expression of traditional real time RT-PCR reference or 'housekeeping' genes is largely unknown, it was essential to identify a stable reference gene for the physiological conditions inherent in the current study. Analysis of putative reference genes was carried out using the geNorm version 3.4 Microsoft Excel add-in (Vandespele et al., 2002). In this study, housekeeper genes analysed included those for β-actin (Bahar, 2006), ubiquitin (Neuvians et al., 2003), glyceraldehyde phosphate dehydrogenase (GAPDH) (Brunswig-Spickenheier and Mukhopadyay, 2003), 18S rRNA (De Kelelaere et al., 2006) and peptidylprolyl isomerase A (*PPIA*) (O'Gorman et al., 2006). The gene which exhibited greatest stability during real time RT-PCR of muscle mRNA samples analysed was β-actin with *M* values ranging from 0.10 to 0.22. GeNorm was used to

determine the optimal number of reference genes for normalisation and based on a recommended cut-off *V* value of 0.15; additional reference genes would not have contributed to a more accurate normalisation factor. Therefore β-actin was used as a single standard reference gene for these experiments.

Details of primer sets used in this study to measure the gene expression of Δ-9 desaturase, SREBP-1c, PPARα and β-actin are listed in Table 2. Primer sequences for Δ-9 desaturase and β-actin were as described by Baher (2006). Sequences for PPARα primers were designed using Primer3 software program based on accession number AF229356. There is no published sequence available for SREBP-1c in the bovine. The human SREBP-1c (NCBI gene ID NM_001005291) displays high sequence homology to bovine cDNA sequence of NCBI gene ID XM-879234. ClustalW web-based software program was used to align these sequences and to design bovine-specific primers for SREBP-1c in the bovine. The primers were subjected to BLAST analysis and were 100% homologous to the bovine sequence.

Table 2. Bovine oligonucleotide primers used for real time RT-PCR.

Gene Name	Primer sequence (5´-3´)	Accession number	Reference
Δ-9 desaturase	Forward: 5´GCTGCTTGTGCGCAAACA3´	AF481915S3	Current study
	Reverse: 5´TCGGCTCTTAGGTCGGATAAATT3´		
SREBP-1c	Forward: 5´ACCGCTCTTCCATCAATGAC3´	XM_879234	Current study
	Reverse: 5´TTCAGCGATTTGCTTTTGTG3´		
PPAR-α	Forward: 5´TTGTGGCTGCTATCATTTGC3´	NM_001034036	Current study
	Reverse: 5´AGAGGAAGACGTCGTCAGGA3´		
β-actin	Forward: 5´CGCCATGGATGATGATATTGC3´	BC142413	Bahar, 2006
	Reverse: 5´AAGCGGCCTTGCACAT3´		

Each real time PCR reaction was carried out in a total volume of 20 μL with 1 μL cDNA (50 ng/μL), 10 μL SYBR Green I master mix (Langanbach, Killarney Road, Bray, Co. Wicklow, Ireland), 1 μL forward and reverse primers (20 ng of each) and 8 μL nuclease-free H_2O. Dissociation curves were examined for the presence of a single PCR product. Real time RT PCR was performed using a Corbett Rotor-Gene™ 3000 quantitative PCR system (Corbett Life Sciences, Sydney, Australia) with the following cycling parameters: 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by amplicon dissociation (95°C for 1 min, 50°C for 45 s, increasing 0.5°/cycle until 95°C was reached). Gene expression results were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The $2^{-\Delta\Delta CT}$ method was used to determine mean fold changes in gene expression between the control and each of FO1, FO2 and FO50. To test the effect of level of dietary n-3 PUFA, the mean relative gene expression level for FO1 and FO2 were compared. The effect of duration of feeding on the relative gene expression of Δ-9 desaturase was

examined by comparing the FO2 and FO2(50) groups. Differences in gene expression results between the CON and the FO2 treatment group for SREBP-1c and PPARα were also calculated using this method (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analysed using the Statistical Analysis Systems software package version 9.1 (SAS Institute, Cary, NC, USA). Data were examined for adherence to normality using PROC UNIVARIATE (SAS, 2001). Data were then analysed to determine the effect of treatment on Δ-9 desaturase, SREBP-1c and PPAR-α expression level using mixed models ANOVA (PROC MIXED, SAS, 2001). In all analyses the individual animal was denoted as the experimental unit and animal within treatment was set as the error term. The statistical model included terms for treatment and block. The Tukey critical difference test was used to determine statistical difference between treatment means. Univariate and stepwise multiple regression analyses were carried out to establish relationships between tissue fatty acid concentrations and relative gene expression of Δ-9 desaturase, SREBP-1c and PPAR-α using PROC REG and PROC STEPWISE of SAS respectively. Relationships were also determined between the relative gene expression of each of the genes measured.

Average daily feed intake was included as a covariate in the statistical model. Furthermore, as Δ-9 desaturase gene expression has previously been shown to be related to adiposity in bovine subcutaneous tissue (Martin et al., 1999), this was tested for and as no effect was detected (P=0.55), it was not necessary to correct gene expression data for tissue fat concentration.

RESULTS

Muscle fatty acid concentrations

The effect of treatment on the concentration of 30 fatty acids in muscle has been described by Kenny et al. (2007) and thus these data are not reported here. Briefly, muscle concentrations of the n-3 PUFA, EPA, DHA, docosapentaenoic acid (DPA), and total n-3 PUFA as well as VA were higher in the three FO treatment groups compared with CON ($P<0.01$), however, there was no effect ($P>0.05$) of treatment on the concentration of cis9, trans11 CLA or total n-6 PUFA. Tissue concentrations of linoleic acid were similar for CON and FO2(50) but were higher for CON compared with either FO1 or FO2 ($P<0.05$); while concentrations of linolenic acid were not affected by treatment ($P>0.05$).

Effect of PUFA on mRNA expression of Δ-9 desaturase

The effect of level and duration of n-3 PUFA supplementation is shown in Figures 1 and 2. Compared to CON, Δ-9 desaturase gene expression was decreased 2.6 fold in animals on FO1 ($P<0.05$), 4.9 fold on FO2 ($P<0.01$) and 4.4 fold on FO2(50) ($P<0.05$) (Figure 1). There was a tendency for level of Δ-9 desaturase gene expression to be lower in animals on FO2 compared with those on FO1 ($P=0.09$) (Figure 2). There was no difference, however, in Δ-9 desaturase gene expression between animals on FO2 compared with those on FO2(50) ($P=0.24$) (Figure 2).

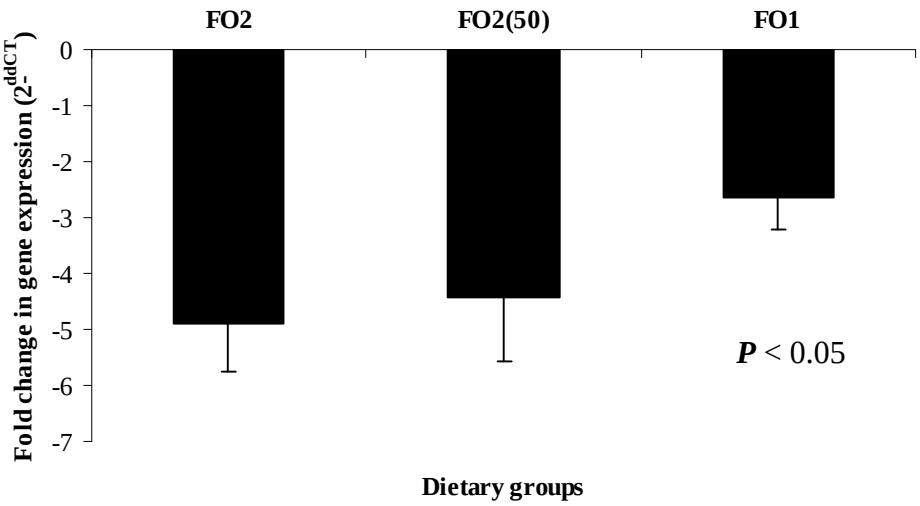


Figure 1. Effect of dietary n-3 PUFA supplementation on Δ-9 desaturase mRNA in *M. longissimus dorsi* of cattle offered FO2, FO2(50) or FO1 diets compared to the control (Fold change ± SEM) (n=10/treatment). **CON**; Control, 6% soyoil for 100 days. **FO2**; 6% soyoil + 2% fishoil for 100 days. **FO2(50)**; 8% palmitic acid for first 50 days and 6% soyoil + 2% fishoil (FO2) for latter 50 days. **FO1**; 6% soyoil + 1% fishoil for 100 days.

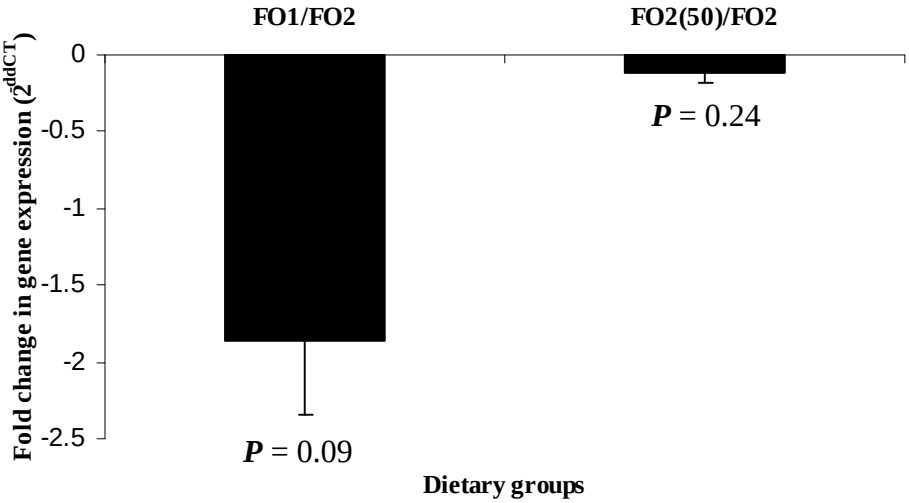


Figure 2. Effect of level (FO1/FO2) and duration of feeding (FO2/FO2(50)) of n-3 PUFA on the expression of Δ-9 desaturase mRNA in *M. longissimus dorsi* of cattle. **FO1**; 6% soyoil + 1% fishoil for 100 days. **FO2**; 6% soyoil + 2% fishoil for 100 days. **FO2(50)**; 8% palmitic acid for first 50 days and 6% soyoil + 2% fishoil (FO2) for latter 50 days.

Effect of PUFA on mRNA expression of SREBP-1c and PPAR-α

Expression of mRNA for SREBP-1c was decreased 2 fold in animals fed the FO2 compared with CON ($P<0.05$) (Figure 3). Dietary supplementation with FO2 did not alter the gene expression of PPAR-α ($P=0.3$) (Figure 3).

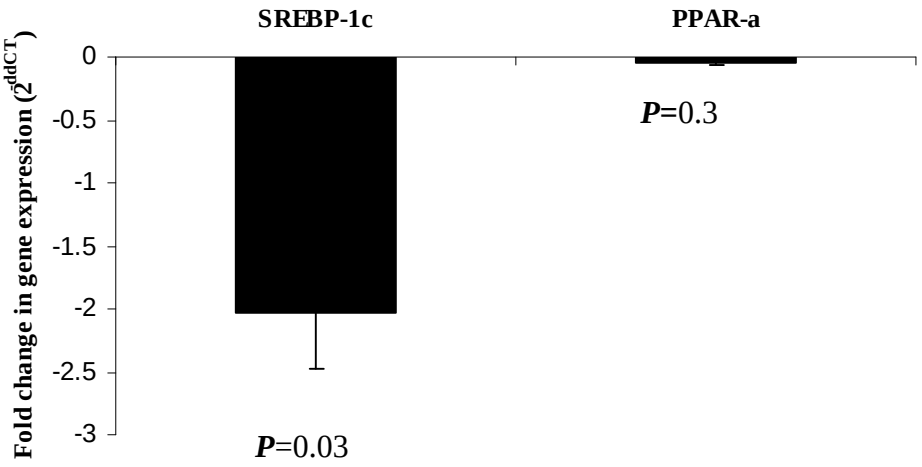


Figure 3. Effect of 2% dietary fish oil supplementation (FO2) compared to CON on mRNA expression of putative transcriptional regulators of Δ-9 desaturase, sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator activated receptor-α (PPAR-α) in *M. longissimus dorsi* of cattle (Fold change ± SEM) (n=10/treatment). **CON**; Control, 6% soyoil for 100 days. **FO2**; 6% soyoil + 2% fishoil for 100 days.

Relationship between tissue fatty acid concentrations and gene expression of Δ-9 desaturase, SREBP-1c and PPAR-α

The relationships between concentration of the following fatty acids; VA, cis9, trans11 CLA, cis10, trans12 CLA, oleic acid, linoleic acid, linolenic acid, EPA, DHA, DPA, total n-3 PUFA, total n-6 PUFA and the ratio of n-3 to n-6 PUFA (n-3:n-6 PUFA); and gene expression for Δ-9 desaturase is presented in Table 3.

Table 3. Regression coefficients for the relationship between tissue fatty acid concentrations and Δ-9 desaturase gene expression

Fatty acid	β_0	β_1	R^2
Vaccenic acid	-0.639	0.164	0.12
CLA 9,11	1.45*	-1.29	0.12
CLA 10,12	0.85***	-3.99	0.03
Oleic acid	-2.99	0.13	0.13
Linoleic	-1.07	0.30*	0.26
Linolenic	4.00*	$-5.83^{(P=0.07)}$	0.18
Eicosapentaenoic	1.99***	-0.83**	0.32
Docosapentaenoic	2.61***	-2.48*	0.24
Docosahexaenoic	1.84***	-4.33**	0.33
Total n-3 PUFA	2.28***	-0.67**	034
Total n-6 PUFA	-1.01	0.24*	0.21
n-6:n-3	-0.25	0.19***	0.55

β_0, β_1 are the regression coefficients for the intercept and linear components respectively.
The probability of a coefficient not being statistically significantly different from zero is denoted as follows: *P<0.05, ** P<0.01 and *** P<0.005.

Similarly, relationships between muscle concentrations of these fatty acid and SREBP-1c gene expression are presented in Table 4.

Table 4. Regression coefficients for the relationship between tissue fatty acid concentrations and SREBP-1c gene expression

Fatty acid	β_0	β_1	R^2
Vaccenic acid	2.29	-0.18$^{(P=0.08)}$	0.17
CLA 9,11	0.83	-0.14*	0.24
CLA 10,12	1.85***	-8.13	0.16
Oleic acid	0.38	0.02	0.01
Linoleic acid	-0.37	0.22**	0.35
Linolenic	2.49*	-2.65	0.10
Eicosapentaenoic	1.53***	-0.35	0.15
Docosapentaenoic	1.65**	-0.81	0.07
Docosahexaenoic	1.55***	-2.23*	0.24
Total n-3 PUFA	1.68***	-0.29$^{(P=0.08)}$	0.17
Total n-6 PUFA	-0.38	0.18**	0.32
n-6:n-3	0.49$^{(P=0.06)}$	0.95***	0.38

β_0, β_1 are the regression coefficients for the intercept and linear components respectively.
The probability of a coefficient not being statistically significantly different from zero is denoted as follows: *P<0.05, ** P<0.01 and *** P<0.005.

There was a negative relationship between Δ-9 desaturase gene expression and the concentrations of EPA, DPA, DHA and total n-3 PUFA (P<0.05). Likewise, a tendency (P=0.07) towards a negative relationship between linolenic acid and Δ-9 desaturase gene expression was observed. A positive relationship was detected between Δ-9 desaturase gene expression and concentrations of linoleic acid (P<0.05), total n-6 PUFA (P<0.05) and n-6:n-3 PUFA (P<0.01). There was no relationship (P>0.05) observed between gene expression for Δ-9 desaturase and any other fatty acid measured.

A negative relationship was detected between SREBP-1c mRNA expression and both DHA (P<0.05) and cis9, trans11 CLA (P<0.05). Furthermore, the negative relationship observed between SREBP-1c gene expression and VA (P=0.08); and total n-3 PUFA (P=0.08), approached statistical significance. Similar to that observed for Δ-9 desaturase, there was a positive relationship between SREBP-1c gene expression and linoleic acid (P<0.01), total n-6 PUFA (P<0.01) and n-6:n-3 PUFA (P<0.01). No relationship was exhibited between EPA or any other fatty acid and SREBP-1c gene expression (P>0.05). No statistically significant relationship existed between PPAR-α gene expression and any of the fatty acids measured (P>0.05).

A highly significant positive relationship was found between Δ-9 desaturase and SREBP-1c gene expression (R^2=0.55; P<0.01), however, no relationship existed between PPAR-α and either Δ-9 desaturase or SREBP-1c gene expression (P>0.05) (Table 5).

Table 5. Regression coefficients for the relationships between concentrations Δ-9 desaturase, SREBP-1c and PPAR-α gene expression

Genes	β_0	β_1	R^2
Δ-9 desaturase vs SREBP-1c	0.67***	0.45***	0.55
Δ-9 desaturase vs PPAR-α	1.06***	-0.05	0.01
SREBP-1c vs PPAR-α	0.88***	0.12	0.02

β_0, β_1 are the regression coefficients for the intercept and linear components respectively.
The probability of a coefficient not being statistically significantly different from zero is denoted as follows: *P < 0.05, ** P < 0.01 and *** P < 0.005.

Following stepwise multiple regression analysis, SREBP-1c gene expression and EPA tissue concentrations explained most of the variation observed in Δ-9 desaturase gene expression (R^2 =0.65) and thus there was no advantage in including further variables in the regression model. There was no relationship between the calculated desaturase index and either Δ-9 desaturase or SREBP-1c gene expression (P>0.05).

DISCUSSION

To the authors knowledge this is the first study to examine the effect of level and duration of n-3 PUFA supplementation on Δ-9 desaturase mRNA expression in the muscle of beef cattle. This study demonstrates for the first time, that dietary n-3 PUFA inhibits expression of the gene which codes for the critical enzyme required to desaturate VA to CLA in bovine muscle tissue. Furthermore, there is evidence from the current study that the degree of inhibition of transcription for this gene is related to the level of dietary n-3 PUFA intake. While the minimum time required to evoke a significant reduction in Δ-9 desaturase mRNA expression was not established in the current study, 50 days was found to be sufficient and extending the supplementation period beyond this did not further depress Δ-9 desaturase expression. This study also investigated the effect of n-3 PUFA supplementation on the expression of mRNA for putative regulators of Δ-9 desaturase, SREBP-1c and PPAR-α. This is the first published study to measure gene expression of SREBP-1c in the bovine and we found transcription of the gene coding for this transcription factor to be significantly decreased in muscle tissue of cattle fed a diet high in n-3 PUFA compared to the control animals. However, supplementation with n-3 PUFA did not alter the gene expression of the other putative regulator of Δ-9 desaturase expression investigated, PPAR-α.

The expression of Δ-9 desaturase is known to be strongly modulated by several nutrients (PUFAs and fructose), drugs (sterculic acid), hormones such as insulin and leptin (Ntambi and Miyazaki, 2004), and cholesterol (Kim et al., 2002). Results of the current study showed that while there was evidence of a negative relationship between VA, the substrate for *de novo* tissue synthesis of cis9 trans11 CLA, and SREBP-1c

gene expression, there was no relationship between VA and Δ-9 desaturase gene expression and hence no evidence of substrate inhibition. This is contrary to the report of Lin et al. (2004), who found that Δ-9 desaturase mRNA abundance was decreased due to the accumulation of VA in mammary gland tissue of lactating mice. While a negative relationship was displayed between cis9, trans11 CLA and SREBP-1c gene expression, there was no evidence from the current study of a CLA mediated inhibition of Δ-9 desaturase gene expression which is in contrast to that postulated by other authors (Keating et al., 2005; Lin et al., 2004). However, the lack of a relationship between CLA and Δ-9 desaturase gene expression in the current study may also have been the result of a lack of sufficient variation in tissue CLA concentrations between treatments.

In the current study there was also no relationship between calculated Δ-9 desaturase activity index (Kelsey et al., 2003) and Δ-9 desaturase gene expression. These results concur with the findings of Archibeque et al. (2005) who also reported that a calculated Δ-9 desaturase index did not reflect actual Δ-9 desaturase enzyme activity in adipose tissue of beef steers.

Daniel et al. (2004) reported that increased oleic acid content of sheep tissue in response to concentrate rich diets is associated with an increase in Δ-9 desaturase gene expression. There was a positive, but non statistically significant, relationship between oleic acid concentrations and Δ-9 desaturase gene expression in the current study. This is contrary to another report in the literature by Keating et al. (2006) who found that the bovine Δ-9 desaturase gene promoter was down-regulated by oleic acid.

The current study established a positive relationship between the n-6 PUFA, linoleic acid, total n-6 PUFA and n-6:n-3 PUFA; and both Δ-9 desaturase and SREBP-1c gene expression. Contrary to this report, the *in vitro* work of Sessler et al. (1996) showed that mRNA expression for Δ-9 desaturase was decreased by addition of the n-6 PUFA, linoleic acid and arachidonic acid, to a murine cell line, in a dose-dependent manner. Furthermore in that study it was found that the half life of Δ-9 desaturase mRNA could be shortened by arachidonic acid which led the authors to suggest that the repressed gene expression was due to a reduction in the stability of its mRNA (Sessler et al., 1996).

There is evidence in the current study of a negative relationship between the parent n-3 PUFA, linolenic acid, and Δ-9 desaturase gene expression. Furthermore, a significant negative relationship was observed between linolenic acid concentration and SREBP-1c gene expression. Similarly, the results of a study with sheep showed a reduction in Δ-9 desaturase gene expression in adipose and liver tissues of lambs fed forage compared with a concentrate based diet (Daniel et al., 2004). The authors attributed this repression in mRNA levels to the higher concentrations of linolenic acid in the forage compared with the concentrate diet. Furthermore, there is some evidence from the work of McGettrick and co-workers (2007), that grazing cattle supplemented with fish oil had lower relative quantities of Δ-9 desaturase mRNA in muscle and adipose tissue. In agreement, it has been demonstrated, using a mouse adipocyte cell line, that linolenic acid inhibited Δ-9 desaturase gene expression (Sessler et al., 1996). Given that α-linolenic acid is the predominant PUFA in grass (Dewhurst et al., 2003), these results may have implications for strategies to further augment of concentrations of CLA in the tissue of cattle reared at pasture.

In the current study, of the variables measured, multiple regression analysis displayed that EPA and SREBP-1c gene expression accounted for most of the variation in expression of the Δ-9 desaturase gene. Indeed, we found a negative relationship between Δ-9 desaturase gene expression and the n-3 PUFA, EPA (C20:5n-3), DHA (C22:6n-3), and linolenic acid (C18:3n-3). These findings are in agreement with those of a previous report showing that feeding a mixture of the n-3 PUFA, EPA, DHA and linolenic acid resulted in a 50% suppression of Δ-9 desaturase mRNA in rat liver (Bellinger et al., 2004). Similarly, Renaville et al. (2006) showed that a reduction in the conversion rate of VA to CLA in a human intestinal cell line following addition of EPA, was attributed to a negative effect of this PUFA on mRNA expression of Δ-9 desaturase.

Nutrients, in particular fatty acids, have been shown to regulate Δ-9 desaturase both at a transcriptional (Renaville et al., 2006) and enzyme activity (Sessler et al., 1996) level. In human cell lines the transcription of Δ-9 desaturase has been shown to be under the control of two transcription factors, SREBP-1c and PPARα (Renaville et al., 2006). The current study also examined the gene expression of these two transcription factors following n-3 PUFA dietary supplementation. Similar to Δ-9 desaturase gene expression, SREBP-1c mRNA abundance was significantly decreased, while PPAR-α mRNA levels remained unchanged.

SREBP-1c is a key regulator of Δ-9 desaturase which mediates its transcriptional activation (Nakamura and Nara, 2002). The present study found that a negative relationship existed between SREBP-1c gene expression and both DHA and cis9, trans11 CLA while the relationship between the expression of this gene and EPA

concentrations, although negative in direction, did not reach statistical significance. Similar to Δ-9 desaturase gene expression, a positive relationship between linoleic acid, total n-6 PUFA and n-6:n-3 PUFA; and SREBP-1c gene expression was displayed. Importantly, there was a highly significant positive relationship displayed between the gene expression of Δ-9 desaturase and its putative transcription factor SREBP-1c indicating that these two genes are co-regulated. As SREBP-1c appears to regulate Δ-9 desaturase gene transcription, it can therefore be suggested that in the bovine, the effect of n-3 PUFA on Δ-9 desaturase mRNA levels is mediated by reduced SREBP-1c gene expression. Furthermore, there is evidence from the current study that the balance between n-6 and n-3 PUFA tissue concentrations is important in the regulation of Δ-9 desaturase, acting through SREBP-1c.

This is the first report of a reduction of SREBP-1c gene expression following n-3 PUFA supplementation in muscle tissue in the bovine species and is consistent with other reports (Renaville et al., 2006) who found that reduced SREBP-1c mRNA levels were associated with decreased gene expression of Δ-9 desaturase in human intestinal cell cultures and proposed that lower levels of SREBP-1c would decrease Δ-9 desaturase gene transcription and expression, which would in turn decrease Δ-9 desaturase activity. In rat hepatic tissue, dietary PUFAs were shown to exert their effects by reducing both mRNA and proteolytic activation of SREBP-1c (Xu et al., 2001; 2002). To support this theory, dietary PUFA have been shown to be associated with a decrease in the formation of mature cleaved SREBP-1c protein in the liver tissue of mice (Yahagi et al., 1999). While Δ-9 desaturase gene expression may also be reduced by leptin, this mechanism has been shown to be independent of SREBP-1c (Biddinger et al., 2006).

PPAR-α has been shown to induce Δ-9 desaturase expression in rats and in pigs (Forman et al., 1997; Cheon et al., 2005). PPARs can directly induce Δ-9 desaturase in cell culture through the peroxisome proliferator response element (PPRE) present in the promoter region (Miller and Ntambi, 1996). As a regulatory relationship has been reported between Δ-9 desaturase and PPAR-α (Nakamura and Nara, 2002), the present study investigated whether reduced expression of Δ-9 desaturase could be associated with alterations in PPAR-α gene expression. However, supplementation of cattle diets with fishoil had no effect on PPAR-α mRNA levels. Furthermore, unlike Δ-9 desaturase and PPAR-α, there was no significant relationship between PPAR-α gene expression and any of the fatty acids measured. In addition, the current study found that no relationship existed between gene expression of Δ-9 desaturase and PPAR-α. Other investigators using human intestinal cell lines also reported that while treatment with EPA did reduce Δ-9 desaturase and SREBP-1c gene expression, PPAR-α mRNA levels remained unaltered. Moreover, a study in mice reported that a reduction in Δ-9 desaturase enzyme activity may be partly dependent on SREBP-1c but most likely independent of PPAR-α (Sampath and Ntambi, 2006). PPAR-α may have an indirect role to play in the regulation of Δ-9 desaturase gene expression (Nakamura and Nara, 2002).

Increased human consumption of CLA and n-3 PUFA is strongly recommended by nutritionists (McRae et al., 2004). CLA in human tissues may be synthesized through the tissue desaturation of VA by Δ-9 desaturase (Turpeinen et al., 2002) and thus increased VA is the human diet. Data presented in the current study has important implications for those ingesting n-3 PUFA as it may have negative effects on *de novo*

synthesis of CLA in human muscle through potential reductions in Δ-9 desaturase gene expression. However, as a positive relationship between n-6 PUFA and particularly the ratio of n-6 to n-3 PUFA concentration, with Δ-9 desaturase gene expression was observed, the correct balance of n-6 to n-3 PUFA concentrations ingested in the diet appears to be of critical importance to achieve optimal Δ-9 desaturase gene expression levels and in turn CLA production in muscle tissue.

Beef which is naturally enriched with CLA and n-3 PUFA could be a good regular source of these important fatty acids and an alternative to expensive nutritional supplements. As such the research presented herein is important in the context of any potential human health benefits of food sources of these fatty acids. It also illustrates the potential interactions between nutrients and gene transcription at tissue level. This has important implications for the development of dietary strategies to augment the concentration of both CLA and n-3 PUFA in ruminant meat. Hence, further work is required to elucidate the molecular and biochemical mechanisms controlling the synthesis and deposition of n-3 PUFA and CLA in muscle to minimize the negative effects of n-3 PUFA supplementation on the Δ-9 desaturase enzyme. This will ultimately provide improved strategies to consistently produce nutritionally enhanced beef.

LITERATURE CITED

AbuGhazaleh, A. A., Schingoethe, D. J., Hippen, A. R., Kalscheur, K. F. and Whitlock, L. A. 2002. Fatty acid profiles of milk and rumen digesta from cows fed fish oil, extruded soybeans or their blend. J. Dairy Sci 85:2266-76.

Archibeque, S. L., Lunt, D. K., Gilbert, C. D., Tume, R. K and Smith, S. B. 2005. Fatty acid indices of stearoyl-CoA desaturase do not reflect actual stearoyl-CoA desaturase enzyme activities in adipose tissues of beef steers finished with corn-, flaxseed-, or sorghum-based diets. J. Anim. Sci. 83:1153-66.

Bahar, B. 2006. Development of isotopic and molecular techniques for the authentication of dietary background information in bovine tissues. Ph.D. Thesis, National University of Ireland, UCD, Dublin.

Bellinger, L, Lilley, C. and Langley-Evans, S. 2004. Prenatal exposure to a maternal low- protein diet programmes a preference for high-fat foods in the young adult rat. Br J Nutr 92:513-20.

Biddinger, S. B., Miyazaki, M., Boucher, J., Ntambi, J. M., and Kahn, C. R. 2006. Leptin suppresses stearoyl-CoA desaturase 1 by mechanisms independent of insulin and sterol regulatory element-binding protein-1c. Diabetes. 55:2032-41.

Brunswig-Spickenheier, B. and Mukhopadhyay, A. K. 2003 Expression of osteopontin (OPN) mRNA in bovine ovarian follicles and corpora lutea. Reprod. Domest. Anim. 38:175-81.

Cheon, Y., Nara, T. Y., Band, M. R., Beever, J. E., Wallig, M. A., Nakamura, M. T. 2005. Induction of overlapping genes by fasting and a peroxisome proliferator in pigs: evidence of functional PPARalpha in nonproliferating species. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288:1525-35.

Daniel, Z. C. T. R., Wynn, R. J. , Salter, A. M. and Buttery, P. J. 2004. Differing effects of forage and concentrate diets on the oleic acid and conjugated linoleic acid content of sheep tissues: the role of steroyl-CoA desaturase. J. Anim. Sci. 82:747-758.

De Ketelaere, A., Goossens, K., Peelman, L. and Burvenich, C. 2006. Technical note: validation of internal control genes for gene expression analysis in bovine polymorphonuclear leukocytes. J. Dairy Sci. 89:4066-9.

Dewhurst, R. J., Scollan, N. D., Lee, M. R. F., Ougham, H. J. and Humphreys, M. O. 2003. Forage breeding and management to increase the beneficial fatty acid content of ruminant products. Proceedings of the Nutrition Society. 62:329-336.

Forman, B. M., Chen, J., Evans, R. M. 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A. 94:4312-7.

Ip, C., Banni, S., Angioni, E., Carta, G., McGinley, J., Thompson, D., Barbano, D. and Bauman, D. 1999. Conjugated linoleic acid-enriched butter alters mammary gland morphogenesis and reduces cancer risk in rats. J. Nutr. 129:2135-2142.
Keating, A. F., Kennelly, J. J. and Zhao, F. Q. 2006. Characterisation and regulationof the bovine steroyl-CoA desaturase gene promoter. Biochem. Biophys. Res. Comm. 344: 233-240.

Keating, A. F., Stanton, C. J., Murphy, J., Smith, T. J., Ross, R. P. and Cairns, M. T. 2005. Isolation and characterisation of the bovine steroyl-CoA desaturase promoter and analysis of polymorphisms in the promoter region in dairy cows. Mamm. Genome. 16:184-193.

Kelsey, J. A., Corl, B. A., Collier, R. J. and Bauman, D. E. 2003. The effect of breed, parity, and stage of lactation on conjugated linoleic acid (CLA) in milk fat from dairy cows. J. Dairy Sci. 86:2588-98.

Kenny, D. A., Kelly, J. P., Monahan, F. J. and Moloney, A. P. 2007. Effect of dietary fish and soya oil on muscle fatty acid concentrations and oxidative lipid stability in beef cattle. J. Anim. Sci. Vol. 85: 911. Abstract.

Kim, H. J., Miyazaki, M., Ntambi, J. M. 2002. Dietary cholesterol opposes PUFA-mediated repression tof the stearoyl-CoA desaturase-1 gene by SREBP-1 independent mechanism. J. Lipid Res. 43:1750-1757.

Lin, X., Loor, J. J. and Herbein, J. H. 2004. Trans10, cis12-18:2 is a more potent inhibitor of *de novo* fatty acids synthesis and desaturation than cis9, trans11-18:2 in the mammary gland of lactating mice. J. Nutr. 134:1362-8.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2(-delta delta CT)) method. Methods 25:402-8.

Martin, G. S., Lunt, D. K., Britain, K. G. and Smith, S.B. 1999. Postnatal development of stearoyl coenzyme A desaturase gene expression and adiposity in bovine subcutaneous adipose tissue. J Anim Sci 77:630-636.

McGettrick, S. A., Maloney, A. P., Monahan, F. J., Sweeney, T. and Mulligan, F. J. 2007. Delta 9 desaturase gene expression in muscle, adipose tissue and liver of beef heifers following supplementation of grass with a concentrate containing sunflower seed and fish oil. J. Anim. Sci. 85:369.Abstract.

MacRae, J O'Reilly, L. and Morgan, P. Desirable characteristics of animal products from a human health perspective. Livestock Production Science. 94: 95-103.

Miller, C. W. and Ntambi, J. M. 1996. Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression. Proc. Natl. Acad. Sci. U S A. 93:9443-8.
Nakamura, M. T. and Nara, T. Y. 2002. Gene regulation of mammalian desaturases. Biochem Soc Trans 30:1076-9.

Neuvians, T. P., Pfaffl, M. W., Berisha, B. and Schams, D. 2003. The mRNA expression of insulin receptor isoforms (IR-A and IR-B) and IGFR-2 in the bovine corpus luteum during the estrous cycle, pregnancy, and induced luteolysis Endocrine 22:93-100.
Ntambi, J. M. and Miyazaki, M. 2004. Regulation of stearoyl-CoA desaturases and role in metabolism. Prog Lipid Res 43:91-104.

O'Gorman, G. M., Park, S. D., Hill, E. W., Meade, K.G., Mitchell, L. C., Agaba, M., Gibson, J. P., Hanotte, O., Naessens, J., Kemp, S. J. and MacHugh, D. E. 2006. Cytokine mRNA profiling of peripheral blood mononuclear cells (PBMC) from trypanotolerant and trypanosusceptible cattle infected with Trypanosusceptible cattle infected with Trypanosoma congolense. Physiol Genomics 28: 53-61.

Parodi, P. 2003. Conjugated linoleic acid in food. In J. Sebedio, W. W. Christie and R. Adolf (ed) Advances in Conjugated Linoelic Acid Research, vol 2, pp: 101-121. AOCS Press, Champaign, IL.

Renaville, B., Mullen, A., Moloney, F., Larondelle, Y., Schneider, Y. J., and Roche, H. 2006. Eicosapentaenoic acid and 3,10 dithia stearic acid inhibit the desaturation of trans-vaccenic acid into cis-9, trans-11-conjugated linoleic acid through different pathways in Caco-2 and T84 cells. Br. J. Nutr. 95:688-95.

Sampath, H. A. and Ntambi, J. M. 2006. Stearoyl-coenzyme A desaturase 1, sterol regulatory element binding protein-1c and peroxisome proliferator-activated receptor-[alpha]: independent and interactive roles in the regulation of lipid metabolism. Curr Opin Clin Nut Met Care 9:84-88.

Sessler, A. M., Kaur, N., Palta, J. P. and Ntambi, J. M. 1996. Regulation of stearoyl-CoA desaturase 1 mRNA stability by polyunsaturated fatty acids in 3T3-L1 adipocytes. J. Biol. Chem. 22:29854-8.

Tabor, D. E., Kim, J. B., Spiegelman, B. M. and Edwards, P.A .1999. Identification of conserved cis-elements and transcription factors required for sterol-regulated transcription of stearoyl-CoA desaturase 1 and 2. J Biol Chem 274:20603-10.

Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, Griinari JM. 2002. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr. 2002 Sep;76(3):504-10.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N.,, De Paepe, A. and Speleman, F. 2002. Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol RESEARCH 0034 Epub.

Wahle, K. W. J., Heys, S. D. and Rorondo, D. 2004. Conjugated linoleic acids: are they beneficial or detrimental to health? Prog Lipid Res 43:553-387.

Xu, J., Cho, H., O'Malley, S., Park, J. H. and Clarke, S. D.2002. Dietary Polyunsaturated fats regulate rat liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different mechanisms. J Nutr. 132:3333-9

Xu, J., Teran-Garcia, M., Park, J. H., Nakamura, M. T. and Clarke, S. D.2001. Polyunsaturated fatty acids suppress hepatic sterol regulatory element-binding protein-1 expression by accelerating transcript decay. J. Biol. Chem. 276:9800-7.

Yahagi, N., Shimano, H., Hasty, A.H., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., Yamada, N. 1999. A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. J Biol Chem. 274:35840-4.

Study 2. Effect of level of eicosapentaenoic acid on the transcriptional regulation of Δ-9 desaturase using a novel *in vitro* bovine intramuscular adipocyte cell culture model

INTRODUCTION

The chemical composition of marbling fat is important from consumer acceptance and human health perspectives. Fatty acid synthesis in ruminant adipose tissue has been studied for decades and the composition, particularly the nutraceutical component of ruminant fat, may be enhanced through dietary manipulation (Scollan et al, 2006). The strategic supplementation of cattle diets can generate beef with increased concentrations of conjugated linoleic acid (CLA) and n-3 polyunsaturated fatty acid (PUFA) concentrations, a lower quantity of fat and a fatty acid profile more compatible with current human dietary recommendations (Moloney *et al*., 2001). More recently, however, it has been shown that the simultaneous accretion of tissue concentrations of n-3 PUFA and conjugated linoleic acid (CLA) appears to be hampered by apparent negative effects of n-3 PUFA on tissue CLA synthesis (Waters *et al.,* 2008). Our group have shown that increases in dietary n-3 PUFA supplementation decreased mRNA levels for Δ-9 desaturase in muscle (Waters *et al*., 2008). Furthermore, tissue concentration of the n-3 PUFA, eicosapentaenoic acid (EPA) was found to exhibit a significant negative relationship with Δ-9 desaturase gene expression. There is also evidence that the transcription factor; sterol regulatory element binding protein (SREBP)-1c controls activation and expression of Δ-9 desaturase in humans (Renaville *et al*., 2006). To date there is no information on the effect EPA specifically on the transcriptional regulation of Δ-9 desaturase in the

bovine. This requires further investigation and understanding of the molecular mechanisms involved, particularly at a cellular level.

Adipogenesis is the process of adipocyte cell development whereby differentiation results in its transformation from a fibroblast-like cell to a lipid-filled cell with the expression of transcription factors and molecular markers for the mature fat cell phenotype and capable of metabolizing lipid (Fernyhough *et al.*, 2005). There appears to be differences in adipocyte development and its regulation in subcutaneous and intramuscular depots (Hausman *et al.,* 2008). The development and maintenance of primary adipocytes in culture and conducting reproducible experiments has been difficult (Viravaidya and Shuler, 2002). It is acknowledged that there is limited information available on optimal culture conditions for bovine adipocytes particularly from different adipose depots and for their differentiation (Grant *et al*., 2008).

In non-ruminants, several preadipocyte cell lines such as 3T3-L1 and Ob 17 have proven to be useful models for the study of adipocyte differentiation (Forrest *et al*., 1983), growth and development (Fernyhough *et al*. (2005), function (Morganstein *et al*., 2008) and diseases characterized by increased fat depots (Natal *et al*., 2008). Importantly, *in vitro* differentiation of adipocytes has been shown to possess many characteristics of adipose cells *in vivo* (Grégoire *et al*., 1998). In ruminants, most studies investigating fatty acid synthesis or accretion *in vitro* have employed tissue explants, slices or fat cells in short-term primary culture (Faulconnier *et al*., 1994; Vernon, 1988). While no mature intramuscular adipocyte cell line has been developed for the bovine species, Aso *et al*., (1995) established the first clonal bovine intramuscular preadipocyte (BIP) line from white adipose tissue of the *Musculus*

longissimus thoracis of Japanese black cattle. Unfortunately the preadipocyte cell line has since been lost. There is no report of the existence of a mature validated intramuscular adipocyte cell line for the bovine species. Therefore, the objective of the current study was to develop and validate a mature bovine intramuscular adipocyte cell line and to employ this resource to examine the effect of level of EPA supplementation on the mRNA expression of Δ-9 desaturase and SREBP-1c genes. An *in vitro* approach was applied to avoid possible confounding effects of inter animals variance in feed intake and adiposity.

MATERIALS AND METHODS

Adipocyte cell isolation

Intramuscular adipose tissue was obtained from the *M. longissimus* thoracis of an Angus X Charlais beef heifer in a licensed abattoir (Jennings Meats, Ballinrobe, Co. Mayo, Ireland) within 20 min of slaughter. The heifer was fed a diet of grass silage supplemented with 3 kg of a cereal based concentrate six weeks prior to slaughter. Adipose tissue was stored in sterile phosphate buffered saline containing Penicillin (5000 IU/ml) (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) and Streptomycin (5mg/ml) (PBS-PS) (Sigma-Aldrich Ireland Ltd) on ice during the 45 min transport to the laboratory. Visible connective and muscle tissue were carefully dissected away from the intramuscular adipose tissue. Adipocytes were isolated using an adaptation of the methods described by Fernyhough *et al.* (2005). Briefly, adipose tissue was washed four times in sterile PBS-PS and was cut into approximately 1 cm^3 pieces. Tissue was subsequently treated with 25 mL of PBS containing glucose (1.5 %) (Sigma-Aldrich Ireland Ltd), bovine serum albumin (0.1 %) (Invitrogen, Carlsbad, California, USA) and collagenase (1.5 %) (Sigma-Aldrich Ireland Ltd) for 1 hour at

37^{o}C with agitation. Following enzymatic digestion, the collagenase was removed by centrifugation for 5 min at 1500 rpm, the tissue was then washed (3 x) in sterile PBS. The upper adipose layer was removed and transferred to 25 cm^3 culture flasks (Sarstedt Ltd., Drinagh, Wexford, Ireland).

Culture of adipocytes

Mature adipocytes were cultured using an inverted ceiling culture method to facilitate adherence of cells to the bottom of culture flasks (Fernyhough *et al*., 2005). The upper adipose layer was transferred to culture flasks (25 cm^3) and filled with 24.5 mL Dulbecco's Modified Eagle's Medium/Nutrient F-12 (DMEM/F12) culture medium (Sigma-Aldrich Ireland Ltd) supplemented with 25 mM $NaHCO_3$ (VWR International Ltd, Lutterworth, Leicestershire, United Kingdom), 10 % fetal calf serum (FCS; Invitrogen, Carlsbad, California, USA), 0.03 % L-glutamine (Sigma-Aldrich Ireland Ltd), 0.009 % Penicillin G (Sigma-Aldrich Ireland Ltd), 0.0145 % Streptomycin Sulphate (Sigma-Aldrich Ireland Ltd), 1 % fungizone (Invitrogen, Carlsbad, California, USA) and inverted in an CB210 CO_2 incubator (Binder GmbH, Tuttlingen, Germany) set at 37 ˚C and 5 % CO_2 with the lid loosened to allow passage of CO_2 into the culture. Following 5 days, unattached cells were removed and the flask was placed in an upright position. Fresh culture medium, which was equilibrated to 37 °C to avoid heat shocking adipocytes, was added to flasks every two to three days and attachment to the bottom of the flask was monitored daily by using a Nikon oxivert inverted light microscope.

Trypsinisation and passaging of adipocytes

Cells were routinely passaged at 70 % confluency. Culture media was removed and cells were incubated in 2 ml 0.05 % trypsin-EDTA (Invitrogen, Carlsbad, California, USA) solution for 5 min at 37° C to enzymatically detach cells from the wall of the flask. Efficiency of cell detachment was monitored under a microscope as prolonged incubation in trypsin can damage cells. Once detachment was complete, 2 ml of FCS was added to the flask to neutralise the trypsin. The cell suspension was then removed and centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the pellet re-suspended in culture medium. Cells were cultured for 30 passages. Adipocytes from all passages were stored in liquid nitrogen.

Storage and thawing of adipocytes

Cells were trypsinised, centrifuged for 5 min at 1500 rpm and excess medium was aspirated and discarded. Cells from one flask (25 cm^3) were frozen down to a 1 ml volume for long term storage. Freezing medium (1mL; 10% DMSO, 90% FCS) (Sigma-Aldrich Ireland Ltd) was added to the cell pellet in a cryovial (Invitrogen, Carlsbad, California, USA). As freezing medium contains dimethyl sulfoxide (DMSO), which is a cryoprotectant and is toxic to cells at temperatures greater than 4 °C, this process was carried out as quickly as possible. Cryovials were stored overnight at -80 °C followed by transfer into liquid nitrogen (-180 °C).

To thaw cells, a cryovial was carefully removed from liquid nitrogen and placed in a beaker of pre-warmed water (37 °C). When thawed, (approximately 2 min), contents were transferred to a centrifugation tube (25 mL) (Sarstedt Ltd., Drinagh, Wexford, Ireland). Culture medium (9 mL), pre-warmed to 37 °C, was added to the centrifuge

tube and the suspension was centrifuged at 1500 rpm for 5 min. The supernatant was discarded, the pellet was re-suspended in 1 mL of culture medium and the sample was repeat pipetted to ensure homogeneity of the sample. 1 mL of the cell suspension was added into a culture flask and 9 mL of pre-warmed culture medium was added to the flask and cells were cultured until approximately 70 % confluency.

Evaluation of lipid accumulation of adipocytes

Both Sudan IV and Oil red O were initially assessed as staining methods to establish accumulation of intracellular lipid in adipocytes. Sudan IV penetrated cells to a greater level and was therefore used throughout this study to determine the ability of adipocytes to accumulate lipid at passage 10, 20 and 30. Briefly, culture medium was removed from the flask and cells were washed with PBS and then with 70 % ethanol. Cells were fixed on a glass slide by the addition of 10 % formalin and incubated for 10 min. Sudan IV stain (5 mL) was added to the slide and cells were incubated for 5 min. Cells were washed with PBS and basophilic structures such as nuclei were counterstained by the addition of 2 mL haemotoxylin for 2-3 min. Cells were finally washed with PBS and examined using a Nikon oxivert inverted light microscope.

Fatty acid analysis

Analysis of fatty acids was conducted in spent media and adipocytes at passage 30 in the development of the cell line using gas chromatography. Fatty acid methyl esters were also measured in spent media and adipocytes following treatment of cells with EPA. Cells were trypsinised, centrifuged for 5 min at 1500 rpm and excess medium was aspirated and analysed separately. Total lipids were extracted from a pellet of adipocytes as well as 1 mL of spent media using chloroform methanol (2:1 v/v) as

described by Folch *et al.* (1957). Methylation was carried out for both adipocytes and spent media using the method described by Park and Goins (1994). The fatty acid methyl esters (FAME) were separated on a CP Sil 88 column (100 m x 0.25 mm i.d., 0.20 μm film thickness; Chrompack, Middleburg, The Netherlands) using a gas liquid chromatograph (3400; Varian, Harbor City, CA, USA) fitted with a flame ionization detector. Helium (37 psi) was used as the carrier gas. The injector temperature was held isothermally at 225 ºC for 10 min and the detector temperature was 250 ºC. The column oven was held at an initial temperature of 140 ºC for 8 min and then programmed to increase at a rate of 8.5 ºC/min to a final temperature of 200 ºC, which was held for 41 min. Data were recorded and analysed on a Minichrom PC system (VG Data System, Manchester, UK). Prior to fatty acid analysis, a hemocytometer was used to determine cell density in flasks. A 10μl sample was introduced into one of the V-shaped wells of the hemocytometer and using a microscope, adipocytes were counted. This data was then used to normalise fatty acid concentrations determined in adipocytes.

RNA isolation

To validate the transcriptional activity of the adipocytes, the expression of markers of fat metabolism was identified by reverse transcription-PCR. Adipocytes were typsinised and cells collected by microcentrifugation at 12,000 rpm for 5 min at 4 ˚C in sterile 1.5 mL eppendorfs. The supernatant was removed and the pellet was immersed and stored in RNAlater (Ambion, Applera Ireland, Dublin) at -20 ºC. Total RNA was isolated from adipocytes using TRIzol reagent and chloroform and subsequently precipitated using isopropanol. RNA quantity was determined by absorbance at 260 nm using the Nanodrop spectrophometer (Labtech International

Ltd., East Sussex, UK). RNA quality was assessed using 28S/18S ratio and RNA integrity number (RIN) on the Agilent Bioanalyzer 2100 with the RNA 6000 nano labchip kit (Agilent Technologies Ireland, Dublin, Ireland).

Reverse transcription PCR and sequence analysis

Reverse transcription-PCR was performed to determine the expression of markers of fat metabolism in the cell line (Table 1). RNA was DNase-treated with RQ1 RNase-free DNase (Promega UK Ltd., Southhampton, UK) and purified using the RNeasy mini kit (Qiagen Ltd, Crawley, UK). 1 μg of RNA was DNase-treated and reverse transcribed to generate cDNA, using the Reverse Transcription system according to manufacturers instructions (Promega UK Ltd., Southampton, UK). cDNA was then quantified using the Nanodrop spectophotometer (Labtech International Ltd., East Sussex, UK), diluted to 50 ng L^{-1} working stocks and stored at -20 ˚C.

Details of primer sets used in this study are listed in Table 6. Primers for RT-PCR were commercially synthesized (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Primers were designed to amplify specific fragments (approximately 200 bp) of the following genes which are adipocyte markers including peroxisome proliferator activated receptor (PPAR)-γ, fatty acid synthase (FAS), fatty acid binding protein (FABP)-4, adipocyte long chain fatty acid binding protein (aP2), CD36, Δ-9 desaturase, the transcription factor sterol regulatory element binding protein (SREBP)-1c, leptin and microsomal triglyceride transfer protein (MTP) (Table 6).

Table 6. Bovine specific oligonucleotide primers used in this study.

Gene Name	Primer sequences	Accession Number	Amplicon size
FAS	F: 5´CTGAGTCGGAGAACCTGGAG3´	AF285607	232 bp
	R: 5´ACAATGGCCTCGTAGGTGAC3´		
CD36	F: 5´TGGCAACCACTTTCATCAGA3´	BC103112	161 bp
	R: 5´CTGGCATTAGAATCCCTCCA3´		
AP2	F: 5´GGCCAAGCTCAAGAAGAAGA3´	NM_001075702	236 bp
	R: 5´AGGGCGAGTCTGAGAAGACA3´		
FABP4	F: 5´CATCTTGCTGAAAGCTGCAC3´	NM_174314.2	202 bp
	R: 5´ACCCCCATTCAAACTGATGA3´		
MTP	F: 5´TGCAGCCATCATTAAAGCAG 3´	NM_001101834	108 bp
	R: 5´TGCCAGTGCTCTGAGAGAGA3´		
Δ-9 desaturase	F: 5**'**CGACGTGGCTTTTTCTTCTC3´	BC112700	190bp
	R: 5´GATACCATGGCACGAGTGTG 3´		
SREBP-1C	F:5´ACCGCTCTTCCATCAATGAC3´	XM_879234	190 bp
	R:5´TTCAGCGATTTGCTTTTGTG3´		
PPAR γ	F: 5'-GTGAAGCCCATTGAGGACAT-3'	NM_181024	149 bp
	R: 5'-AGCTGCACGTGTTCTGTCAC-3'		
Leptin	F: 5'-CTGTTCCAGCTCCCTCTCAC-3'	NM_173928.2	105 bp
	R: 5'-CATGATGCTCCCTGGATTCT-3'		
HNF4α	F: 5'-GCTCCATGGTGTTCAAGGAT-3'	NM_001015557	119 bp
	R: 5'-CAGCACCAACTCATCAAGGA-3'		

aP2; adipocyte lipid binding protein, FABP-4; fatty acid binding protein-4, FAS; fatty acid synthase, MTP; microsomal triglyceride transfer protein, PPAR-γ; peroxisome proliferator activated receptor-gamma, SREBP; sterol regulatory element binding protein.

The liver specific hepatocyte nuclear factor-4 (HNF4) was applied as the negative control (Sladek, 1993). Cycling parameters applied in PCR were as follows: 95˚C for 2 min and 30 cycles of 95˚C for 30 s, 60˚C for 30 s, 72˚C for 30 sec (Eppendorf Master Cycler, Unitech, Dublin, Ireland), followed by 72˚C for 5 min and PCR products were stored at 4˚C. Analysis of amplified PCR products (10 μL) was carried out on 1% agarose gels using 1X Tris acetate EDTA (TAE) running buffer, pH 8.3 in a model EC-360-M Maxicell gel system (EC apparatus Corporation, St. Petersburg, USA). Agarose gels were stained using 5μg/ml of ethidium bromide. DNA was

observed using a UV Transilluminator (254 nm) and the image was photographed using a Bio ID Gel Documentation system (Vilber Lourmat, Cedex, France). All amplified PCR products generated were purified using the PCR purification kit (Roche, Basel, Switzerland) and sequenced (Macrogen, Nucleics Pty Ltd, Bendigo, Australia) and analysed using the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI) web page, to verify their identity.

Eicosapentaenoic acid preparation and incubation of adipocytes

The novel adipocyte cell line was subsequently used to determine the effects of n-3 fatty acid, EPA on the transcriptional regulation of Δ-9 desaturase in an *in vitro* bovine intramuscular adipocyte cell culture model. EPA, stock solution (50 mg/500µl; Cayman Chemical, Ann Arbor, MI) was diluted to 1 mM in pre-heated sterile DMEM/F12 medium containing 33 mg mL^{-1} fatty acid-free BSA. The remaining ethanol was evaporated under a gentle stream of nitrogen and the EPA was stored at -20 ºC. The solution was incubated for 2 h at 37 ºC to ensure binding of EPA to BSA. This 1 mM stock solution was then further diluted to 100 µM and 50 µM in their respective culture media. Ethanol was used as a vehicle to incorporate EPA into the culture medium and was added at the same concentration to the control (0 µM EPA).

Mature adipocytes (passage 30) were removed from storage and grown for 21 days to maximum confluency, n = 60 flasks (75 mL). Prior to treatment, cells were serum starved for a 24 h period and were subsequently incubated in a serum-free medium containing 1 % insulin, selenium and transferrin as substitutes for fetal calf serum. Following 24 h, the medium was replaced with serum-free medium containing one of three concentrations of EPA *viz* 0 µM (control), 50µM or 100µM, n = 16 (8 for fatty

acid analysis and 8 for gene expression analysis) for each treatment based on EPA concentrations recorded in muscle tissue. Following the 24 h incubation, cells and media were harvested. Cells were collected using trypsin-EDTA and spun at 1500 rpm for 5 min. Supernatant was collected and stored at -20 ºC for fatty acid analysis. One of the duplicate cell pellets were immersed and stored in 1 mL RNA later at -20 ºC while the other duplicate was snap frozen and stored at -80 ºC for fatty acid analysis. Analysis of the fatty acid methyl esters in the adipocytes was carried out using gas chromatography as described above.

Gene expression analysis

RNA was isolated, its quality analysed and cDNA synthesized as described above. Primers were designed to amplify specific fragments of Δ-9 desaturase and SREBP-1c Table 1). β-actin was used as a single standard reference gene for this experiment (Bahar, 2006). Primers were first tested using end point PCR to optimize amplification conditions. All amplified PCR products from this study were also sequenced to verify their identity.

Each real time PCR reaction was carried out in a total volume of 20 μL with 1 μL cDNA (50 ng/μL), 10 μL SYBR Green I master mix (Langanbach, Killarney Road, Bray, Co. Wicklow, Ireland), 1 μL forward and reverse primers (20 ng of each) and 8 μL nuclease-free H_2O. Dissociation curves were examined for the presence of a single PCR product. Real time RT PCR was performed using a Corbett Rotor-Gene™ 3000 quantitative PCR system (Corbett Life Sciences, Sydney, Australia) with the following cycling parameters: 95°C for 15 min followed by 40 cycles of 95°C for 30

s, 60°C for 30 s, 72°C for 30 s, followed by amplicon dissociation (95°C for 1 min, 50°C for 45 s, increasing 0.5°/cycle until 95°C was reached). Gene expression results were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

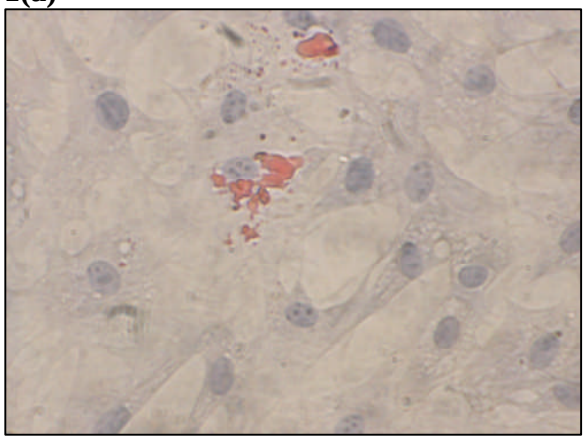
Data were analysed using the Statistical Analysis Systems software package version 9.1 (SAS Institute, Cary, NC, USA). Data were examined for adherence to normality using PROC UNIVARIATE (SAS, 2001). Data were then analysed to determine the effect of EPA treatment on fatty acids in spent media and adipocytes; and Δ-9 desaturase and SREBP-1c expression level using mixed models ANOVA (PROC MIXED, SAS, 2001). The Tukey critical difference test was used to determine statistical difference between treatment means. Regression analyses were carried out to establish relationships between supplemental EPA concentrations and relative gene expression of Δ-9 desaturase and SREBP-1c using PROC REG; SAS 2001.

RESULTS

Staining and microscopy

Staining and microscopy analysis showed that isolated cells accumulated lipid over time. Figures 4 (a-c) clearly demonstrated that fat droplets increased in number and size over time with large fat-filled vesicles observed at 30 passages. Cell nuclei were stained blue with haemotoxylin identifying basophilic structures such as ribosomes, nucleus, and the cytoplasmatic regions rich in RNA demonstrating the functionality of the cell line. Adipocyte cell doubling time was recorded as 2-3 days.

1(a)



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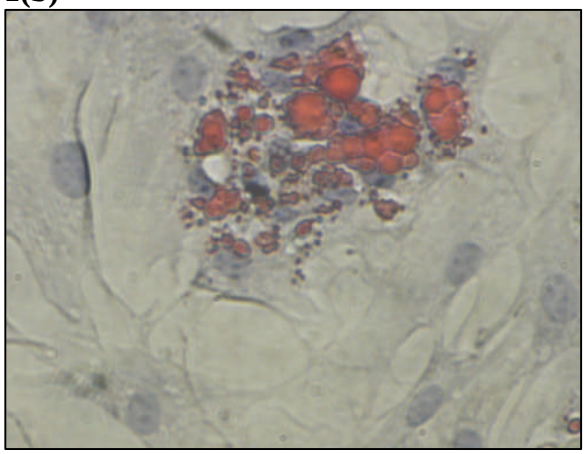

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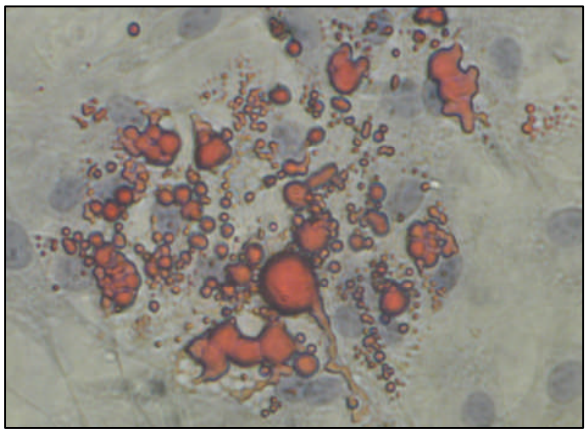


Figure 4. Analysis of lipid accumulation in primary intramuscular adipocyte cell line at passage (a) 10 (b) 20 and (c) 30 using Sudan staining and microscopy. The lipid droplets are stained red with Sudan IV and nuclei counterstained with haemotoxylin.

Fatty acid analysis

Fatty acid analysis of spent media and adipocytes is presented in Table 7. Thirteen fatty acids ranging from tetradecanoic acid (C14:0) to polyunsaturated fatty acid, docosohexanoic acid (C22:6) were easily detected and measured. Fatty acids including long chain n-3 PUFAs such as EPA and DHA were reliably separated, identified and quantified by gas chromatography.

Table 7. Concentrations of fatty acids detected in adipocytes and spent media

Fatty acid	Adipocytes	Spent media
C14:0	0.056 ± 0.0039	0.0024 ± 0.00029
***cis* C14:0**	0.023 ± 0.0031	0.027 ± 0.0026
C16:0	0.054 ± 0.0017	0.029 ± 0.0032
***trans*-9 C16:1**	0.035 ± 0.0031	0.0163 ± 0.23
***cis*-9 C16:1**	0.033 ± 0.0028	ND
C18:0	0.0743 ± 0.00185	0.0449 ± 0.001
***cis*-9 C18:1**	0.019 ± 0.0014	0.0078 ± 0.0006
***cis*-9, *cis*-12 C18:2**	0.028 ± 0.001	0.0011 ± 0.0005
***cis*-9,*cis*-12, *cis* -15 C18:3**	0.014 ± 0.002	0.008 ± 0.003
C20:4	0.11 ± 0.026	0.009 ± 0.0008
C20:5	0.125 ± 0.0026	0.013 ± 0.001
C22:5	0.037 ± 0.004	0.003 ± 0.0001
C22:6	0.024 ± 0.005	0.0004 ± 0.00001

Fatty acid results are presented as mg of fatty acid mL^{-1} of media and mg 10^{-7}cells. These are mean (± SE) for 8 replicates. ND; non-detectable.

Fatty acid methyl ester analysis in spent media and adipocytes following treatment with EPA is presented in Table 8.

Table 8 (a) Effect of dietary EPA supplementation on adipocyte fatty acid concentration (mg 10^{-7}cells).

Fatty acid	Full name	Control	50µM	100µM	SEM	Linear	Quadratic
C14:1	Myristoleic acid	0.03	0.065	0.091	0.011	0.053	0.01
C16:0	Palmitic acid	0.0658	0.1151	0.1672	0.03	0.052	0.003
C16:1	Palmitelaidic acid	0.041	0.130	0.066	0.013	0.201	0.001
C16:1	Palmitoleic aicd	0.0875	0.241	0.213	0.0051	0.251	0.497
C18:0	Stearic acid	0.091	0.16	0.23	0.0022	0.042	0.002
C18:1	Oleic aicd	0.023	0.041	0.057	0.009	0.193	0.054
C18:2	Linoleic acid	0.034	0.059	0.071	0.0011	0.143	0.093
C18:3	α-Linoleic acid	0.009	0.034	0.044	0.0046	0.004	0.003
C20:4	Arachidonic acid	0.130	0.23	0.27	0.062	0.287	0.240
C20:5	Eicosapentaenoic acid	0.13	0.6	0.84	0.005	0.000	0.006
C22:5	Docosapentanoic acid	0.044	0.12	0.15	0.014	0.004	0.003
C22:6	Docosahexanoic acid	0.022	0.074	0.096	0.0098	0.004	0.003

Table 8 (b) Effect of dietary EPA supplementation on media fatty acid concentration (mg mL^{-1}).

Fatty acid	Full name	Control	50µM	100µM	SEM	Linear	Quadratic
C14:1	Myristoleic acid	0.0027	0.0017	0.0020	0.00034	0.217	0.156
C16:0	Palmitic acid	0.029	0.033	0.027	0.0033	0.747	0.180
C16:1	Palmitelaidic acid	0.0016	0.0018	0.0016	0.00028	1.000	0.563
C16:1	Palmitoleic aicd	ND	ND	ND	ND	ND	ND
C18:0	Stearic acid	0.045	0.044	0.045	0.0017	0.947	0.729
C18:1	Oleic aicd	0.0078	0.0094	0.0086	0.0015	0.731	0.528
C18:2	Linoleic acid	0.0011	0.0011	0.00097	0.00086	0.307	0.676
C18:3	α-Linoleic acid	0.001433	0.00095	0.00137	0.00014	0.743	0.022
C20:4	Arachidonic acid	0.0009	0.00083	0.0008	0.000099	0.499	0.828
C20:5	Eicosapentaenoic acid	0.00013	0.00073	0.0032	0.000071	0.227	0.430
C22:5	Docosapentanoic acid	0.00033	0.00043	0.0026	0.0012	0.210	0.448
C22:6	Docosahexanoic acid	0.0004	0.0003	0.0003	0.00023	0.762	0.847

Fatty acid results are presented as mg of fatty acid mL^{-1} of media and mg 10^{-7}cells. ND: Non detectable.

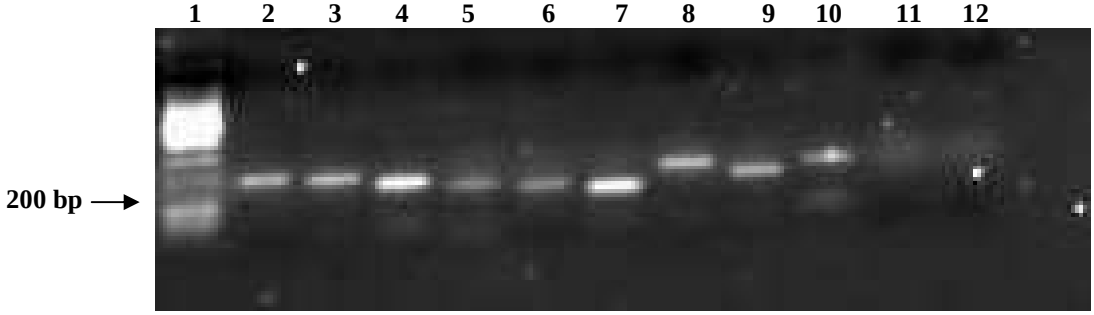
Results show that EPA treatment of adipocytes resulted in a significant increase in concentration of myristoleic acid, palmitic acid, stearic acid, α-linoleic acid, docosopentanoic acid and docosahexanoic acid with increasing concentrations of supplemental EPA (P<0.05). The concentration of palmitelaidic acid was significantly greatest at 50 µM EPA treatment (P<0.05). Of note, treatment with EPA caused a cellular accumulation of EPA methyl esters, with a significant increase in cells treated with 50 and 100µM EPA compared with the control (P<0.05). There was a trend towards increased linoleic acid (P=0.08) and steric acid (P=0.06) concentration in the 100 µM EPA treatment group compared to the control. In the media, there was

significant increase in EPA concentration of supplemental EPA (P<0.05). Remaining fatty acids analysed showed no significant changes.

Reverse-transcriptase PCR and sequence analysis

High quality total RNA was successfully isolated from adipocytes. Results of 28S/18S ratios for all samples were between 1.8 and 2 and RNA integrity numbers (RIN) of 9 to 10. In addition, yields of RNA recovered from 25 mL flasks were in the region of 12 μg per flask. Markers of fat metabolism PPAR-γ, FAS, FABP-4, aP2, CD36, Δ-9 desaturase, leptin, SREBP-1c and MTP were all shown to be expressed at passage 10, 20 and 30 using reverse transcription-PCR (Figure 5). No expression of the negative control HNF4 was observed. PCR products were sequenced and subjected to BLAST analysis to establish their identity. In the case of all PCR products sequenced, DNA sequences were 100 % identical to published sequences.

5(a)



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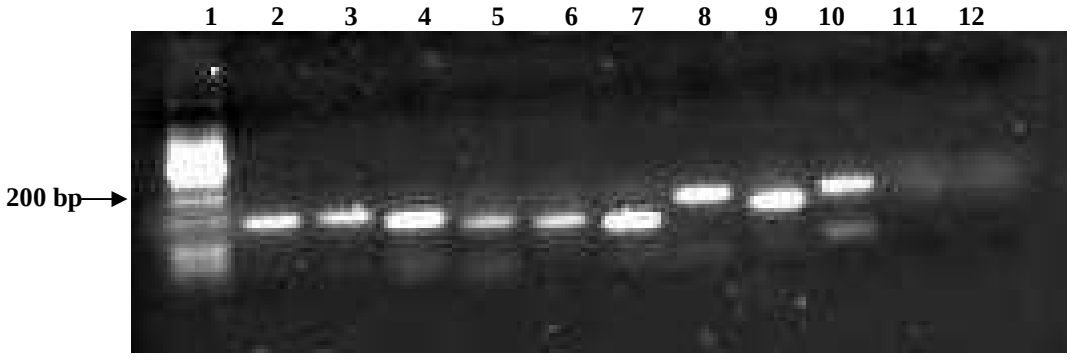

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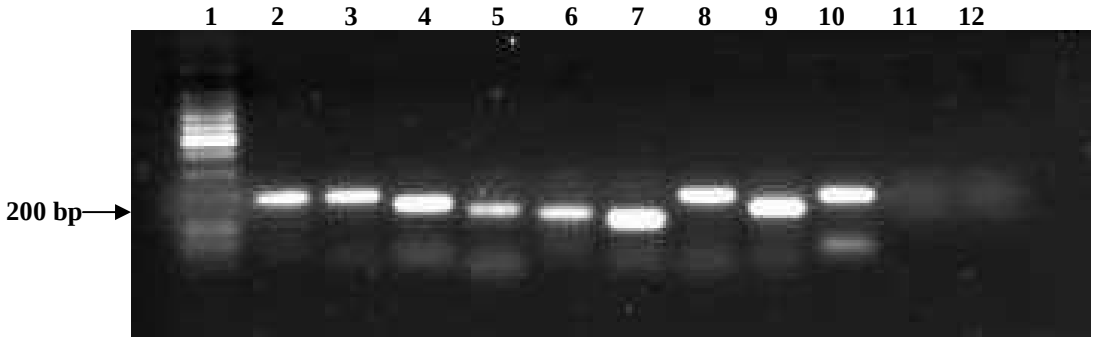
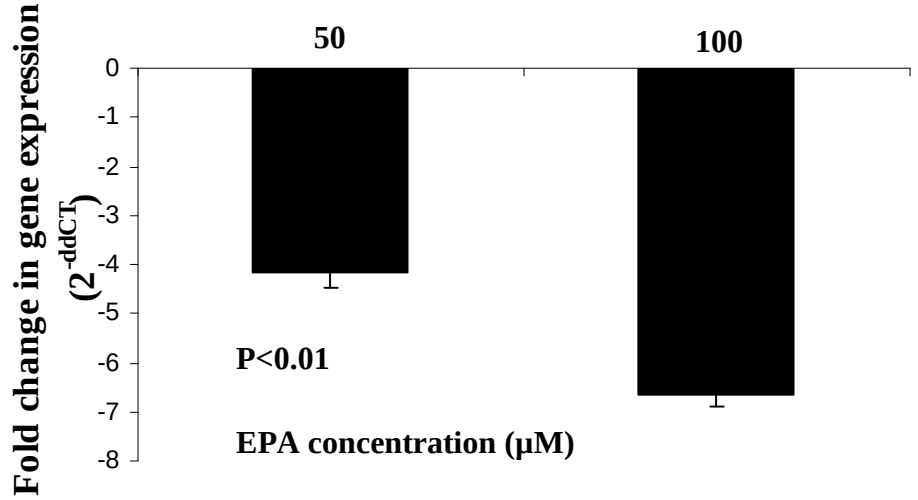


Figure 5. Reverse transcription PCR analysis of marker genes involved in fatty acid metabolism in bovine intramuscular adipocyte cells line. Lane 1; 100 bp ladder, **Lane** 2; Δ-9 desaturase **Lane** 3; SREBP-1c, **Lane** 4; CD36, **Lane** 5; PPAR-γ, **Lane** 6; MTP, **Lane** 7; Leptin, **Lane** 8; aP2, **Lane** 9; FABP-4, **Lane** 10; FAS, **Lane** 11, HNF4α **Lane** 12; water control. **(a)** passage 10, **(b)** passage 20 and **(c)** passage 30. aP2; adipocyte lipid binding protein, FABP-4; fatty acid binding protein-4, FAS; fatty acid synthase, MTP; microsomal triglyceride transfer protein, PPAR-γ; peroxisome proliferator activated receptor-gamma, SREBP; sterol regulatory element binding protein.

Gene expression analysis

Compared with the control, expression of (a) Δ-9 desaturase and (b) SREBP-1c mRNA was decreased (a) 5 and 7 fold and (b) decreased 6 and 18 fold respectively following supplementation with 50μM and 100μM EPA (Figure 6).

6(a).



6(b).

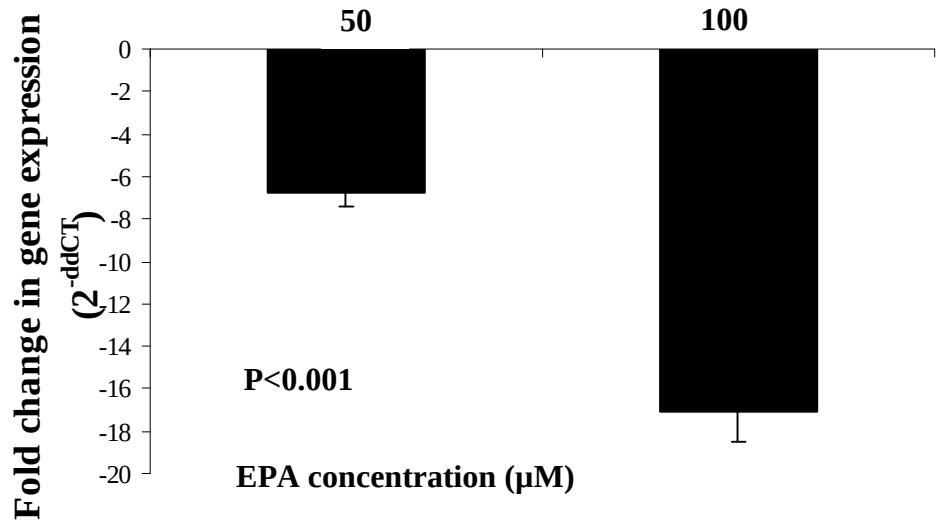
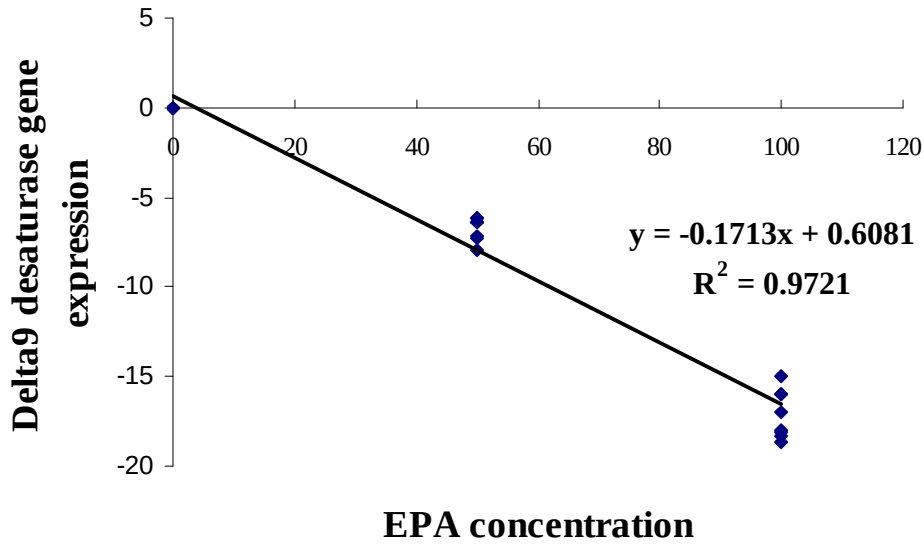


Figure 6. Effect of concentration of EPA in culture media on the gene expression of (a) Δ9 desaturase and (b) SREBP-1c in an *in vitro* intramuscular adipocyte cell culture model. Gene expression results expressed as fold change ± SEM.

There was a strong negative relationship between gene expression of both Δ-9 desaturase and SREBP-1c and EPA concentration (R^2 = 0.97 and 0.98, respectively, *P*<0.0001; Figure 7). There was a positive relationship observed between SREBP-1c and Δ-9 desaturase gene expression (R^2 = 0.90; *P*<0.0001).

7(a)



7(b)

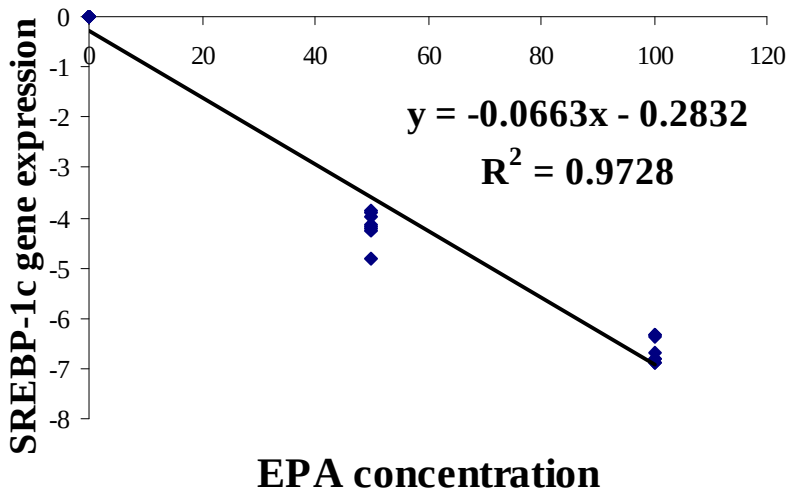


Figure 7. Regression analysis of EPA concentration versus gene expression of (a) SREBP-1c and (b) Δ9-desaturase.

DISCUSSION

A biologically functional bovine intramuscular primary adipocyte cell line has been developed and validated. The cell line was confirmed to display *in vivo* adipocyte functionality as determined by both histological and molecular methods. Furthermore, it has been shown that EPA treatment decreased gene expression of both Δ-9 desaturase and SREBP-1c *in vitro* which is consistent with our earlier findings *in vivo* (Waters *et al*., 2007).

A mature bovine intramuscular adipocyte cell line resource is of huge importance in implementing hypothesis driven research and in the investigation of the biochemical and molecular mechanisms of tissue fatty acid synthesis, metabolism and accretion in beef cattle. The increasing cost of large animal experiments, increased legislative requirements on the ethical use of animals (Radzikowski, 2006), together with considerable inter animal variation in feed intake (Nkrumah *et al*., 2007), hepatic function (Dorroch *et al*., 2001), resistance to disease (Morris, 2007) and inherent fatty acid concentrations in cattle even within breeds fed the same diets (Pitchford *et al*., 2002) results in marked difficulties in investigating dietary effects on tissue fatty acid accretion. While there is notable genetic variation associated with beef fatty acid composition (Zhang *et al*., 2008), there is also variation in the expression of a number of genes controlling fatty acid constituents of beef, such as sterol regulatory element binding protein (SREBP) (Hoashi *et al*., 2007) and Δ-9 desaturase (Lehnert *et al*., 2006). Consequently, the use of more controlled environments to test specific biological hypotheses is desirable.

Microbial contamination constitutes one frequent problem encountered when culturing cells, especially as it has been shown to impede cell cycle, triggering cell death under various conditions (Darin *et al*., 2003). Fortunately no contamination was encountered during the course of this study. It was also noted that the novel cell line developed in this study was a primary culture and did not spontaneously immortalize. Results clearly show that the isolated mature intramuscular bovine adipocytes retain the ability to accumulate lipid in culture. The cell line was fully differentiated with adipocytes containing vesicles filled with lipid droplets. Two histological staining techniques were initially carried out to evaluate the cellular composition of the intramuscular adipocyte cell culture. Oil red O and Sudan IV were used specifically to stain triglycerides present in cells. In the current study, Oil red O stain was not fully absorbed by the cells and proved an inefficient staining method. Sudan IV stain was found to be a superior method for staining lipid, targeting triglycerides present in the cells and confirming the presence of lipid within the intracellular vesicles. Comparative studies by Dux *et al*. (1981), evaluating simple stains for typing skeletal muscle fibres, also showed that Sudan stain was superior to Oil Red O. Optimum staining was obtained using both fixed and unfixed sections using this method.

In the current study, high quality total RNA was successfully isolated from adipocytes with 28S/18S ratios of 1.8-2 and a RIN number of 9-10, indicating that the cell line was transcriptionally active. Yields of RNA recovered from a 25 cm^3 flask were in the region of 12 μg per flask which is sufficient for real time PCR studies and microarray analysis and would therefore not require amplification which potentially introduces some degree of bias in gene expression analyses (Li *et al*., 2003).

PPAR-γ (Kershaw *et al*., 2007), FAS (Kuhajda *et al*., 1994), FABP-4 (Wolnicka-Glubisz, 2005), aP2 (Cabrero *et al*., 2001), CD36 (Sun and Yang, 2003), Δ-9 desaturase (Su *et al*., 2004), leptin (Zhou *et al*., 1999), SREBP-1c (Seo *et al*., 2004) and MTP (Stalenhoef *et al*., 1995) were selected as adipocyte markers due to their roles in fatty acid metabolism and synthesis, lipid transport, adipogenesis and differentiation. All were shown to be expressed using reverse transcription-PCR. HNF4, a liver-enriched transcription factor which plays a critical role in transcriptional regulation of many liver specific gene promoters (De Simone and Cortese, 1992, Sladek, 1993), was applied as a negative control in the current study and was not detected. Thus, mRNA expression of adipocyte markers and the failure to amplify the negative control in combination with the ability of the cells to accumulate lipid over time confirm the primary adipocyte cell line was mature and functional at passage 30. It therefore seems possible that mature adipocytes may not be a terminally differentiated cell form, as previously thought. Instead, these adipocytes may be capable of re-entering the cell cycle and forming proliferative-competent precursor cells like preadipocytes, adipofibroblasts, or even other forms of cells, similar to the finding of Dodson *et al*., (2005).

Gas chromatography analysis showed that fatty acids long chain n-3 PUFAs could be easily detected and measured in both adipocytes and spent media, ranging from tetradecanoic acid (C14:0) to polyunsaturated fatty acid, docosohexanoic acid (C22:6). This demonstrates that adipocytes could be supplemented with various fatty acids and their incorporation into adipocytes could be monitored by GC analysis. The ability to measure these fatty acids *in vitro* is of huge benefit in lipid research using cell lines, particularly in assessing strategies to improve the fatty acid quality of beef.

Treatment of adipocytes with EPA resulted in significant accumulation in concentration of the fatty acids; myristoleic acid, palmitic acid, stearic acid, α-linoleic acid, EPA, docosopentanoic acid and docosahexanoic acid. Cellular accumulation of EPA methyl esters validated its uptake into cells. The concentration of palmitaleadic acid was increased in the 50μM treatment. These results indicate that following EPA supplementation cells underwent an altered state of fatty acid metabolism. As the concentration of docosopentanoic and docosahexanoic acid was increased, it appears that a process of fatty acid elongation may have occurred following EPA supplementation. EPA was increased in the media following treatment indicating that EPA was present in excess concentrations and not all was incorporated into adipocytes.

This is first report demonstrating that EPA treatment of bovine intramuscular adipocytes decreased gene expression of both Δ-9 desaturase and SREBP-1c *in vitro.* Even short term exposure of cells to EPA decreased the expression of both Δ-9 desaturase and SREBP-1c genes in a dose dependent manner, seen also in the human model study by Renaville (2006) investigating the effects of EPA on Δ-9 desaturase and SREBP-1c in intestinal cell *in vitro*. Acute (24hr) treatment with EPA reduction in mRNA Δ-9 desaturase expression was possibly mediated through a decrease in gene expression of SREBP-1c. Furthermore, acute treatment with EPA resulted in EPA uptake and altered fatty acid profiles of intramuscular adipocytes. These results are consistent with other *in vivo* findings (Waters et al., 2007), thus validating the functionality of the *in vitro* model. This data has significant implications for both ruminant and human nutrition. Bovine intramuscular adipocyte cell line is important for future studies of lipid metabolism in cattle. This is an important resource in

elucidating limitations associated with simultaneous augmentation of tissue n-3 PUFA and CLA and unravelling molecular mechanisms involved. It will also permit future investigations on the cellular mechanisms involved controlling differences in fat depots and important traits like marbling in beef. Moreover, this resource can be used in RNA-interference mediated research to gain a greater understanding of the role of various genes and the molecular pathways and mechanisms they control. This technology has recently been applied in the 3T3-L1 adipocytes in mice (Zhou *et al*., 2008).

In vitro cell line based studies have been instrumental in advances in fatty acid research. They are a rapid, cost effective method of carrying out various hypothesis driven research studies prior to investing in large animal experiments. In terms of marbling, differences in the development preadipocytes among adipose depots in cattle are poorly understood. Grant *et al.* (2008) developed an *in vitro* cell culture system to examine differences in differentiation between intramuscular and subcutaneous bovine preadipocytes. Many *in vitro* cell lines have produced vital results in investigating factors which effect *de novo* tissue synthesis of CLA. De La Torre *et al*., (2005) found that vaccenic acid was highly metabolised by bovine hepatocytes followed by its conversion to CLA.

With mandatory legislation controlling the use of animal models for experimentation, research is becoming restrictive with governments only granting licences to those who demonstrate the need to conduct the research. There is increasing pressure on scientists to use non-animal techniques, such as cell culture methodologies, to perform preliminary research (Matfield, 1996). This novel cell line resource will

facilitate the more economical, rapid screening of research hypotheses circumventing experimental animals.

LITERATURE CITED

Aso H, Abe H, Nakajima I, Ozutsumi K, Yamaguchi T, Takamori Y, Kodama A, Hoshino FB and Takano S 1995. A preadipocyte clonal line from bovine intramuscular adipose tissue: nonexpression of GLUT-4 protein during adipocyte differentiation. Biochemical and Biophysical Research Communications 213, 369-75.

Bernlohr DA, Bolanowski MA, Kelly TJ Jr and Lane MD 1985. Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. Journal of Biological Chemistry 260, 5563-7.

Cabrero A, Alegret M, Sánchez RM, Adzet T, Laguna JC and Vázquez M. 2001. Bezafibrate reduces mRNA levels of adipocyte markers and increases fatty acid oxidation in primary culture of adipocytes. Diabetes 50, 1883-90.

Darin N, Kadhom N, Brière JJ, Chretien D, Bébéar CM, Rötig A, Munnich A, Rustin P. 2003. Mitochondrial activities in human cultured skin fibroblasts contaminated by Mycoplasma hyorhinis. BMC Biochem. 4:15.

De La Torre A, Gruffat D, Chardigny JM, Sebedio JL, Durand D, Loreau O and Bauchart D 2005. *In vitro* metabolism of rumenic acid in bovine liver slices. Reproduction, Nutrition and Development 45, 441-51.

De Simone V, Cortese R. 1992. Transcription factors and liver-specific genes. Biochim Biophys Acta. 1132(2):119-26.

Dodson, M.V., M.E. Fernyhough, J.L. Vierck, and G.J. Hausman. 2005. Adipocytes may not be a terminally differentiated cell type: Implications for animal production. Animal Science 80(3):239-240.

Dorroch U, Goldammer T, Brunner RM, Kata SR, Kühn C, Womack JE and Schwerin M 2001. Isolation and characterization of hepatic and intestinal expressed sequence tags potentially involved in trait differentiation between cows of different metabolic type. Mammalian Genome 12, 528-37.

Dux L, Mesaros, MG, Rohan J, Gajdos L, Jakab G and Guba F. 1981. The value of simple lipid stains for typing skeletal muscle fibers. Histochemical Journal 13, 63-71.

Faulconnier Y, Thévenet M, Fléchet J and Chilliard Y 1994. Lipoprotein lipase and metabolic activities in incubated bovine adipose tissue explants: effects of insulin, dexamethasone, and fetal bovine serum. Journal of Animal Science 72, 184-91.

Fernyhough ME, Vierck Jl, Hausman GJ, Mir PS, Okine EK and Dodson, MV 2005. Cytotechnology 46, 163-172.

Folch J, Lees M and Sloane Stanley GH 1957. A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry 226, 497-509.

Forrest C, Grimaldi P, Czerucka D, Negrel R, and Alihaud G. 1983. In vitro 19, 344-354.

Grant AC, Ortiz-Colòn G, Doumit ME and Buskirk DD 2008. Optimization of in vitro conditions for bovine subcutaneous and intramuscular preadipocyte differentiation. Journal of Animal Science 86, 73-82.

Gregoire FM, Smas CM and Sul HS 1998. Understanding adipocyte differentiation. Physiological Reviews 78,783-809.

Hausman GJ, Poulos SP, Pringle TD, Azain MJ. 2008. The influence of thiazolidinediones on adipogenesis in vitro and in vivo: potential modifiers of intramuscular adipose tissue deposition in meat animals. J Anim Sci. In Press.

Hoashi S, Ashida N, Ohsaki H, Utsugi T, Sasazaki S, Taniguchi M, Oyama K, Mukai F and Mannen H 2007. Genotype of bovine sterol regulatory element binding protein-1 (SREBP-1) is associated with fatty acid composition in Japanese Black cattle. Mammalian Genome 18, 880-6.

Kershaw EE, Schupp M, Guan HP, Gardner NP, Lazar MA and Flier JS. 2007. PPARγ regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. American Journal of Physiology, Endocrinology and Metabolism 293, 1736-1745.

Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD, Pasternack GR. 1994. Fatty acid synthesis: a potential selective target for antineoplastic therapy. Proc Natl Acad Sci U S A. 91(14):6379-83.

Lehnert SA, Byrne KA, Reverter A, Nattrass GS, Greenwood PL, Wang YH, Hudson NJ, Harper GS. 2006. Gene expression profiling of bovine skeletal muscle in response to and during recovery from chronic and severe undernutrition. J. Anim. Sci. 84(12):3239-50.

Li J, Adams L, Schwartz SM and Bumgarner RE. 2003. RNA amplification, fidelity and reproducibility of expression profiling. Comptes Rendus Biologies 326, 1021-30.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2(-delta delta CT)) method. Methods 25:402-8.

Matfield M. 1996. The ethics of animal research. A special lecture to the Japanese Association of Laboratory Animal Science 6th June 1995. Experimental Animals 45, 209-15.

Morganstein DL, Christian M, Turner JJ, Parker MG and White R 2008. Conditionally immortalized white preadipocytes: a novel adipocyte model. Journal of Lipid Research 49, 679-85.

Moloney AP, Mooney MT, Kerry JP and Troy DJ 2001. Producing tender and flavoursome beef with enhanced nutritional characteristics. The Proceedings of the Nutrition Society 60, 221-9.

Morris CA. 2007. A review of genetic resistance to disease in Bos taurus cattle. Veterinary Journal 174, 481-91.

Natal C, Fortuño MA, Restituto P, Bazán A, Colina I, Díez J and Varo N 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. American journal of Physiology. Endocrinology and Metabolism 294, 52-60.

Nkrumah JD, Sherman EL, Li C, Marques E, Crews DH Jr, Bartusiak R, Murdoch B, Wang Z, Basarab JA and Moore SS 2007. Primary genome scan to identify putative quantitative trait loci for feedlot growth rate, feed intake, and feed efficiency of beef cattle. Journal of Animal Science 85, 3170-81.

Pitchford WS, Deland MP, Siebert BD, Malau-Aduli AE and Bottema CD 2002. Genetic variation in fatness and fatty acid composition of crossbred cattle. Journal of Animal Science 80, 2825-32.

Radzikowski C 2006. Protection of animal research subjects. Science and Engineering Ethics 12, 103-110.

Renaville B, Mullen A, Moloney F, Larondelle Y, Schneider YJ & Roche HM. (2006). Eicosapentaenoic acid and 3,10 dithia stearic acid inhibit the desaturation of trans-vaccenic acid into cis-9, trans-11-conjugated linoleic acid through different pathways in Caco-2 and T84 cells. *Br J Nutr* **95,** 688-95.

Scollan N, Hocquette JF, Nuernberg K, Dannenberger D, Richardson I, Moloney A. 2006. Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. Meat Science. 74, 17-33.

Seo JB, Moon HM, Noh MJ, Lee YS, Jeong HW, Yoo EJ, Kim WS, Park J, Youn BS, Kim JW, Park SD and Kim JB 2004. Adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c regulates mouse adiponectin expression. Journal of Biological Chemistry 279, 22108-17.

Sladek F. M. 1993 Orphan receptor HNF-4 and liver-specific gene expression. Receptor. 3(3):223-32.

Stalenhoef AF, Armstrong VW, Steinmetz A, Taskinen MR and Zechner R. 1995. The 17th annual meeting of the European Lipoprotein Club. Arteriosclerosis, Thrombosis, and Vascular Biology 15, 543-9.

Su X, Han X, Yang J, Mancuso DJ, Chen J, Bickel PE, Gross RW. 2004. Sequential ordered fatty acid alpha oxidation and Delta9 desaturation are major determinants of lipid storage and utilization in differentiating adipocytes. Biochemistry. 43(17):5033-44.

Sun YR and Yang Z. 2003. Advances of nuclear translation factor: peroxisome proliferator-actived receptor gamma2. Yi Chuan 25, 713-7.

Waters SM,. Kelly JP, O Boyle P, Moloney AP and DA Kenny 2008. Effect of level and duration of dietary n-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of Δ-9 desaturase in muscle of beef cattle. Journal of Animal Science. In Press.

Waters SM, Killeen AP, Fitzgerald, A, Kenny DA and Hynes AC. Effect of level of eicosapentaenoic acid on the transcriptional regulation of Δ-9 desaturase in an *in vitro* bovine intramuscular adipocyte cell culture model. Proceedings from the 3rd International Symposium on Animal Functional Genomics, Edinburgh, United Kingdom 7–9 April 2008, p50.

Vernon RG and Taylor E 1988. Insulin, dexamethasone and their interactions in the control of glucose metabolism in adipose tissue from lactating and nonlactating sheep. Biochemical Journal 256, 509-14.

Viravaidya K and Shuler ML 2002. The effect of various substrates on cell attachment and differentiation of 3T3-F442A preadipocytes. Biotechnology and Bioengineering 78, 454-8.

Zhang S, Knight TJ, Reecy JM and Beitz DC 2008. DNA polymorphisms in bovine fatty acid synthase are associated with beef fatty acid composition. Animal Genetics 39, 62-70.

Zhou QL, Jiang ZY, Holik J, Chawla A, Hagan GN, Leszyk J and Czech MP. 2008. Akt substrate TBC1D1 regulates GLUT1 expression through the mTOR pathway in 3T3-L1 adipocytes. Biochemistry Journal 411, 647-55.