

MOLECULAR BIOLOGY

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

Dietary supplementation with a blend of oils rich in long chain omega-3 polyunsaturated fatty acids (n-3 PUFA) and linoleic acid have a synergistic effect on the accumulation of ruminal and tissue concentrations of trans vaccenic acid (VA). VA is the main substrate for Δ -9 desaturase which is responsible for *de novo* tissue synthesis of the cis 9, trans 11 isomer of CLA. n-3 PUFA supplementation appear to enhance ruminal synthesis of VA, but then inhibits its conversion to CLA using this dietary regime in beef cattle. We have previously shown that increased tissue concentrations of n-3 PUFA reduces the mRNA expression for Δ -9 desaturase, however, the biochemical mechanisms regulating this is not known. Although human Δ -9 desaturase has been shown to be under the control of two transcription factors, sterol regulatory element binding protein (SREBP)-1c and peroxisome proliferator activated receptor α (PPAR α), there is no information on this process in the bovine. The objective of this study was to investigate the influence of dietary n-3 PUFA on the gene expression of two putative regulators of Δ -9 desaturase, SREBP-1c and PPAR α in bovine muscle.

Young beef bulls (n=24) were individually fed diets containing 6% soya oil, in combination with either 0 (CON) or 2 per cent (FO2) of a high n-3 fishoil product containing 39% eicosapentaenoic acid (EPA) and 24% docosahexanoic acid (DHA). Animals were fed for 100 days. Samples of *M. longissimus dorsi* were harvested 30 min. post mortem and stored at -80°C. Total RNA was isolated from fragmented frozen muscle tissue using TRIzol reagent and real time RT-PCR was performed to measure the relative expression of SREBP and PPAR α in muscle. Fold changes in gene expression between CON and FO2 were calculated (Livak and Schmittgen, 2001). In a previous study (Waters et al., 2007) using the same tissues we have shown that muscle concentrations of both EPA and DHA were higher ($P < 0.001$) in FO2 compared with CON. Concentrations of VA were higher in FO2 compared with CON with no effect of treatment on the concentration of cis 9, trans 11 CLA. Expression of mRNA for Δ -9 desaturase was decreased 4.6 fold in FO2 compared with CON. In the current study, data were analysed using mixed models methodology (PROC MIXED, SAS 2001). Univariate multiple regression analyses was carried out to establish relationships between tissue fatty acid concentrations and relative gene expression of Δ -9 desaturase, SREBP-1c and PPAR- α (PROC REG, SAS).

Expression of mRNA for SREBP was decreased 2.1 fold in animals fed the FO2 diet compared to the control ($P < 0.05$) (Figure 41). Dietary supplementation with FO2 did not alter the expression of PPAR- α ($P = 0.3$) (Figure 42). A positive relationship existed between Δ -9 desaturase and SREBP-1c gene expression ($R = 0.75$; $P < 0.01$), however, no relationship was displayed between PPAR- α and both Δ -9 desaturase and SREBP-1c gene expression ($P > 0.05$). A significant negative relationship existed between both VA and EPA and SREBP-1c gene expression ($P < 0.05$) while no relationship was observed between PPAR- α mRNA expression and any FA measured ($P < 0.05$).

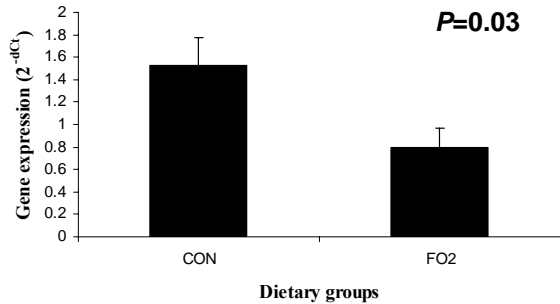


Figure 41. SREBP-1c relative gene expression in bovine muscle

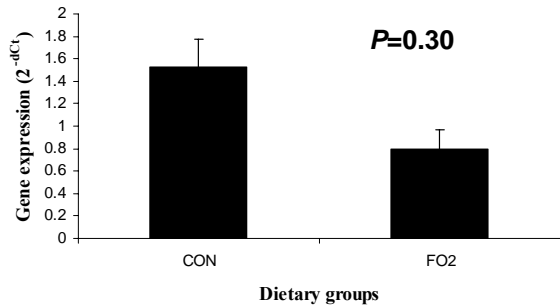


Figure 42. PPAR- α relative gene expression in bovine muscle

Simultaneous enhancement of CLA and n-3 PUFA concentrations in bovine muscle may be hindered by negative effects of dietary n-3 PUFA supplementation on tissue Δ -9 desaturase gene expression, mediated through reduced mRNA transcription for SREBP-1c.

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Effect of supplementation of bovine intramuscular adipocytes with eicosapentaenoic acid on the transcriptional regulation of the Δ -9 desaturase gene

The potential human health promoting effects of omega-3 polyunsaturated fatty acids (n-3 PUFA) and the ruminant derived *cis*-9, *trans*-11 isoform of conjugated linoleic acid (CLA) have been well publicised. Most CLA in ruminant, as well as human tissues is synthesised through the tissue desaturation of vaccenic acid (VA) by Δ -9 desaturase. However, although concentrations of VA are readily accentuated in bovine muscle through the ruminal synergism between dietary n-3 PUFA and linoleic acid, concurrent increases in CLA have not been realised. We have reported that this may be a consequence of n-3 PUFA, and in particular eicosapentaenoic acid (EPA), mediated inhibition of mRNA expression for both Δ -9 desaturase and its transcriptional regulator sterol regulatory element binding protein (SREBP)-1c. However, whether n-3 PUFA intake inhibits the transcriptional regulation of Δ -9 desaturase and SREBP-1c in a dose dependent fashion is unknown. The objective of the current study, therefore, was to utilise a recently developed bovine intramuscular adipocyte cell line 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 employed to avoid possible confounding effects of inter animal variance in feed intake and adiposity.

Bovine adipocytes were derived from an intramuscular adipocyte cell line developed. Cells were cultured in 75 ml flasks (n=8 per treatment group) and reached maximal confluency as observed by phase microscopy. Prior to treatment, cells were starved of serum for 24h and incubated in a serum-free medium containing 1% insulin, selenium and transferrin as substitutes for fetal calf serum. Following 24h, this medium was replaced with serum-free medium containing one of three concentrations of EPA viz 0 μ M (control), 50 μ M or 100 μ M. Ethanol was used as a vehicle to incorporate EPA into the culture medium and was added at the same concentration to the control. Cells were monitored for a further 24h and subsequently trypsinised and centrifuged at 1500 rpm for 5 min. The supernatant was removed, the pellet covered with 1 ml RNAlater® (Ambion) and samples stored at -20°C. RNA was extracted using TRIzol reagent. cDNA was produced by reverse transcription. Δ -9 desaturase and SREBP-1c mRNA expression was measured by quantitative real time RT-PCR. PCR products were sequenced to verify their identity. The $2^{-\Delta\Delta CT}$ method was used to determine mean relative fold changes in gene expression between the control and both 50 μ M and 100 μ M treatment groups. Data was analysed using mixed models ANOVA (PROC MIXED, SAS 2001) and regression methodologies (PROC REG; SAS 2001).

High quality RNA was isolated from all flasks with RIN and 28S/18S values of 9-10 and 1.8-2.0, respectively. Approximately 10.5 μ g RNA per flask was recovered. Compared with the control, expression of Δ -9 desaturase mRNA was decreased 5 and 7 fold, respectively following supplementation with 50 μ M and 100 μ M EPA (Figure 43a). Similarly, gene expression of SREBP-1c was decreased 6 and 18 fold in cells supplemented with 50 and 100 μ M EPA, respectively (Figure 43b). Regression analysis showed a strong positive relationship between SREBP-1c and Δ -9 desaturase gene expression ($\beta_0 = -9.60$; $\beta_1 = 3.99$; $R^2 = 0.90$; $P < 0.0001$). Even short term exposure to n-3 PUFA down-regulated transcription of both genes. Results are consistent with other *in vivo* findings, thus further validating the functionality of the *in vitro* model.

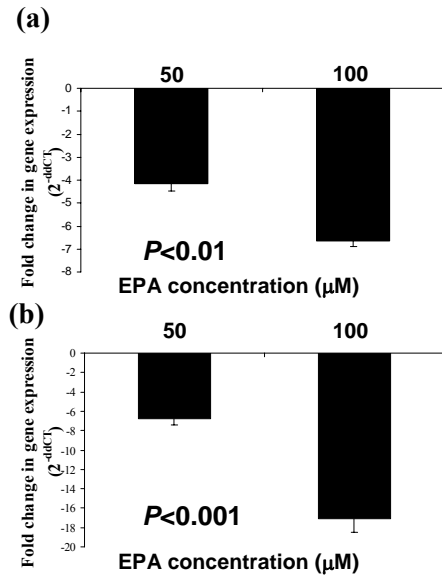


Figure 43. Effect of concentration of EPA in culture media on the gene expression of (a) $\Delta 9$ desaturase and (b) SREBP-1c in an *in vitro* intramuscular adipocyte cell culture model. Gene expression results expressed as fold change \pm SEM.

Exposure of bovine intramuscular adipocytes to the long chain n-3 PUFA, EPA decreases the expression of both Δ -9 desaturase and SREBP-1c genes in a dose dependent manner and has significant implications for both ruminant and human nutrition.

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Effect of dietary n-3 polyunsaturated fatty acids on gene expression in the bovine uterus using microarray technology

Modern high-yielding dairy cows display poor reproductive performance. The greatest increment of reproductive wastage is due to early embryo loss within the first two weeks of insemination. Nutrition plays a key role in reproduction and there is emerging evidence that dietary long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) may act as specific potent regulators of some reproductive events. The establishment of pregnancy is reliant on an orchestrated interaction between the embryo and its uterine environment and involves critical processes such as nutrient transfer, immune function and cellular signalling. While n-3 PUFAs, particularly eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), are known to be regulators of gene transcription in many tissues, their role as mediators of uterine function has not been well studied. Microarray technology has provided the opportunity to examine the simultaneous expression of thousands of genes within a cell type of interest, thus yielding static as well as dynamic information on these genes. The objective of this study was to use a 23K oligonucleotide bovine microarray (Affymetrix[®]) to examine the effects of dietary supplementation of n-3 PUFA on bovine uterine endometrial gene expression and to relate changes in gene expression to key biochemical pathways using novel bioinformatic tools.

Reproductively normal crossbred beef heifers (n=24) were individually fed a straw and barley/beet pulp based concentrate and supplemented with a rumen protected source of either saturated fatty acid (palmitic acid; Low PUFA) or high n-3 PUFA (High PUFA 273g) per head per day for 45 days. Both diets were isolipid and isonitrogenous. Following slaughter, uterine endometrial tissue was harvested from all animals and stored at -80°C. Analysis of fatty acid methyl esters in endometrial tissue was carried out using gas chromatography. Based on endometrial n-3 PUFA concentration, total RNA was extracted using TRIzol reagent, from endometrial tissue from the seven highest animals on High and the seven lowest on Low PUFA. RNA quantity was determined spectrophotometrically and quality was assessed using the Agilent Bioanalyzer 2100. Gene profiling was carried out using the Affymetrix GeneChip® Bovine Genome array with 23,000 gene transcripts. Protocols for RNA labeling and scanning were as outlined in the Affymetrix Expression Analysis Technical Manual. RNA was hybridized to the microarray gene chip. Microarray data was normalised and statistical analysis of gene expression was carried out using the Puma method. Data were analysed using Ingenuity Pathway analysis (Ingenuity Systems®, www.ingenuity.com).

Combined endometrial concentrations of EPA and DHA were more than two-fold higher in the High compared to the Low PUFA group ($P < 0.05$), while EPA concentrations alone were more than four-fold higher in the high group ($P < 0.01$). A platform consisting of a total of 5787 annotated genes was used to compare the effects of the high PUFA diet versus the low PUFA diet. Human homologue Entrez Gene IDs could be assigned to 4926 of the 5787 differentially expressed bovine genes. These 4926 genes were mapped to the Ingenuity Pathway Analysis database. 3712 were found to be network eligible, from this 1350 were up-regulated and 2362 were down-regulated. Of the 3712 genes that were network eligible a total of 418 genes were significantly differentially expressed ($P < 0.05$) of which 256 were up-regulated and 162 were down-regulated. Fold changes ranged from a minimum of -13.53 to a maximum fold change of 13.17. There were significant changes in the expression of genes involved in a number of important functions, including embryonic development, reproduction, immune function, protein synthesis and cell death (Figure 44).

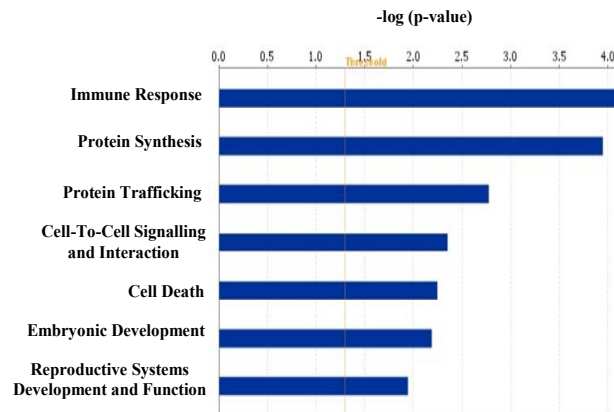


Figure 44. Effect of n-3 PUFA on expression of genes [-log(p-value)] regulating key biochemical processes in the bovine uterine endometrium.

Animals supplemented with a high PUFA diet showed differential endometrial expression of genes involved in regulating key biological processes that could potentially influence bovine fertility.

RMIS No. 5679

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Effect of Holstein-Friesian cow genotype on expression of genes in the GH-IGF axis during early and mid-lactation

It has been shown that the New Zealand Holstein Friesian (NZHF) has lower milk production but superior fertility compared to the North American Holstein Friesian (NAHF) strain in a pasture-based milk production system. The energy demands of early lactation exceed energy intake resulting in a 5-10 week period of negative energy balance (NEB). During positive energy balance, pulsatile release of growth hormone (GH) from the pituitary gland stimulates expression of insulin-like growth factor (IGF-I) in hepatocytes, resulting in increased IGF-I mRNA abundance in liver tissue, and increased circulating concentrations of IGF-I. During NEB, however, the GH-IGF axis becomes uncoupled and circulating concentrations of IGF-I decline precipitously. It is hypothesised that differences in fertility may be due to greater negative energy balance (NEB) during early lactation, and a longer period of preferential partitioning of nutrients to milk production for the NAHF strain. These changes are likely to impact components of the somatotrophic axis. The objective of this study was to examine the effect of cow genotype on the expression of genes in the GH-IGF axis during early and mid-lactation.

Ten mature NAHF cows and 10 mature NZHF cows were selected as representative of their respective strains within the Moorepark strain comparison study. Liver tissue was collected from all cows at 35 and 150 days postpartum by puncture biopsy as described by Smith et al. (1997), snap frozen in liquid nitrogen, and stored at -80°C . Cows were offered *ad libitum* grass silage

plus 8 kg of concentrate per day at the time of the first biopsy and *ad libitum* zero grazed pasture plus 4 kg of concentrate per day at the time of the second biopsy. Total RNA was extracted using the TRIzol reagent and purified using the RNeasy minkit (Qiagen). Real-Time RT-PCR analysis was performed on genes involved in the IGF system using the ABI 7500 Fast Real-Time PCR System with the SYBR Green Master Mix (Applied Biosystems, Warrington UK). All samples were assayed in the same run thereby eliminating inter-assay variation, and the specificity of the reaction products was confirmed by melt curve analysis and gel electrophoresis. PCR products were sequenced to confirm their identity. Real-Time RT-PCR data were log transformed for normalisation of variances and were analysed using PROC mixed in SAS (SAS, 2003) with terms for day and strain and their interaction included in the model. Expression of the exogenous control, the kanamycin resistance gene was used as a covariate in the analysis.

Mean systemic concentrations of (IGF-1) were higher in NZHF than NAHF during the post-transition period (97.5 vs 77.5 ng/mL) ($P<0.05$). The results of the real-time RT-PCR analysis are summarised in (Table 66). IGF-1 was the only gene that was significantly affected by cow genotype; mRNA abundance was 1.6 times greater in the NZ breed ($P<0.05$). The abundance of acid labile subunit (ALS) mRNA tended ($P=0.06$) to be greater in NZ cows compared to NA cows. Across genotypes, mRNA abundance of IGFBP-1, IGFBP-2 and GHR1A decreased significantly from day 35 to 150, whereas trends for increased IGF-I and ALS mRNA were observed during this period. None of the genes analysed demonstrated a significant Strain*Time effect (Values not shown).

Although several of the genes involved in the IGF system remained unchanged across genotypes, the NZ cows had greater abundance of IGF-I mRNA and tended to have increased expression of ALS (critical to IGF-1 stability). The decrease in IGFBP-1 and IGFBP-2 mRNA abundance from day 35 to day 150 is consistent with improving energy balance status. Consistent with greater IGF-I mRNA abundance, the NZ strain also exhibited greater circulating IGF-I concentrations, which may play a role in achieving superior reproductive performance. Holstein-Friesian cow genotype and stage of lactation have significant effects on the GH-IGF axis.

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Table 66: Real Time RT-PCR analysis of genes involved in the synthesis and stability of IGF-1.
Values are back-transformed least square means followed by the 95% confidence limits and are expressed as fg per µg of reversed transcribed RNA.

Gene	STRAIN		Fold change	P value	DAYS POSTPARTUM			
	NAHF	NZHF			35	150	Fold change	P value
IGF-1	0.32 (.23-.44)	0.51 (.37-.72)	1.6	< 0.05	0.32 (.22-.47)	0.51 (.34-.76)	1.6	0.15
GHR (tot)	0.51 (.37-.71)	0.47 (.33-.66)	1.1	0.71	0.6 (.42-.88)	0.4 (.27-.58)	1.5	0.15
GHR1A	0.03 (.02-.04)	0.03 (.02-.04)	-	0.65	0.04 (.03-.05)	0.02 (.02-.03)	2.0	< 0.05
IGFBP1	4.3 (2.8-6.8)	4.3 (2.7-6.8)	-	0.98	7.7 (4.9-12.3)	2.4 (1.5-3.8)	3.2	< 0.01
IGFBP2	3.4 (2.6-4.4)	3.1 (2.3-4.1)	1.1	0.62	5.5 (4.0-7.5)	1.9 (1.4-2.6)	2.9	< 0.001
IGFBPALS	0.06 (.04-.09)	0.1 (.07-.14)	1.7	0.06	0.06 (.04-.09)	0.1 (.07-.16)	1.7	0.11

