FINAL REPORT TO EBLEX, HCC AND QMS

Plant based strategies to improve the nutritional value of beef for the consumer [PROBEEF]

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Executive summary

Beef is a highly nutritious and valued food, a source of high biological value protein and micronutrients (including vitamins A, B_6, B_{12}, D, E, iron, zinc and selenium). However, often these positive attributes have often been overshadowed due to the prominence given to several negative attributes, including the perception that beef contains high amounts of fat which is rich in saturated fat, associations between red meat and cancer and non-nutritional issues such as animal health scares (BSE) and food chain pathogens (*E. coli* 0157). Beef is a natural carrier of beneficial *omega* (*n*-3) PUFA (eicosapentaenoic acid (EPA, 20:5*n*-3) and docasahexaenoic acid (DHA; 22:6*n*-3)) and also conjugated linoleic acid (CLA) and is an important vehicle for the delivery of beneficial fats through to man. Enhancing the levels of these fatty acids in beef will improve the nutritional value of this important meat for the consumer. This project addressed plant-based strategies to increase the content of *n*-3 fatty acids and CLA in beef and concomitantly to assess the implications of such increases on aspects of meat quality. Associated studies examined the interactions between plant structure and composition and the important processes of lipolysis and biohydrogenation in the rumen.

The extent of genetic variation in perennial ryegrass in total lipid content and the fatty acid composition of this lipid and identifying and mapping QLTs for desirable fatty acids was investigated in a ryegrass mapping population over a 3-year period and 2-cuts per season. The major fatty acid identified in grass were 16:0; 18:0; 18:1n-9; 18:2n-6 and 18:3n-3. As expected 18:3n-3 and 16:0 were the dominant fatty acids. A significant effect of genotype and of cut, but little evidence of genotype by cut interaction was noted. Broad sense heritabilities of concentrations of all the fatty acid constituents in the foliage of perennial ryegrass, except oleic acid were 0.5 or above. Such high heritabilities **suggest that these traits can indeed be improved genetically**. Seasonal and environmental effects play a substantial role, but the significant contribution of the genetic component to the phenotypic variation in fatty acid content is encouraging for the prospects of genetic improvement of qualitative and quantitative aspects of fatty acid content in forage grasses for ruminant nutrition. Significant QTLs for linolenic acid (18:3*n*-3) and total fatty acids were found on linkage group 1 (LG). Furthermore, QTLs for linoleic acid (18:2*n*-6) were found on LGs 2 and 5, while there were strong associations with stearic acid (18:0) on LGs 4 and 7. The generation of a dense genetic map has also allowed to pinpoint the genomic regions underlying the traits, and the markers provide interesting leads for further analysis of these traits.

Lipolysis of dietray lipids and subsequent biohydrogenation of released fatty acids has a large effect on the ability to beneficially enhance the fatty acid composition of beef. Studies focused on two key areas related to plants (1) fate of plant chloroplasts in the rumen and (2) role of plant secondary components in altering lipolysis and biohydrogenation. *Omega*-3 rich plant phospholipids are abundant within the chloroplast membranes and understanding the fate of chloroplasts within the rumen may open further opportunities to enhance the fatty acid composition of beef. Additionally the 'Alpine factor', whereby animals receiving alpine pasture have reduced biohydrogenation, may be linked to secondary plant metabolites, including PPO, saponins, tannins and catecholamines. These compounds potentially may alter lipolysis or biohydrogenation to enhance the fatty acid composition of beef. Studies demonstrated that in animals fed on "chloroplast rich" feeds such as grass, rumen protozoa relative to bacteria are rich in polyunsaturated fatty acids (PUFA) and that this was related to their ability to engulf chloroplasts. A study assessed whether increasing intra-protozoal chloroplast resulted in increased throughput of PUFA to the duodenum by comparing flow of protozoa to the duodenum post feeding of a diet low in chloroplast (straw:concentrate) and high in chloroplast (fresh grass). It was found that feeding a fresh grass diet to the steers resulted in a higher protozoal chloroplast content but did not result in their increased contribution to PUFA present at the duodenum. For reasons which are currently unclear, protozoa on grass diet were retained in the rumen. Developing **strategies to increase intra-protozoal chloroplast flow to the duodeum would increase flow of beneficial fatty acids to the small intestine and through to muscle**.

Studies confirmed that feeding red clover, compared to perennial ryegrass, containing the plant secondary component "polyphenol oxidase" (PPO) reduced lipolysis. However, PPO-containing cocksfoot did not reduce lipolysis, suggesting limited potential for grass PPO relative to red clover PPO to alter lipid profiles in beef.

The effects of plant secondary compounds (Catecholamines, Saponins, polyphenol oxidase (*Trifolium pratenses* (wildtype red clover) vs. *Trifolium pratenses* (genetically modified PPO gene 1 silenced red

clover) and tannin (*Lotus Japonicus* (Low tannin) vs. *Lotus pedunculatus* (High tannin)) on lipolysis, biohydrogenation and the rumen microbial ecosystem were assessed in *in vitro* batch culture. The studies demonstrated that (1) saponin (deodorase) was the most effective in reducing biohydrogenation and (2) that for maximum benefit of PPO in red clover it is essential that both the "substrate for the enzyme" and the enzyme are present.

Two large scale beef production studies were conducted to assess the ability of plant-based strategies to produce beef with a fatty acid composition which is more consistent with current human health recommendations and consumer requirements. The first study, 40 Belgium Blue steers were fed on grass-based diets from weaning through to commencing a 120 day finishing period. In this period cattle were fed on either grass silage or barley straw/concentrate with/without a lipid rich plant extract (referred to as PX). The amount of total lipid, neutral lipid, phospholipid, saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in loin steaks did not vary between dietary treatments, but the amount of PUFA, *n*-6 and *n*-3 in the total lipid of meat were differentially affected by diet. Animals receiving the forage-based diets had the lower amount of *n*-6 fatty acids, but higher concentrations of *n*-3 fatty acids (18:3n-3, EPA and DHA), compared to those fed on straw and concentrate. Addition of PX to concentrate or forage increased the amount of *n*-3 fatty acids (18:3*n*-3) in muscle total lipid, with subsequent improvements in the *n*-6:*n*-3 ratios. This study confirmed the **benefits of grass feeding relative to concentrate in improving the content of omega-3 fatty acids in beef lipids**.

The first, and rate-limiting, step of the conversion of 18:3*n*-3 to its long chain derivatives, EPA, DPA and DHA, is elongation to 18:4*n*-3 (stearidonic acid). Provision of a diet rich in 18:4*n*-3 may further enhance the incorporation of EPA, DPA and DHA in beef. The second beef study, involved 32 Charalois steers, examined the effects of feeding an oil containing stearidonic acid (18:4*n*-3; source echium oil; *Echium plantagineum*) relative to linolenic acid (18:3*n*-3; source linseed oil). **Addition of echium oil or linseed oil had no effect on the concentrations of total lipid, neutral lipid, phospholipid, SFA, MUFA or PUFA, compared to feeding forage alone.** Additionally, the ratio of *n*-6:*n*-3 fatty acids, P:S were similarly unaffected by diet, as was the concentration of EPA+DHA in the total lipid of *M. longissiumus*. In both studies described above, diet had little effect on colour shelf-life or sensory properties of the beef.

A series of studies were conducted to examine the effects of altering the fatty acid profile of beef lipids (beef from forage *versus* concentrate fed animals) on plasma lipids and lipoproteins and the development of atherosclerosis in an animal model, using the ApoE*3 Leiden mouse. The effect of adding different oils (linseed oil, fish oil, rapeseed oil or echium oil) to the diet of the mice were also examined. The differences in the fatty acid composition of forage *versus* concentrate fed beef were enough to induce some significant differences in mouse tissue fatty acids but had no overall effect on the development of atherosclerosis. Supplementing beef with relatively modest amounts of unsaturated fatty acid markedly reduced plasma cholesterol and development of atherosclerosis. This effect was seen with all of the oils studied, but surprisingly, rapeseed oil (relatively rich in oleic and linoleic acid) was more potent than any of the *n*-3 PUFA rich oils studied. While extrapolation of results in this animal model to humans should be done with caution, the results suggest that reducing the proportion of SFA in beef may be fundamentally more important that the type of fatty acid they are replaced with.

In conclusion, nutritional quality is an increasingly important factor contributing to food product quality. Much attention has been placed on increasing the content of *n*-3 PUFA in beef and other foods as increased consumption of long chain *n*-3 PUFA would be beneficial in improving health and well-being and reducing disease in man. Green forage rich in the 18:3*n*-3 is an important tool to increasing delivery of *n*-3 PUFA through the ruminant animal into meat. As the 18:3*n*-3 is the building block of the long chain *n*-3 PUFA (EPA and DHA) feeding forage can increase these beneficial PUFA in meat. However, the levels of *n*-3 PUFA, 18:3*n*-3, EPA and DHA achieved by forage feeding fall below the level required to be able to claim that beef is either a "source" or "rich-in" *n*-3 PUFA (based on recommendations of the European Food Safety Authority). Hence, it is essential that the two main factors influencing the levels of *n*-3 PUFA in beef lipids are further addressed, namely (1) strategies to enhance levels of 18:3*n*-3 in forage and subsequent delivery into the animal and (2) increased ability to reduce lipolysis and/or biohydrogenation in the rumen. Recent progress in genetic control of lipids in perennial ryegrasses is likely to help significantly. Increased knowledge of the fate of the lipid rich

chloroplast in the rumen represents a very exciting opportunity to deliver more beneficial *n*-3 PUFA from rumen through to the small intestine and hence to meat lipids.

Background

Quality and the requirement for nutritionally-improved beef

The quality of food is becoming increasingly important to consumers. For meat, the definition of quality is becoming increasingly complex as it encompasses not merely the physical aspects of the meat such as tenderness, juiciness, flavour but also incorporates more recent issues such as safety, traceability, healthiness and production environment. Consumers are progressively more aware of the relationships between diet and health, particularly in relation to cancer and atherosclerosis. Knowledge of these relationships has augmented consumer interest in the nutritional quality of food such that this is becoming a more important dimension of product quality (Scollan et al., 2006).

Beef is considered to be a highly nutritious and valued food. The importance of meat as a source of high biological value protein and micronutrients (including for example vitamins A, B_6, B_{12}, D, E, iron, zinc, selenium) is well recognised. However, over the last 10-15 years, these positive attributes have often been overshadowed due to the prominence given to several negative attributes. The latter include the perception that beef contains high amounts of fat which is rich in saturated fat, associations between red meat and cancer and non-nutritional issues such as animal health scares (BSE) and food chain pathogens (*E. coli* 0157).

Beef is a natural carrier of beneficial *n*-3 PUFA (eicosapentaenoic acid (EPA, 20:5*n*-3) and docasahexaenoic acid (DHA; 22:6*n*-3)) and also CLA and is an important vehicle for the delivery of beneficial fats through to man. Enhancing the levels of these fatty acids in beef will improve the nutritional value of this important meat for the consumer.

Fat content and fatty acid composition of beef

Intramuscular fat (IMF) is the most important fat depot in relation composition and fatty acids for man. It consists on average (proportions), of 0.45 - 0.48, 0.35 – 0.45 and up to 0.05 of total fatty acids as SFA, monounsaturated fatty acids (MUFA) and PUFA, respectively. The polyunsaturated : saturated fatty acid (P:S) ratio for beef is typically low at around 0.1 (Scollan et al., 2006), except for double muscled animals which are very lean (<1% IMF) where P:S ratios are typically 0.5-0.7 (Raes et al., 2001). The *n*-6:*n*-3 ratio for beef is beneficially low, typically less than 3, reflecting the considerable amounts of beneficial *n*-3 PUFA in beef, particularly 18:3*n*-3 and the long chain PUFA, EPA and DHA. Beef also contains CLA and in particular the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. The anticarcinogenic and antiatherogenic effects of *cis*-9, *trans*-11 and the anti-obesity effects of *trans*-10, *cis*-12 have been well documented (Belury, 2003).

Manipulating the fatty acid composition of beef

Plant-based strategies are the most appropriate and sustainable approach to increasing the content of *n*-3 PUFA in beef. Importantly for UK beef production systems, forage is a very important component of the diet and is a cheap and abundant source of *n*-3 PUFA. The transfer of 18:3*n*-3 from forage through to meat is dependent on two important processes, (1) increasing the level of 18:3*n*-3 in the forage (and hence into the animal) and (2) reducing the extent of ruminal biohydrogenation (Scollan et al., 2006).

Studies in Aberystwyth have shown that grass variety, stage of grass growth and method of preservation (silage and hay; extent of wilting etc) influence the concentration of 18:3*n*-3 in grass and clover (Dewhurst et al., 2006). Little research has been conducted to assess the extent of genetic variation, which may exist in either total lipid content, or the fatty acid composition of this lipid and hence the potential opportunity to select grasses for increased fatty acids. If sufficient variation exists then this would provide the opportunity to select for grasses higher in lipid and a higher proportion of 18:3*n*-3 would enhance delivery of this fatty acid into the food chain.

Reduction of the extent of biohydrogenation in the rumen may further enhance incorporation of dietary PUFA into meat. Dietary PUFA are rapidly hydrogenated in the rumen by the microbes, resulting in the production of SFA (principally 18:0) but also in the formation of CLA and trans monoene (principally TVA) intermediates. This is one of the main reasons why ruminant fats are highly

saturated. Lipolysis in the rumen is a prerequisite for the microbial hydrogenation (biohydrogenation) of unsaturated fatty acids. The extent to which biohydrogenation is 'complete' influences the amount of SFA produced in the rumen but also the amount of CLA and TVA. This project examined a range of plant factors, which influence lipolysis and biohydrogenation in the rumen. Increasing our understanding and developing methods of altering lipolysis and biohydrogenation of dietary PUFA in the rumen is essential in terms of providing new opportunities for enhancing the fatty acid composition of beef and other ruminant products.

Measuring the impact of nutritionally enhanced food on human disease

Few studies which have enhanced the content of *n*-3 PUFA in animal projects such as milk and beef have then established the benefits of these nutritionally enhanced foods on either (1) animal models of disease, for example cardiovascular disease or (2) direct studies with man examining impact on indicators of cardiovascular disease (i.e. blood fatty acid profiles). One of the few studies by Noakes et al. (1996) demonstrated that PUFA modified milk resulted in a significant 0.28-mmol/L (4.3%) lowering of total cholesterol ($P < 0.001$). Most of this decrease was in LDL cholesterol, which decreased by 0.24 mmol/L ($P < 0.001$) whereas HDL cholesterol and triacylglycerols remained essentially unchanged. This alteration in the fatty acid profile of dairy products, if applied to population typical of developed Western countries, represents a potential strategy to lower the risk of coronary heart disease without any appreciable change in customary eating patterns.

Considerable evidence suggests that diets rich in *n*-3 PUFA are associated with reduced risk of developing atherosclerotic cardiovascular disease in human populations. Mechanisms underlying these effects have not been fully elucidated but may include reductions in plasma lipids, anti-inflammatory and anti-thrombotic effects. Experiments designed to further investigate such mechanism indicate that different animal models respond in different ways. For example, *n*-3 PUFA actually increase plasma cholesterol and triacylglycerol, and thereby development of atherosclerosis, in cholesterol- fed hamsters, but not rats (Lin et al., 2005). In transgenic mouse models, *n*-3 PUFA appear to specifically protect against atherosclerosis in the LDL receptor knock-out (LDLr-/-) mouse but not the apoE knock-out (apoE-/-) mouse (Zampolli et al., 2006). While both plasma cholesterol and triacylglycerol were decreased by n-3 PUFA in the LDLr-/- animals, both lipids were increased in the apoE-/- strain. By contrast, fish oil has been shown to decrease plasma cholesterol and triacylglycerol in the apoE*3 Leiden mouse model (van Vlijmen et al., 1998). This later strain expresses the human apoE*3 Leiden gene, resulting in impaired lipoprotein clearance and a lipoprotein profile similar to humans. Such mice readily develop diet-induced hyperlipidemia and atherosclerosis and are highly responsive to changes in the fatty acid profile of the diet (de Roos et al., 2005). This mouse model is an attractive option for studying the impact of altering the fatty acid profile of beef lipids on plasma lipids and lipoproteins and the development of atherosclerosis.

Objectives

The overall objective was to investigate key factors influencing the delivery of beneficial lipids from plants (forage) through to beef muscle. The aim was to enhance the nutritional value of beef for the consumer by improving the content of beneficial fatty acids using safe and natural (forage) strategies. The research was conducted under the five objectives:

1. To assess the degree of variation in both total lipid and individual fatty acids in perennial ryegrass and identify associated QTLs.
2. To examine interactions between plant components and events in the rumen (lipolysis and biohydrogenation) which determine the ability of different plants to manipulate the fatty acid composition of beef.
3. To assess the ability of plant-based strategies (based on PUFA-rich grasses) to produce beef with a fatty acid composition which is more consistent with current human health recommendations and consumer requirements, containing higher amounts of *n*-3-PUFA, CLA and lower levels of saturated fatty acids.
4. To assess the impact of strategies imposed in objective 3 on the fatty acid composition of beef, (including PUFA, CLA, *trans*-fatty acids) and colour shelf life and sensory attributes of beef and beef products.
5. To assess the impact of altering the fatty acid profile of beef lipids on plasma lipids and lipoproteins and the development of atherosclerosis in an animal model.

Objective 1: *To assess the degree of variation in fatty acid composition of perennial ryegrass*.

Background
Estimating the heritability of variation in fatty acid components of lipids in the foliage of perennial ryegrass is a prerequisite to identifying the prospects for genetic improvement of these traits. If the heritability is significant, breeding is possible.

A dense genetic map can help to improve the resolution of QTL mapping, so the F1 mapping family consisting of 96 segregating individuals, was used as a test-bed for increasing the density of the map. In addition to the 56 SSR (microsatellite) markers already on the map, over 450 single nucleotide (SNP) markers were added to the map using restriction associated DNA (RAD) marker technology (Baird et al., 2008). The improved map was used in the subsequent QTL analysis.

Main Objectives
The work seeked to quantify the heritability of fatty acid constituents in perennial ryegrass, and secondly, to identify the most significant QTL associated with concentration of fatty acids. Such information will lay the foundations for enhancing those constituents for the benefit of forage quality.

This is, to our knowledge, the first analysis and identification of quantitative trait loci of fatty acids in perennial ryegrass, including the first determination of heritability of this trait in forage grasses. Additionally, as this analysis was based exclusively on field evaluations over a period of three years, it represents a realistic platform for future genetic improvement, candidate gene identification and genetic dissection of this complex and important trait.

Results
Experiment 1 outcomes
Plant material: Progeny from a cross between genotypes of an old variety Aurora and a recently developed high sugar grass variety AberMagic was used in this study. RAD marker and fatty acid analysis was performed on the two parents and 95 progeny. The plants were grown in the field near IBERS as spaced plants, with management cuts every 6 weeks and annual application of 250 kg N ha^{-1}. Two replicate clones of each genotype was planted in a randomised design in two blocks. Leaf material was harvested for fatty acid analysis in June and September of 2008, 2009 and 2010.

Analysis of fatty acids: Fatty acid content was determined from 1 g of freeze-dried material using heneicosanoic acid methyl ester (C21:0) as an internal standard (Sigma-Aldrich Co, St Louis, MO, USA) and a one-step extraction-transesterification procedure. Fatty acid methyl esters (FAME) were separated and quantified using a gas chromatography.

RAD marker identification: DNA was extracted from leaf tissue using the DNeasy 96 Plant Kit (QIAGEN, Crawley, West Sussex, UK) and quantified via picogreen detection analysis. RAD marker sequencing and identification was carried out by Floragenex (Eugene, Oregon, USA) using an Illumina HiSeq2000 NGS platform (Baird *et al.* 2008).

Genetic map, QTL detection and statistical analyses: The genetic map was constructed using the JoinMap4 programme, and the QTL analysis was carried out using the programme MapQTL 5. A more detailed description of these analyses as well as the statistical methods used for determining heritability can be seen in Hegarty *et al.* (2013). Suffice it to say that the experiment was treated as a split-plot design in time with cuts as subplots. A linear mixed model was used to estimate variance components and broad sense heritability. The analysis was carried out using REML (restricted maximum likelihood) analysis in Genstat (14^{th} edition;VSN International Ltd, Hemel Hempstead, UK).

Results
Phenotypic analysis: The means and standard deviations for the five major fatty acid consitutents (16:0; 18:0; 18:1n-9; 18:2n-6 and 18:3n-3) for each of the three years is shown in Table 1. 18:2n-6 and 18:3n-3 constituted by far the largest percentage of the total, and their values were highest in year 2, while the three minor constituents tended to increase for every cut. Table 1 also shows that the five constituents measured here made up nearly all the fatty acids present in the samples. A variance component analysis using the REML model across all six cuts demonstrated a significant effect of genotype and of cut, but little evidence of genotype by cut interaction (Table 2).

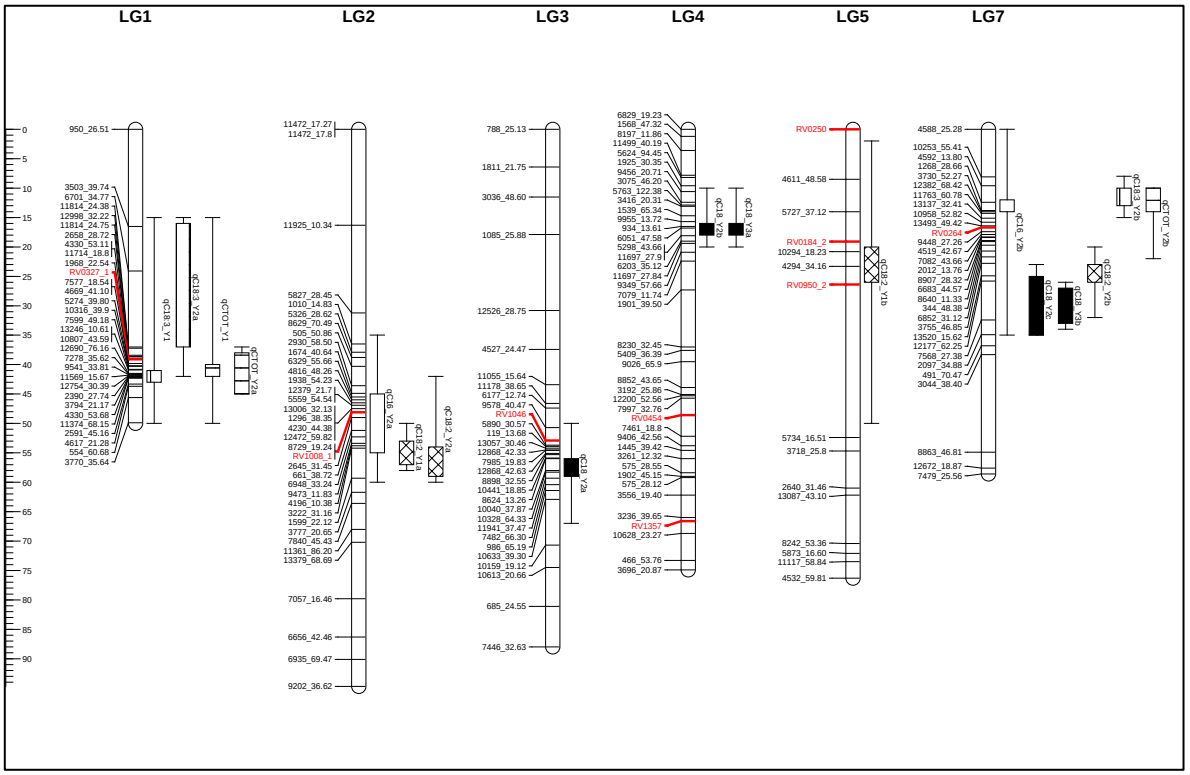

Figure 1. QTL of fatty acid constituents at different years. The bars indicate the extent of the QTL by composite interval mapping, and the line extensions indicate the interval mapping QTL. SSR markers are highlighted in red.

QTL analysis: The RAD markers and the SSR markers were used to construct a linkage map consisting of seven linkage groups comprising of 434 markers and spanning a total of 505 cM. A QTL analysis consisting of a combination of interval mapping and composite interval mapping revealed the presence of significant QTLs on six of the seven linkage groups of perennial ryegrass. The QTLs for the different fatty acid constituents and years are summarised in Figure 1. The major findings are that significant QTLs for linolenic acid (18:3*n*-3) and total fatty acids were found on linkage group 1 (LG). Furthermore, QTLs for linoleic acid (18:2*n*-6) were found on LGs 2 and 5, while there were strong associations with stearic acid (18:0) on LGs 4 and 7.

Message: The main conclusions of this work is that the broad sense heritabilities of concentrations of all the fatty acid constituents in the foliage of perennial ryegrass, except oleic acid were 0.5 or above. Such high heritabilities suggest that these traits can indeed be improved genetically. Seasonal and environmental effects play a substantial role, but the significant contribution of the genetic component to the phenotypic variation in fatty acid content is encouraging for the prospects of genetic improvement of qualitative and quantitative aspects of fatty acid content in forage grasses for ruminant nutrition. The generation of a dense genetic map has also allowed us to pinpoint the genomic regions underlying the traits, and the markers provide interesting leads for further analysis of these traits. A more complete description of this work is given by Hegarty et al., 2013.

Table 1. Mean and standard deviation (mg $(gDW)^{-1}$) of fatty acid components in the perennial ryegrass Aurora x AberMagic population. The values for each year are means of two cuts.

Year	Population	n	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	CTOT
All	F1	1095	4.26 ±0.620	0.39 ±0.078	0.38 ±0.139	2.73 ±0.633	15.02 ±4.015	25.03 ±5.253
All	Aurora-17	13	3.92 ±0.693	0.38 ±0.103	0.46 ±0.270	2.65 ±0.846	12.09 ±3.708	22.95 ±8.706
All	AberMagic	13	4.01 ±0.579	0.39 ±0.073	0.46 ±0.133	2.94 ±0.705	11.61 ±2.889	22.42 ±6.123
1	F1	340	3.77 ±0.513	0.36 ±0.073	0.35 ±0.116	2.71 ±0.447	14.66 ±2.440	23.41 ±3.141
1	Aurora-17	3	2.89 ±0.140	0.32 ±0.036	0.42 ±0.082	2.52 ±0.267	9.12 ±1.305	16.58 ±1.059
1	AberMagic	3	3.26 ±0.544	0.34 ±0.041	0.33 ±0.044	2.69 ±0.420	10.17 ±2.385	18.29 ±3.141
2	F1	378	4.20 ±0.389	0.39 ±0.060	0.37 ±0.123	3.14 ±0.372	18.03 ±3.193	28.96 ±4.581
2	Aurora-17	4	3.88 ±0.428	0.42 ±0.135	0.58 ±0.501	3.30 ±1.047	14.53 ±4.336	29.59 ±12.764
2	AberMagic	4	3.91 ±0.220	0.42 ±0.075	0.57 ±0.119	3.56 ±0.558	13.44 ±2.874	27.10 ±8.462
3	F1	377	4.75 ±0.513	0.42 ±0.088	0.40 ±0.167	2.35 ±0.732	12.35 ±3.861	22.55 ±5.128
3	Aurora-17	6	4.45 ±0.247	0.39 ±0.102	0.40 ±0.059	2.28 ±0.722	11.96 ±3.311	21.71 ±4.718
3	AberMagic	6	4.44 ±0.301	0.40 ±0.079	0.46 ±0.117	2.64 ±0.698	11.11 ±2.929	21.37 ±3.741

Table 2. Variance components ± standard error and broad sense heritabilities (H_B^2) of the five fatty acids (C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2 linoleic acid; C18:3, linolenic acid) and the total (TOT) across all cuts. σ_g^2, σ_t^2, $\sigma_{g.t}^2$, σ_r^2, $\sigma_{g.r}^2$ and σ_e^2represent the genotype, cut, genotype by cut, replicate and genotype by replicate and error variance components respectively. Negative values were treated as zero.

	Trait					
	C16:0	C18:0	C18:1	C18:2	C18:3	TOT
σ_g^2	0.0235 ± 0.00602	0.0010 ± 0.00018	0.0009 ± 0.00064	0.0303 ± 0.00790	1.2258 ± 0.24908	1.4414 ± 0.39168
σ_t^2	0.2381 ± 0.15118	0.0038 ± 0.00239	0.0007 ± 0.00049	0.2288 ± 0.14519	9.3603 ± 5.93766	13.5114 ± 8.58929
$\sigma_{g.t}^2$	-0.0145 ± 0.00824	0.0001 ± 0.00010	0.0018 ± 0.00074	-0.0236 ± 0.00748	-1.5640 ± 0.32383	-1.8656 ± 0.69248
σ_r^2	0.0003 ± 0.00087	0.0001 ± 0.00008	0.0004 ± 0.00067	0.0170 ± 0.02475	0.8416 ± 1.20795	1.4583 ± 2.09690
$\sigma_{g.r}^2$	-0.0010 ± 0.00505	0.0001 ± 0.00006	0.0024 ± 0.00071	0.0136 ± 0.00680	-0.2418 ± 0.19495	-0.5384 ± 0.38208
σ_e^2	0.1876 ± 0.01249	0.0019 ± 0.00013	0.0135 ± 0.00091	0.1796 ± 0.01194	8.2347 ± 0.54628	16.4033 ± 1.08969
H_B^2	***0.59***	***0.81***	***0.25***	***0.57***	***0.63***	***0.50***

Objective 2: *Interactions between plant components and events in the rumen (lipolysis and biohydrogenation) which determine the ability of different plants to manipulate the fatty acid composition of beef.*

Background
Studies focused on the use of grass and/or clover and their cellular composition to beneficially manipulate the fatty acid composition of beef by altering lipolysis and biohydrogenation in the rumen. Phospholipids are abundant within the chloroplast membranes and understanding the fate of chloroplasts within the rumen may open further opportunities to enhance the fatty acid composition of beef. Additionally the 'Alpine factor', whereby animals receiving alpine pasture have reduced biohydrogenation, may be linked to secondary plant metabolites, including PPO, saponins, tannins and catecholamines. These compounds potentially may alter lipolysis or biohydrogenation to enhance the fatty acid composition of beef.

Main Objectives
Experiment 2a and b: Assessed *in vitro* the rate of ingestion, digestion and ejection of chloroplast within the rumen protozoa as well as assessing *in vivo* the protection afforded by this intracellular location on PUFA flow to the duodenum.

Experiment 2a: *In vitro* studies were conducted to enhance understanding of the fate of PUFA-rich chloroplasts in the rumen and in particular the relationship between chloroplasts and rumen protozoa. Intact chloroplasts were prepared from spinach (Spinacea oleracea) leaves using standard techniques by Mills and Joy (1980). The harvested chloroplasts were fed to both *Epidinia* spp. and *Entodinia* spp. Intracellular and extracellular chlorophyll as well as the fatty acid content of the protozoa were monitored at time intervals of 0, 0.5, 1, 2, 4 and 8 h. The intracellular location in protozoa of auto-fluorescing chloroplast were assessed using fluorescence confocal microscopy.

Experiment 2b: *In vivo* – Four Hereford × Friesian steers prepared with rumen and duodenal cannulae will be offered a restricted diet of fresh grass (high chloroplast diet) and hay (low chloroplast diet). Duodenal flow of 18:2*n*-6 and 18:3*n*-3 will be calculated under both diets. Rumen protozoa will also be collected and purified, under both diets, in order to both quantify the intracellular chlorophyll content and as a standard for quantitative PCR (qPCR). Protozoal flow to the duodenum under both diets will be quantified using qPCR. Knowing the chlorophyll content of the protozoa within the rumen and their flow to the duodenum an estimate of the flow of intracellular chloroplast to the duodenum will be made. The relationships between PUFA fatty acid and protozoa to the duodenum, thus providing evidence for a link to intracellular chloroplast, were undertaken using canonical correlation analysis.

Experiment 3: Assessed the effect of grass PPO on protecting 18:3*n*-3 and 18:4*n*-4 supplied from *Echium spp.* oil across the rumen. Six Hereford × Friesian steers prepared with rumen and duodenal cannulae fed on red clover silage (high PPO), perennial ryegrass (intermediate grass PPO) or cocksfoot (high grass PPO) silage each supplemented with *Echium spp.* oil in a paired 3 x 3 Latin square design. Duodenal flows of fatty acids were determined and the extent of biohydrogenation calculated in each diet and the protection offered to 18:4*n*-3 determined.

Experiment 4: Studies, *in vitro*, assessed the effect of plant secondary compounds (PPO, saponins, tannins and catecholamines) on *n*-3 PUFA biohydrogenation and the rumen microbial ecosystem. Key microbial species involved in biohydrogenation will be assessed using both denaturing gradient gel electrophoresis (DGGE) and qPCR. The relationships between fatty acid metabolism and the microbial species were established using canonical correlation analysis.

Results
Experiment 2a outcome: *A series of in vitro* experiments were conducted with the objective to enhance understanding of the fate of PUFA-rich chloroplasts in the rumen and in particular the relationship between chloroplasts and rumen protozoa. A number of unforeseen technical problems have hampered the success of these *in vitro* approaches. Studies initially attempted to rid protozoa of as much intracellular chloroplasts as possible so that at time 0 h they would only contain very low amounts of intracellular chloroplast prior to addition of chloroplasts. Firstly we fractionated protozoa and then incubated them anaerobically to monitor the time it takes for them to egest their intracellular chloroplasts. Protozoal density, viability and the presence of intracellular chloroplast over a 24 h time

course were monitored. Protozoal viability dropped steadily with few viable cells visible post 6 h, and microscopy showed that protozoa had much intracellular chloroplast present even after 24h. Following on, we tried to replace intracellular 18:3*n*-3 rich chloroplasts with a 16:3*n*-3 rich chloroplast by feeding them maize post-fractionation. This experiment showed again protozoa are not very active post 6h, so removal of C18:3*n*-3 rich chloroplasts and the experiment itself need to be conducted within this time, which is not feasible. We then decided to obtain protozoa from cows fed straw (low 18:3*n*-3 and chlorophyll) 1 week before experimentation, and use these directly post-fractionation in the experiments. Experiments were set up *in vitro* with a ratio of 1:100 (1×10^4 protozoa/mL: 1×10^6 chloroplasts/mL). Given the experimental design necessary and the amount of protozoa we could extract from rumen fluid we were limited to protozoal densities of 1×10^4 protozoa/mL. Unfortunately, using these densities, fatty acids and chlorophyll were undetectable. Thus due to viability and density issues we concluded that monitoring ingestion/digestion/egestion events *in vitro* would not work. Subsequently we embarked upon an *in vivo* experiment whereby six Hereford × Friesian steers (mean live weight 250 kg), were fed straw and concentrate for 14 d before switching to fresh grass for one day only (just the morning feed). Feed was sampled every day for the straw:concentrate diet and pooled weekly (frozen), whilst a grass sample was taken on the morning of the experiment only (approximately 1 kg) and frozen. Protozoa, planktonic and attached bacteria were harvested -1 h before the change in diet and 2 and 6 h post the change. Chlorophyll and fatty acid content of each microbial fraction was analysed and fixed samples were monitored for intracellular chloroplasts using confocal microscopy and transmission electron microscopy. The straw concentrate diet had more ADF, NDF, 18:1*n*-9, and less WSC, oil, 16:0, 18:2*n*-6 and 18:3*n*-6 compared to the fresh grass diet as expected (See Appendix 1 Table 1). Fatty acid data showed that protozoa were significantly enhanced in 16:0, 18:0, 18:2*n*-6, 18:3*n*-6, 18:1 *trans*-11 content 2 h post feeding of fresh grass and levels of each of these fatty acids became somewhat reduced at 6 h although 16:0, 18:0 and 18:3*n*-3 intra-protozoal concentrations remained significantly higher than values obtained 1 h before feeding fresh grass (Table 3). Microscopy data showed that after 2 h of fresh grass feeding, protozoa had substantially more intracellular chloroplasts which remained high after 6 h of feeding, thus fatty acid data was correlated to intra-protozoal chloroplast content (data not shown). These data illustrate that protozoal uptake of mainly plant bound chloroplasts occurs quickly and intracellular levels of chloroplasts are maintained for at least 6 h. Thus protozoa rapidly become a major reservoir of chloroplasts and subsequently human health beneficial fatty acids.

Table 3: Fatty acid content (mg g^{-1} DM) of protozoa, at various time intervals pre- and post-feeding on fresh perennial ryegrass.

	Time (h)			SED	*P*
Fatty acid	-1	2	6		
18:3*n*-3	0.052[a]	0.604[b]	0.511[b]	0.062	**<0.001**
18:2*n*-6	0.955[a]	2.805[b]	1.586[a]	0.298	**<0.001**
18:2 *cis*-9, *trans*-11	0.316[a]	0.298[a]	0.334[a]	0.070	0.879
18:1 *trans*-11	1.166[a]	2.792[c]	1.681[b]	0.206	**<0.001**
18:0	5.728[b]	7.479[c]	4.516[a]	0.400	**<0.001**
16:0	5.281[a]	11.625[c]	8.201[b]	0.585	**<0.001**

Experiment 2b outcome: Protozoal contribution to duodenal *n*-3 PUFA flow due to intracellular chloroplast content was assessed. Six Holstein × Friesian steers were fed, in a two-period change-over design, either straw:concentrate (60:40; dry matter basis; S:C, low chloroplast) or fresh perennial ryegrass (PRG; high chloroplast). Following 12 d adaptation to diet, ruminal protozoal and whole duodenal samples were obtained. Nitrogen and fatty acid content of whole duodenum and rumen protozoal samples were assessed and protozoal 18S rDNA qPCR performed, enabling calculation of protozoal nitrogen flow (Table 4). Protozoal fatty acid data and microscopical observations revealed that protozoa were enriched with 18:3*n*-3 following PRG feeding, compared with the S:C diet, due to increased intracellular chloroplast content (Table 2; Appendix 1). However, duodenal protozoal 18S rDNA concentration post PRG feeding was low indicating rumen retention of the protozoa. The consequence of this rumen retention of protozoa following fresh grass feeding was that little 18:3*n*-3 or indeed any other fatty acid flowed to the duodenum compared with values obtained following straw:concentrate feeding (Table 5).

Message: Clearly nutrition influences the 18:3*n*-3 content of protozoa; the challenge ahead is to increase protozoal flow to the small intestine, whilst maintaining sustainable rumen densities. This work has been published (Huws, *et. al.* (2012).

Table 4. Daily intakes and duodenal flows of dry matter (DM), organic matter (OM) and nitrogen (N) in steers straw:concentrate (S : C) or fresh perennial ryegrass (PRG) (g/d, unless stated).

	Diet		SED	*P*
	S : C	PRG		
Intake				
DM (kg/d)	8.80	9.42	0.54	NS
Total N	191	207	11.8	NS
Water soluble carbohydrate (WSC)	607	1488	52.2	**<0.001**
Neutral detergent fibre (NDF)	4136	4481	255	NS
Acid detergent fibre (ADF)	2439	2571	149	NS
Fatty acid intake				
12:0	0.28	0.26	0.02	NS
14:0	0.75	1.10	0.10	**0.007**
16:0	27.0	36.0	1.80	**0.008**
16:1*n*-7	1.4	0.4	0.07	**<0.001**
18:0	3.26	2.73	0.20	**0.05**
18:1*n*-9	54.0	4.2	2.59	**<0.001**
18:2*n*-6	50.7	35.0	3.21	**0.008**
18:3*n*-3	15.1	153	3.71	**<0.001**
Duodenal flow				
DM (kg/d)	12.2	12.1	0.74	NS
OM (kg/d)	11.8	11.5	0.63	NS
Total N	125	169	31.5	NS
Protozoal N	34.4	0.70	9.84	**0.027**

NS = Not Significant (*P*>0.10)

Table 5. Total fatty acid and protozoal-related fatty acid duodenal flow in steers fed straw:concentrate (S : C) or fresh perennial ryegrass (PRG).

	Duodenal flow (g/d)				Protozoal flow (g/d)				Contribution*	
	S : C	PRG	SED	*P*	S : C	PRG	SED	*P*	S : C	PRG
14:0	2.29	1.72	0.21	**0.024**	0.26	0.00	0.07	**<0.001**	11.4	0.33
15:0	1.44	1.27	0.15	**<0.001**	0.32	0.00	0.19	**<0.001**	52.8	3.15
16:0	24.9	25.5	1.30	**0.008**	5.36	0.16	3.91	0.114	21.5	0.63
17:0	1.32	1.56	0.15	**<0.001**	0.13	0.00	0.09	**<0.001**	9.85	0.23
18:0	88.6	102	9.77	**0.006**	8.76	0.26	7.66	0.193	9.89	0.25
18:1 *trans*-11	5.20	24.0	2.34	**<0.001**	1.36	0.07	0.90	**<0.001**	26.2	0.29
18:2*n*-6	10.2	2.21	0.33	**<0.001**	1.32	0.02	0.88	**<0.001**	12.9	0.09
18:3*n*-3	1.66	3.06	0.23	**<0.001**	0.14	0.01	0.11	**<0.001**	8.43	0.33
cis-9, *trans*-11 CLA	0.10	0.08	0.02	**<0.001**	0.08	0.00	0.134	**<0.001**	80.0	2.00
trans-10, *cis*-12 CLA	0.00	0.06	0.01	**<0.001**	0.00	0.00	0.00	**<0.001**	50.0	0.00
Total fatty acids	173	196	15.7	**0.002**	23.8	0.46	16.4	**0.096**	13.8	0.23

*Contribution of protozoa to fatty acid duodenal flow (protozoal fatty acid flow/total fatty acid flow *100%).

Experiment 3 outcome: The study investigated protection of PUFA across the rumen in grasses with contrasting PPO activity. Six Hereford × Friesian steers (mean liveweight 350-450 kg), prepared with rumen and duodenal cannulae were allocated to either: red clover silage (RC); cocksfoot silage (CF) or perennial ryegrass silage (PRG). All silages were fed at a fixed rate of 15 g DM/kg live weight. The experiment consisted of two 3 × 3 Latin-Squares, each period was 21 d, consisting of 14 d adaptation to the diet, 4 d for faecal collection, 2 d for duodenal sampling and 1 d for rumen sampling. All silages were well preserved with mean dry matter (DM) of 34.4, 55.3 and 45.4% for CF, PRG and RC, respectively. PPO activity in the silages was low due to deactivation but was higher (P<0.05) in CF than either PRG or RC, 0.15, 0.05 and 0.08 μkatal/g DM, respectively. Protein bound phenol (mg/g DM) as a measure of the degree of oxidation and therefore PPO protection was as expected: highest on RC (15.9), lowest on PRG (10.1) with CF intermediate (12.2). DM and fatty acid intake along with fatty acid flow and biohydrogenation are reported in Table 6. As animals did not consume all offered forage on the grass silage diets there were differences in intake with subsequent differences in fatty acid flow to the duodenum. Flow of C18:0 was comparable across treatments with highest flows of C18:1 trans, CLA and C18 PUFA on RC with PRG intermediate and CF the lowest. Biohydrogenation of C18 PUFA was significantly lower on RC compared to the two grass silages with CF higher than PRG. Differences in intake were due to animals not eating their whole ration.

Table 6. Effect of grass silage with contrasting PPO levels versus red clover silage on intake and ruminal fatty acid metabolism.

	CF	PRG	RC	SED	*P*
DM Intake (kg/d)	5.87[a]	6.58[b]	7.55[c]	0.197	**<0.001**
Fatty acid intake (g/d)					
C18:0 stearic	1.52[a]	2.62[b]	4.04[c]	0.150	**<0.001**
C18:2*n-6* linoleic	16.6[a]	21.0[b]	32.5[c]	0.84	**<0.001**
C18:3*n-3* linolenic	43.5[a]	55.1[b]	59.6[b]	2.21	**<0.001**
Total fatty acids	93.4[a]	119[b]	144[c]	4.71	**<0.001**
Duodenal flow (g/d)					
C18:0 stearic	71.1	75.7	77.1	4.48	NS
C18:1 trans	8.97[a]	12.6[ab]	13.5[b]	0.73	**<0.001**
C18:2 CLA	0.22[a]	0.26[a]	0.79[b]	0.027	**<0.001**
C18:2*n-6* linoleic	1.55[a]	2.54[b]	7.22[c]	0.290	**<0.001**
C18:3*n-3* linolenic	2.43[a]	4.44[b]	13.5[c]	0.527	**<0.001**
Total fatty acids	148[a]	160[a]	187[b]	8.1	**<0.01**
Biohydrogenation (%)					
C18:2*n-6* linoleic	90.6[c]	87.9[b]	77.9[a]	0.72	**<0.001**
C18:3*n-3* linolenic	94.4[c]	92.1[b]	77.6[a]	0.43	**<0.001**

Values with different superscripts ([abc]) differ significantly (P<0.01).

As previously reported RC resulted in a lower biohydrogenation of C18 PUFA than grass silages. CF with its higher levels of grass PPO did not result in elevated levels of C18 PUFA escaping the rumen over the control PRG with lower levels of PPO.

Message: This may suggest that grass PPO has limited potential in improving lipid profiles within ruminant products, although other factors between the grasses cannot be ignored within the present study, which may have contributed to the observed response.

Experiment 4 outcome: The effect of plant secondary compounds on lipolysis, biohydrogenation and the rumen microbial ecosystem were assessed in *in vitro* batch culture. The study consisted of two experiments (Experiment A. Catecholamines and Saponins; Experiment B. Polyphenol oxidase and Tannins). Experiment A consisted of 36 incubations each with 1 g DM of freeze-dried *Lolium perenne* as the basal feed with or without either catecholamine (dopamine 100 ug/g FW) or saponin (1% deodorase). Within Experiment B 48 incubations were used with four treatments: *Trifolium pratenses* (wildtype red clover) vs. *Trifolium pratenses* (genetically modified PPO gene 1 silenced red clover) and *Lotus Japonicus* (Low tannin) vs. *Lotus pedunculatus* (High tannin). Each of the treatments were incubated (in triplicate) in rumen inoculum at 39°C over 4 time points (0, 2, 6 and 24 h). Batch cultures were harvested by thorough homogenisation and samples were analysed for total lipid and lipid fractions to determine level of lipolysis and C18 PUFA biohydrogenation. Samples were also taken for profiling of the microbiota, using RNA as a marker. Saponins, bound phenols and tannins all resulted in a significant reduction in lipolysis in batch culture. There was no effect of catecholamines or red clover differing in PPO content (data not shown). With saponins and tannins this had a direct effect in reducing 18:3*n*-3 biohydrogenation, whereas for bound phenols there was a time lag before the effect was shown (Table 7). Saponins and bound phenols appeared to have a greater effect during early fermentation (2-6 h) whereas the tannin effect was maintained throughout the incubations (Table 8). This may be as a result of depletion of the plant secondary metabolite (PSM) or adaptation of the microbial population. Bacterial diversity revealed changes in the bacterial community following saponin addition which are in line with the fatty acid data in that dendograms showed a clustering of 2 and 6 h samples separately to control samples but at 24 h similarity between saponin and control samples were observed (see Figure 1 Appendix 1). Reverse-transcription of RNA to cDNA for the condensed tannin experiments revealed limited bacterial activity and thus retrieval of cDNA of a high enough concentration for bacterial diversity analysis was not possible. It is likely that the condensed tannins present within *L. pedunculatus* were highly toxic to the rumen microflora.

Message: In summary, this experiment illustrates that deodorase was the most promising for beneficially altering rumen lipid metabolism.

Table 7. Biohydrogenation (%) of 18:3*n*-3 in grass with and without saponins or catecholamines over time in batch culture.

Time	Control	Catecholamine	Saponin	Treat. SED	Treat. Sig
2	43.9[b]	40.2[b]	24.9[a]		
6	45.7[b]	52.2[b]	32.2[a]	2.57	***

24	56.9	55.5	50.8	
SED Time			2.57	
Sig. Time			***	
SED Time*Treatment			4.45	
Sig. Time*Treatment			NS	

[a,b] Differing superscripts indicates significant differences within rows at ***P<0.001. NS, non significant

Table 8. Biohydrogenation (%) of 18:3*n*-3 in Lotus with high and low tannin content over time in batch culture.

Time	Tannin+	Tannin-	Treat. SED	Treat. Sig
2	0.00[a]	17.2[b]		
6	8.00[a]	23.2[b]	2.20	***
24	16.6[a]	26.5[b]		
SED Time		2.69		
Sig. Time		**		
SED Time*Treatment		3.81		
Sig. Time*Treatment		NS		

[a,b] Differing superscripts indicates significant differences within rows at **P<0.01, ***P<0.001. NS, non significant

Objective 3: *To assess the ability of plant-based feeding strategies to produce beef with enhanced content of beneficial fatty acids.*

Background

This objective examined the ability to enhance the fatty acid composition of beef, in particular to increase deposition of 18:3*n*-3 and the long chain C20 and C22 fatty acids, EPA, DPA and DHA in beef, through a plant-based feeding strategy. Feeding grass, rather than concentrates, provides an enriched 18:3*n*-3 diet for cattle and can increase the amount of *n*-3 fatty acids in beef. However the effects of long-term feeding of fresh or ensiled grass, rather than concentrate-feeding, on beef fatty acid composition had not been assessed. The first, and rate-limiting, step of the conversion of 18:3*n*-3 to its long chain derivatives, EPA, DPA and DHA, is elongation to 18:4*n*-3 (stearidonic acid). Provision of a diet rich in 18:4*n*-3 may further enhance the incorporation of EPA, DPA and DHA in beef.

Main Objectives

Experiment 5 objective: To assess the effect of a "lifetime" nutritional feeding strategy based on grass either fed fresh or conserved as silage with/without concentrate enriched in *n*-3 PUFA. Initially, linseed oil was going to be the source of *n*-3 PUFA enrichment, however following consultation with DEFRA and devolved bodies, a plant extract (PX) produced from the liquid fraction of fresh lucerne (*Medicago sativa* L.) that had been extracted, heat-treated and dried was used instead.

Experiment 6 objective: To assess the effect of feeding plant oils rich in 18:4*n*-3 PUFA (from *Echium spp*.) on the fatty acid composition and meat quality of beef steers.

The beef production experiments described under objective 3 also formed the experimental basis for objective 4. The nutritional composition of the diets, the animal production data (growth rate and feed intake) and carcass composition were examined under objective 3. The fatty acid composition and meat quality (shelf-life characteristics and sensory assessment) were assessed under objective 4.

Results

Experiment 5 outcome: A "lifetime" nutritional feeding trial was completed at Aberystwyth University, using 40 Belgium Blue steers to assess the effect of supplementation of a forage based diet with a plant-based source rich in 18:3 *n*-3. The PX extract, produced by Desialis, resulted in a product that was ~10% oil, containing 1.6 and 4% linoleic and α-linolenic acid, respectively. The PX was fed as a crumb or incorporated into a concentrate feed. Cattle were initially fed *ad libitum* perennial ryegrass as silage or as summer grazing, before assignment to one of five diets (n=8/diet; mean age at start of experiment diet = 506 d); 1) *ad libitum* grass silage only (GS), 2) *ad libitum* grass silage plus 75 g PX/dry matter intake (DMI; GS-LPX), 3) *ad libitum* grass silage plus 150 g PX/DMI (GS-HPX), 4) restricted straw and concentrate (40:60 on a DM basis; S-CC), 5) restricted straw plus concentrate containing 25% PX and additional vitamin E (~300 mg/kg), (40:60 on a DM basis; S-PXC). The

composition of these diets is shown in Table 9. Animals were maintained on the diets until achieving fat class 3L (average number of days on experimental diet = 118 d), whereupon they were slaughtered at the University of Bristol. Carcasses were assessed, and the *M. longissimus* sampled for analysis of fatty acid composition and sensory assessment (under Objective 4).

Table 9. Chemical composition and fatty acid composition (g/kg DM, unless otherwise stated) of experimental feeds.

	Feed				
	Control Concs	PX Concs	PX Crumb	Straw	Silage
Chemical composition					
Dry matter (g/kg fresh matter)	879.4	878.9	924.7	842.7	364.8
Total nitrogen (TN)	37.4	36.6	85.8	7.9	24.9
Water-soluble carbohydrates (WSC)	92.1	62.5	20.8	15.1	125.2
Neutral detergent fibre (NDF)	217.8	237.2	13.7	807.7	499.2
Acid detergent fibre (ADF)	93.2	82.5	39.0	521.4	308.7
Ether extract (EE)	64.3	64.1	137.0	9.4	38.8
Ammonia nitrogen (g/kg TN)	-	-	-	-	85.4
pH	-	-	-	-	3.92
Feed fatty acid composition					
Total	72.2	47.4	86.0	4.2	25.6
12:0	0.6	0.3	0.5	0.06	0.1
14:0	0.6	0.8	2.0	0.3	0.2
16:0	22.0	9.2	15.5	1.2	4.5
18:0	2.1	0.8	2.1	0.1	0.3
18:1*n*-9	18.6	5.0	2.7	0.2	0.5
18:2*n*-6	23.0	18.0	14.8	0.6	3.4
18:3*n*-3	3.4	11.1	42.5	0.7	14.7

The total feed intake of cattle on the S-CC and S-PXC diets was greater than that of animals receiving forage-based diets (Table 10). However, the live weight gain, age at slaughter, half carcass weight and carcass conformation and fatness scores were similar across all diets (Table 10). The successful equalisation of growth rates and carcass conformation across the diets avoided any confounding effects of variations in animal fatness and slaughter age upon the fatty acid composition of the IMF (measured under objective 4). Feed intake of fatty acids varied across the diets, with the S-CC animals having the highest intake of 16:0, 18:0 and 18:2*n*-6, and the lowest intake of 18:3*n*-3 compared to all other diets (Table 10). Forage-based diets resulted in a higher feed intake of 18:3*n*-3 compared to concentrate-based diets, but whilst animals receiving the GS-HPX diet had the greatest feed intake of 18:3*n*-3, the lowest feed intake of 16:0, 18:0 and 18:2*n*-6 was achieved by animals fed silage alone (GS) (Table 10). Incorporation of PX into a concentrate feed (S-PXC), resulted in increased feed intake of 18:3*n*-3 and reduced intake of 16:0, 18:0 and 18:2*n*-6, compared to S-CC animals; whereas the reverse was true when S-PXC diet was compared to silage-based diets (Table 10). Thus, in terms of providing a dietary intake low in SFA and 18:2*n*-6, and rich in 18:3*n*-3, the S-PXC diet provided an intermediate level of dietary supply of fatty acids, compared to S-CC and forage-based diets (± PX); where the GS diet provided the lowest SFA feed intakes and increased intake of 18:3*n*-3 (compared to concentrate). Addition of PX to forage diets incrementally further increased 18:3*n*-3 intake, but also that of 18:2*n*-6 and SFA.

Table 10. Feed intake and animal performance of Belgium Blue cattle fed control concentrates (S-CC), straw + PX-concentrate (S-PXC), grass silage (GS), silage + 75 g PX/kg silage DMI (GS-LPX), or silage + 150 g PX/kg silage DMI (GS-HPX).

	Diet					SED	*P*
	S-CC	S-PXC	GS	GS-LPX	GS-HPX		
Feed intake kg DM/day							
Total intake	10.5^{a}	10.6^{a}	8.3^{b}	8.9^{b}	8.7^{b}	0.32	**<0.001**
Concentrate or PX intake	6.8	6.8	-	0.6	1.1	NA	NA
Forage intake	3.6^{a}	3.8^{a}	8.3^{b}	8.3^{b}	7.7^{b}	0.29	**<0.001**
Fatty acid intake (g/kg DMI)							
Total	507.7^{a}	338.6^{b}	212.0^{c}	261.5^{d}	287.9^{e}	8.35	**<0.001**
14:0	5.2^{a}	6.3^{b}	1.4^{c}	2.5^{d}	3.4^{e}	0.11	**<0.001**
16:0	154.5^{a}	67.3^{b}	36.9^{c}	45.8^{d}	50.6^{d}	1.74	**<0.001**
18:0	15.1^{a}	6.3^{b}	2.8^{c}	3.9^{d}	4.7^{e}	0.16	**<0.001**
18:1*n*-9	127.3^{a}	34.6^{b}	3.9^{c}	5.5^{c}	6.5^{c}	0.95	**<0.001**
18:2*n*-6	158.8^{a}	124.6^{b}	28.4^{c}	36.9^{d}	42.0^{e}	1.75	**<0.001**
18:3*n*-3	25.4^{a}	78.2^{b}	121.6^{c}	146.2^{d}	158.0^{e}	4.00	**<0.001**

Animal performance							
Live weight gain (kg/d)	1.21	1.06	1.02	1.11	1.08	0.93	0.392
Age at slaughter (d)	627	626	622	624	624	9.27	0.989
Half carcass cold weight (kg)	178.0	163.5	170.9	172.9	170.0	5.90	0.205
Conformation	73.75	73.75	77.50	85.00	73.75	6.77	0.399
Fatness	55.63	46.88	61.25	63.75	55.00	8.15	0.294

[abcde] Means with different superscript differ significantly, within row. NA = non-applicable.

Experiment 6 outcome: Thirty-two Charolais cross-bred steers were allocated to one of four dietary treatments (n=8 animals/diet): 1) grass silage *ad libitum* (Control), 2) grass silage *ad libitum* plus 1.5% Echium oil (Northstar Lipids Ltd., UK) / silage DMI (Echium 1.5%), 3) grass silage *ad libitum* plus 3.0% Echium oil / silage DMI (Echium 3%), or 4) grass silage *ad libitum* plus 3.0% linseed oil / silage DMI (Linseed 3%). Oils were mixed with sugar beet (1 kg) before feeding. Liveweight was monitored every 14 days and the mean number of days on experimental diets was 95 ± 8 d. Animals were slaughtered when they achieved fat class 3L. Carcasses were assessed, and the *M. longissimus* sampled for analysis of fatty acid composition and sensory assessment (under Objective 4).

The feed intake of forage was similar across diets, however as expected there was significant variation of feed intake of oil (Table 11). The live weight gain of animal receiving 3% echium oil was greater than animals on the control (oil free diet), however the age at slaughter (which was performed once animals achieved fat class 3L), half carcass cold weight and conformation and fatness scores were similar across diets, which indicates that any variations in muscle fatty acid composition were not affected by confounding influences of carcass fatness or age (Table 11).

Table 11. Animal performance and carcass classification of Charolais cross steers fed different diets.

	Diet				SED	*P*
	Control	Echium 1.5%	Echium 3%	Linseed 3%		
Forage intake[b]	7.68	7.76	7.79	7.69	0.34	0.983
Oil intake[c]	-	117[a]	234[b]	231[b]	0.01	**<0.001**
Live weight gain (kg/d)	0.71[a]	0.79[ab]	0.91[b]	0.81[ab]	0.06	**0.033**
Age at slaughter (d)	777	777	776	782	10.81	0.936
Half carcass cold weight (kg)	194.4	194.6	197.2	192.8	7.78	0.925
Conformation	92.5	77.5	77.5	77.5	8.96	0.263
Fatness	89.4	83.1	95.6	83.8	8.55	0.439

[ab] Means with different superscript differ significantly, within row.
[b] Intakes expressed as kg DM/day.
[c] Intake expressed as mL/day.

Objective 4: *To assess the impact of plant-based feeding strategies on meat quality: fatty acid composition, colour shelf-life and sensory.*

Background
Production systems, and in particular diet, can alter the fatty acid composition of meat, but also may affect the shelf life and sensory characteristics due to variations in the susceptibility to oxidative breakdown and production of volatile compounds during cooking. Cattle fed a grass-based diet not only deposit more *n*-3 PUFA in meat, but also plant-derived anti-oxidants such as vitamin E, which can result in beef products that have increased oxidative stability. Beef colour is also affected by oxidative stability, where red oxymyoglobin is oxidised to form metmyoglobin, resulting in meat with a brown colour that consumers find undesirable. The potential of plant-based feeding strategies to generate beef products with beneficially enhanced fatty acid composition, whilst also maintaining or improving the shelf-life and sensory characteristics is of great interest.

Main Objectives
Experiment 5 objective: To assess the effects of the "lifetime" feeding strategy, described in objective 3, on meat quality of beef.

Experiment 6 objective: To assess the effect of feeding plant oils rich in 18:4 *n*-3 PUFA (from *Echium spp*.), as described in objective 3, on meat quality.

Results
Experiment 5 outcome: After slaughter steaks were sampled from the *M. longissimus* (loin) for assessment of fatty acid composition and shelf-life and sensory characteristics. Fatty acid composition was assessed within the separate neutral lipid and phospholipid fractions, using gas chromatography. Identification of the individual CLA isomers was achieved using silver ion high pressure liquid chromatography. The shelf life characteristics were examined by quantitating the amount of vitamin E and thiobarbituric acid reacting substances (TBARS) present in meat, following storage of loin steaks in modified atmosphere packaging (MAP, O_2:CO_2 75:25) on a simulated retail display unit. Meat colour, immediately post-slaughter (bloom) and during shelf-life storage in MAP, was measured as Chroma, calculated from the L*, a* and b* co-ordinates. To assess the eating quality of beef, 10 trained taste panellists tasted grilled loin steaks that had previously been conditioned for 14 days in a vacuum pack at 1˚C, before being cooked to an internal temperature of 74 ˚C. Assessment of eating quality was based upon 0-100 line scales (0 = nil, 100 = extreme intensity) or 8-point category scales for abnormal beef flavour intensity, beef flavour intensity (1 = extremely weak to 8 = extremely strong), juiciness (1 = extremely dry to 8 = extremely juicy) and texture (1 = extremely tough to 8 = extremely tender).

Fatty acid composition: The amount of total lipid, neutral lipid, phospholipid, SFA and MUFA in loin steaks did not vary between dietary treatments, but the amount of PUFA, *n*-6 and *n*-3 in the total lipid of meat were differentially affected by diet (Table 12). Animals receiving the forage-based diets had the lower amount of *n*-6 fatty acids, but higher concentrations of *n*-3 fatty acids, compared to S-CC animals, and vice versa (Table 12). Addition of PX to concentrate or forage increased the amount of *n*-3 fatty acids in muscle total lipid, with subsequent improvements in the *n*-6:*n*-3 ratios (Table 12).

The variations in *n*-6:*n*-3 ratios reflect the concentrations of individual fatty acids in the neutral lipid and phospholipid fractions. For example, the S-CC diets were associated with high concentrations of 18:2*n*-6 and *n*-6 group long chain fatty acids (Table 13). In contrast, the forage-based diets resulted in greater deposition of 18:3*n*-3 in both lipid fractions, and the *n*-3 group long chain fatty acids, including EPA, DPA and DHA, in the phospholipid, with the highest concentrations associated with the GS-HPX diet (Table 13). Incorporation of PX into a concentrate feed (S-PXC) improved the deposition of 18:3*n*-3, but not EPA, DPA or DHA, compared to the S-CC diet; although EPA deposition was similar between GS and S-PXC diets (Table 13). Importantly, 18:3*n*-3 concentrations in the neutral lipid of muscle from animals fed the S-PXC diet was greater than animals on the GS diet, despite the GS animals having a higher feed intake of 18:3*n*-3 than S-PXC animals (identified in objective 3). This indicates an increased efficiency of transfer of 18:3*n*-3 from feed to muscle for the PX extract, compared to grass, possibly due to reduced biohydrogenation of PX fatty acids in the rumen.

Individual CLA isomers were also assessed in the total lipid from *M. longissimus.* Of the thirteen individual CLA isomers identified within the total lipid fraction, only CLA *trans-11, trans*-13, and CLA

trans-11, trans-13 were affected by diet; where addition of PX to forage increased deposition compared to the S-CC diet (P<0.05, data not shown). Within the phospholipid fraction, CLA *cis-9, trans*-11 was also moderated by diet, but the major proportion of this CLA isomer was found in the neutral lipid where no effect of diet was observed (Table 13).

Table 12 Summary of fatty acid composition of the *M. longissimus* of Belgium Blue cattle fed different diets.

	Diet					SED	*P*
	S-CC	S-PXC	GS	GS-LPX	GS-HPX		
Concentrations (mg/100 g muscle)							
Total lipids	2531.6	1999.0	2551.3	2510.4	2433.2	379.3	0.586
Neutral lipids	2028.0	1542.5	2066.6	2021.3	1930.9	363.7	0.595
Phospholipids	503.7	456.5	484.7	489.1	502.3	22.3	0.235
SFA[A]	1059.4	806.1	1054.9	1012.9	990.1	174.9	0.593
MUFA[B]	1017.3	729.4	1061.3	1031.0	966.1	163.2	0.272
PUFA[C]	220.6^{ab}	250.8^{b}	163.5^{c}	192.5^{ac}	205.7^{a}	13.6	**<0.001**
Sum *n*-6[D]	174.9^{a}	179.0^{a}	91.4^{b}	105.6^{b}	111.6^{b}	9.2	**<0.001**
Sum *n*-3[E]	45.7^{a}	71.8^{b}	72.1^{b}	86.9^{bc}	94.1^{c}	5.3	**<0.001**
Health indices							
EPA+DHA	11.35^{a}	13.93^{ab}	16.71^{bc}	18.41^{cd}	21.11^{d}	1.24	**<0.001**
P:S[F]	0.13^{a}	0.23^{b}	0.08^{a}	0.11^{a}	0.13^{a}	0.02	**<0.001**
n-6:*n*-3	3.86^{a}	2.50^{b}	1.26^{c}	1.22^{c}	1.19^{c}	0.10	**<0.001**

[abcd] Means with different superscript differ significantly, within row (P<0.05).
[A] SFA, (12:0 + 14:0 + 16:0 + 18:0).
[B] MUFA, (16:1 + t18:1 + 9c18:1 + 11c18:1 + 20:1).
[C] PUFA, (18:2*n*-6 + 18:3*n*-3 + 20:3*n*-6 + 20:4*n*-6 + 20:4*n*-3 + 20:5*n*-3 +22:4*n*-6 + 22:5*n*-3 + 22:6*n*-3).
[D] *n*-6, (18:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6).
[E] *n*-3, (18:3*n*-3 + 20:4*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3).
[F] P:S, (18:2*n*-6 + 18:3*n*-3)/(12:0 + 14:0 + 16:0 + 18:0).

Table 13. Fatty acid concentration (mg/100 g muscle) in the lipid fractions of *M. longissimus* from Belgium Blue cattle fed different diets.

	Diet					SED	*P*
	S-CC	S-PXC	GS	GS-LPX	GS-HPX		
Neutral lipids							
14:0	59.4	41.6	62.7	56.0	52.3	41.5	0.417
16:0	577.4	424.2	595.0	553.4	525.4	104.6	0.517
18:0	293.9	228.8	278.4	283.1	289.6	55.8	0.774
18:1*trans*	71.8	59.8	52.0	58.5	64.9	12.2	0.573
18:1*n*-9	734.7	533.3	766.3	753.7	697.5	131.2	0.395
18:2*n*-6	29.4^{ab}	32.9^{b}	15.9^{c}	18.6^{ac}	20.0^{ac}	4.0	**<0.001**
18:3*n*-3	6.5^{a}	15.5^{b}	9.3^{ac}	13.6^{bc}	15.2^{b}	2.0	**<0.001**
18:2 *cis*-9, *trans*-11 (CLA)	13.6	12.2	11.6	13.1	12.7	2.4	0.935
Phospholipids							
14:0	1.2	0.8	1.3	1.1	1.2	0.2	0.309
16:0	76.1	64.3	70.0	69.7	71.0	3.9	0.080
18:0	50.3	45.5	46.4	48.8	49.8	2.1	0.128
18:1*trans*	3.9^{a}	3.5^{ab}	3.1^{b}	3.6^{ab}	4.0^{a}	0.3	**0.019**
18:1*n*-9	90.1^{a}	47.7^{b}	113.3^{c}	96.2^{ac}	96.3^{ac}	6.2	**<0.001**
18:2*n*-6	91.9^{a}	101.6^{a}	40.8^{b}	51.5^{b}	52.7^{b}	4.4	**<0.001**
18:3*n*-3	7.3^{a}	20.2^{bc}	17.7^{b}	24.7^{cd}	26.1^{d}	1.7	**<0.001**
18:2 *cis*-9, *trans*-11 (CLA)	1.15^{ab}	1.12^{a}	1.34^{ab}	1.35^{b}	1.40^{b}	0.1	**0.012**
20:3*n*-6	11.6^{a}	8.8^{b}	5.0^{c}	5.6^{c}	6.3^{c}	0.5	**<0.001**
20:4*n*-6	37.3^{a}	32.2^{b}	27.0^{c}	27.2^{c}	29.7^{bc}	1.7	**<0.001**
20:4*n*-3	2.0^{a}	2.9^{a}	4.2^{b}	4.8^{b}	5.2^{b}	0.4	**<0.001**
20:5*n*-3 (EPA)	9.7^{a}	12.2^{ab}	14.1^{bc}	15.9^{cd}	18.1^{d}	1.1	**<0.001**
22:4*n*-6	3.2^{a}	2.0^{b}	1.9^{b}	1.8^{b}	1.9^{b}	0.2	**<0.001**
22:5*n*-3 (DPA)	16.7^{a}	17.3^{a}	21.5^{b}	22.9^{b}	23.7^{b}	1.1	**<0.001**
22:6*n*-3 (DHA)	1.6^{a}	1.6^{a}	2.4^{b}	2.3^{b}	2.8^{b}	0.2	**<0.001**

[abcd] Means with different superscript differ significantly, within row (P<0.05).

Shelf-life and sensory characteristics: There was an effect of diet on the bloomed colour characteristics, where steaks from GS-HPX animals had lower colour intensity than steaks from animals fed S-CC (Table 14). However, by day 1 of simulated retail display, no differences were identified between the diets (see Appendix 1 Figure 1). Colour saturation did decrease over time for treatments, but was always greater than the retail cut-off of 18 (see Appendix 1 Figure 2). There was

a significant interaction between time and diet, probably as a result of higher colour saturation of steaks from S-PXC animals on day 7 of display, compared to S-CC and GS fed animals (see Appendix 1; Figure 2, Table 14).

The maintenance of high colour saturation by S-PXC diet likely reflects greater oxidative stability of these steaks, which had the lowest TBARS and the highest vitamin E concentration, compared to other diets (Table 14). The GS diet was also associated with lower TBARS, compared to S-CC, although similar amounts of vitamin E were identified between these two groups (Table 14). Incorporation of PX into a forage diet was associated with increased the TBARS value compared to grass alone, possibly as a result of higher concentrations of PUFA with increase dietary PX. In contrast, meat from animals fed S-PXC had lower TBARS than S-CC animals, probably due to the additional dietary supply of vitamin E fed to the S-PXC animals. However for an effect of oxidisation of beef on taste, TBARS values need to be > 2 mg malonaldehyde/kg meat (Campo et al., 2006). In the present study, TBARS was always< 2 mg/kg meat from all animals, irrespective of diet.

The eating quality of steaks varied little across the different diets, although significant differences were noted for texture, and the terms 'greasy' and 'bloody' (Table 15). Animals receiving the GS diet produced steaks associated with the lowest score for 'texture', and the highest scores for 'greasy' and 'bloody' (Table 15). Significantly better 'texture' scores were apparent across the other 4 diets, whereas GS scored significantly more poorly against the S-CC, S-PXC and GS-LPX diets on the term 'greasy', and against the S-CC and S-PXC diets on the term 'bloody'. Whilst the term greasy has been associated with variations in overall fatness, animal fatness was controlled across diets in this study. Additionally, the 'overall liking' was not affected by animal diet.

Message: Therefore, moderating the diets fed to cattle had minimal impact upon the eating quality of beef but significant effects on the fatty acid composition.

Table 14. The colour oxidative stability of *M. longissimus* steaks from Belgium Blue cattle fed different diets.

	Diet					SED	*P*
	S-CC	S-PXC	GS	GS-LPX	GS-HPX		
Bloomed colour							
*L**	40.28	39.96	40.33	40.45	40.11	1.2	0.996
*a**	20.83^{a}	19.22^{ab}	19.76^{ab}	20.39^{ab}	17.96^{b}	0.9	**0.026**
*b**	9.58^{a}	8.88^{ab}	9.00^{ab}	9.60^{a}	7.53^{b}	0.6	**0.014**
Hue	24.62^{a}	24.74^{a}	24.43^{ab}	25.19^{a}	22.51^{b}	0.7	**0.009**
Chroma	22.93^{a}	21.18^{ab}	21.72^{ab}	22.54^{a}	19.49^{b}	1.1	**0.022**
Oxidative capacity							
TBARS, day 10^{A}	1.19^{a}	0.27^{b}	0.55^{c}	0.76^{ac}	1.25^{a}	0.1	**<0.001**
Chroma, day 7	23.5^{a}	24.8^{b}	23.2^{a}	23.8^{ab}	23.9^{ab}	0.4	**0.009**
Vitamin E^{B}	3.2^{a}	7.7^{b}	3.9^{ac}	4.4^{c}	4.2^{ac}	0.4	**<0.001**

^{abc} Means with different superscript differ significantly, within row (P<0.05).
^{A} mg malonaldehyde/kg meat. Values are geometric means from back-transformations with the SED of $\log_{10}$ values (Log_{10} transformations used for analysis).
^{B} mg/kg muscle.

Table 15. Eating quality of grilled beef loin steak, cooked to 74°C internal endpoint temperature, from Belgium Blue cattle fed different diets. Values are the means derived from analysis of variance with diet and assessor as factors, with 8 replicates.

	Diet					*P*
Attribute	S-CC	S-PXC	GS	GS-LPX	GS-HPX	
8 point category scale						
Texture	4.46^{a}	4.52^{a}	3.84^{b}	4.52^{a}	4.51^{a}	**0.0012**
Juiciness	4.43	4.16	4.59	4.39	4.58	0.08
Beef flavour intensity	4.58	4.70	4.46	4.87	4.87	0.16
Abnormal flavour intensity	2.93	2.78	2.96	2.70	2.52	0.24
100mm line scale						
Greasy	12.09^{a}	10.71^{a}	15.20^{b}	12.43^{a}	12.64^{ab}	**0.024**
Bloody	6.20^{a}	4.35^{a}	9.93^{b}	6.87^{ab}	7.32^{ab}	**0.048**
Livery	14.17	14.48	16.42	14.70	13.94	0.72
Metallic	17.80	17.52	18.99	18.48	19.91	0.79
Bitter	8.20	7.86	11.22	7.25	7.99	0.32
Sweet	11.29	10.59	12.42	11.22	10.46	0.60
Rancid	2.12	2.88	4.70	2.49	0.78	0.20
Fishy	6.54	4.46	6.42	5.17	4.86	0.38
Acidic	13.80	15.71	15.35	14.29	16.14	0.88
Cardboard	20.97	18.45	22.84	19.10	19.22	0.33
Vegetable/Grass	14.03	11.55	14.65	15.45	14.25	0.46
Dairy	11.46	10.45	12.99	11.93	13.06	0.42
Hedonic						

Overall liking	44.20	45.38	44.48	48.35	49.99	0.15

[abcd] Means with different superscript differ significantly, within row (P<0.05).

Experiment 6 outcome: The fatty acid composition, shelf life characteristics and eating quality were assessed as described previously for experiment 5.

Fatty acid composition: Addition of echium oil or linseed oil had no effect on the concentrations of total lipid, neutral lipid, phospholipid, SFA, MUFA or PUFA, compared to feeding forage alone (Table 16). Additionally, the ratio of *n*-6:*n*-3 fatty acids, P:S were similarly unaffected by diet, as was the concentration of EPA+DHA in the total lipid of *M. longissiumus* (Table 16).

Dietary treatment also had little impact upon the fractionated lipid components. Addition of 3% echium to the diet enhanced the deposition of 18:1*trans* and *cis*-9, *trans*-11 CLA in both the neutral and phospholipid fractions, and also increased 18:4*n*-3 in the neutral lipid fraction (Table 17). The other major fatty acids, including the long chain C20 fatty acids of the phospholipid, were unaffected by dietary treatment (Table 17). Examination of the individual CLA isomers of *M. longissiumus* identified that 6 of the 13 detected CLA isomers, including *cis*-9, *trans*-11 CLA which has recognised health benefits for humans (Belury, 2002), were increased by feeding 3% echium oil compared to forage feeding alone (see Appendix 1 Table 3). Deposition of *trans*-10, *cis*-12 CLA, which has been associated with anti-obesity effects in the human diet, was not affected by dietary treatment in the present study (see Appendix 1 Table 3). Addition of 1.5% echium oil or linseed oil increased deposition of *trans*-7, *trans*-9 CLA and *trans*-11, *trans*-13 CLA, respectively. For all other CLA isomers, addition of 1.5% echium oil or linseed oil had an intermediate effect upon CLA isomer concentration compared to feeding 3% echium oil or forage feeding alone (see Appendix 1 Table 3). It is postulated that the higher concentration of 18:1 trans and CLA in muscle may indicate that both echium and linseed oils, which were ruminally unprotected, were heavily biohydrogenated in the rumen.

Shelf-life and sensory characteristics: The colour saturation of meat from all animals declined over time, as expected (see Appendix 1 Figure 3). There was no effect of dietary treatment on the colour saturation of meat, nor an interaction between diet and time, indicating a similar decline of colour saturation over time (see Appendix 1 Figure 3). All treatments maintained colour saturation level above the threshold for acceptable meat colour for at least 15 days of simulated retail display, but by day 18, all treatments had a colour saturation of <18 (see Appendix 1 Figure 3).

There was also no effect of diet upon the oxidative stability, as measured by TBARS, chroma on day 10 of simulated retail display or vitamin E content of muscle (Table 18). Dietary treatment also had little impact upon the eating quality, with only significant variation in the terms 'vegetable/grass' and 'dairy', where feeding cattle linseed oil resulted in meat with a stronger intensity of the term 'vegetable/grass' and feeding 3% echium oil enhanced the attribute 'dairy' (Table 19). However the overall liking was similar between treatments.

Message: In conclusion, addition of echium oil, or linseed oil, did not enhance the profile of long chain C20 fatty acids in the meat, but also had little impact upon the shelf-life characteristics or eating quality of beef.

Table 16. Summary of fatty acid composition of the *M. longissimus* of Charolais cross steers fed different diets.

	Diet					
	Control	Echium 1.5%	Echium 3%	Linseed 3%	SED	*P*
Concentrations mg/100 g muscle						
Total lipid	3138.0	4034.4	3964.3	3378.4	572.3	0.334
Total NL	2678.4	3553.8	3495.8	2912.3	566.2	0.340
Total PL	459.6	480.6	468.4	466.1	19.25	0.742
SFA[A]	1328.4	1785.5	1717.9	1454.4	267.6	0.293
MUFA[B]	1311.6	1648.8	1643.8	1359.4	253.7	0.402
PUFA[C]	164.7	175.3	173.3	175.3	9.09	0.613
Sum *n*-6[D]	78.2	85.5	85.3	83.7	4.40	0.330
Sum *n*-3[E]	86.5	89.7	88.0	91.6	5.05	0.767
Health indices						
P:S[F]	0.06	0.06	0.06	0.07	0.009	0.755
n-6:*n*-3	0.91	0.95	0.97	0.92	0.029	0.154

EPA+DHA[G]	23.4	23.1	20.8	24.6	1.645	0.116

[A] SFA, (12:0 + 14:0 + 16:0 + 18:0).
[B] MUFA, (16:1 + t18:1 + 9c18:1 + 11c18:1 + 20:1).
[C] PUFA, (18:2*n*-6 + 18:3*n*-3 + 18:4*n*-3 + 20:3*n*-6 + 20:4*n*-6 + 20:4*n*-3 + 20:5*n*-3 +22:4*n*-6 + 22:5*n*-3 + 22:6*n*-3).
[D] *n*-6 PUFA (18:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 22:4*n*-6).
[E] *n*-3 PUFA (18:3*n*-3 + 18:4*n*-3 + 20:4*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3).
[F] P:S, (18:2*n*-6 + 18:3*n*-3)/(12:0 + 14:0 + 16:0 + 18:0).
[G] mg/100 g muscle.

Table 17. Fatty acid concentration (mg/100 g muscle) in the lipid fractions of *M. longissimus* from Charolais cross steers fed different diets.

	Diet					
	Control	Echium 1.5%	Echium 3%	Linseed 3%	SED	*P*
Neutral lipids						
14:0	86.8	130.3	127.0	104.8	24.0	0.253
16:0	761.1	1030.4	1003.3	818.5	167.7	0.304
16:1	120.0	164.1	158.4	127.3	28.3	0.324
18:0	375.5	514.8	480.6	428.0	77.9	0.318
18:1*trans*	37.9[a]	71.7[ab]	112.4[c]	73.7[b]	13.0	**<0.001**
18:1*cis*-9	993.8	1237.2	1203.9	1002.6	207.3	0.517
18:2*n*-6	18.6	23.6	22.9	20.1	3.3	0.388
18:3*n*-3	13.2	16.0	15.3	14.1	2.2	0.591
CLA[A]	9.2[a]	14.8[a]	23.1[b]	14.3[a]	3.0	**<0.001**
18:4*n*-3	1.1[a]	1.6[ab]	2.3[b]	1.9[ab]	0.4	**0.026**
Phospholipids						
14:0	0.889	0.993	1.110	0.834	0.324	0.838
16:0	60.899	63.302	60.824	57.610	3.59	0.479
16:1	11.732	13.318	12.155	10.849	0.975	0.108
18:0	41.298	42.832	42.206	42.308	1.878	0.874
18:1*trans*	1.880[a]	2.782[b]	4.572[c]	2.972[b]	0.326	**<0.001**
18:1*n*-9	106.095	114.144	106.280	102.652	6.24	0.323
18:2*n*-6	32.383	34.357	36.250	35.496	1.844	0.197
18:3*n*-3	18.069	18.927	19.688	20.440	1.113	0.196
CLA[A]	0.770[a]	1.091[a]	1.642[b]	1.081[a]	0.118	**<0.001**
18:4*n*-3	0.244	0.226	0.225	0.219	0.036	0.910
20:3*n*-6	4.519	4.342	4.382	4.425	0.313	0.949
20:4*n*-6	19.978	20.118	18.602	20.880	1.142	0.269
20:4*n*-3	3.822	3.526	3.918	3.639	0.337	0.652
20:5*n*-3	18.979	18.410	16.899	19.806	1.306	0.178
22:4*n*-6	1.215	1.278	1.342	1.149	0.107	0.322
22:5*n*-3	23.346	22.358	21.898	23.069	1.321	0.685
22:6*n*-3	3.664	3.747	3.110	3.757	0.369	0.263

[abcd] Means with different superscript differ significantly, within row.
[A] CLA, 18:2*cis*-9, *trans*-11 CLA.

Table 18. The oxidative stability of *M. longissimus* steaks from Charolais cross steers fed different diets.

	Diet				SED	*P*
	Control	Echium 1.5%	Echium 3%	Linseed 3%		
Lipid Oxidation						
TBARS, day 10[A,B]	0.62	0.52	0.44	0.46	0.075	0.213
Chroma, day 10	22.1	22.7	22.4	22.2	0.562	0.663
Vitamin E[C]	5.88	6.18	5.92	6.03	0.460	0.914

[abcd] Means with different superscript differ significantly, within row.
[A] mg malonaldehyde/kg meat.
[B] Values are geometric means from back-transformations with the SED of Log_{10} values (Log_{10} transformations used for analysis).
[C] mg/kg muscle.

Table 19. Eating quality of grilled beef loin steak, cooked to 74°C internal endpoint temperature, from Belgium Blue cattle fed different diets. Values are the means derived from analysis of variance with diet and assessor as factors, with 8 replicates.

	Diet				*P*
Attribute	Control	Echium 1.5%	Echium 3%	Linseed 3%	
8 point category scale					
Texture	5.14	4.77	4.75	5.22	0.0809

Juiciness	5.41	5.42	5.42	5.34	0.9620
Beef flavour intensity	5.23	5.05	5.19	5.20	0.5185
Abnormal flavour intensity	2.47	2.69	2.47	2.80	0.1125
100mm line scale					
Greasy	19.08	20.02	19.66	17.39	0.4223
Bloody	15.05	14.39	14.08	14.13	0.9703
Livery	11.25	11.64	13.14	12.08	0.7005
Metallic	15.58	14.59	16.30	17.48	0.4794
Bitter	7.73	7.02	6.77	9.00	0.5938
Sweet	16.09	13.55	16.73	14.77	0.3302
Rancid	0.75	0.86	0.44	0.55	0.4076
Fishy	2.92	3.19	2.69	2.97	0.6640
Acidic	9.84	11.09	10.11	11.38	0.7270
Cardboard	11.89	13.11	13.45	14.39	0.2863
Vegetable/Grass	12.47^{b}	13.63^{ab}	13.11^{b}	15.81^{a}	**0.0440**
Dairy	26.72^{ab}	23.55^{b}	29.86^{a}	25.02^{b}	**0.0228**
Hedonic					
Overall liking	57.59	55.00	58.02	54.58	0.5092

^{abcd} Means with different superscript differ significantly, within row.

Objective 5: *To assess the effect of nutritionally-enhanced beef on plasma lipids and lipoproteins and the development of atherosclerosis in animal models.*

Background
This objective examined the effects of modified beef fat on atherosclerosis in a mouse model, using the ApoE*3 Leiden mouse as the model species to examine the effect of dietary modified beef fat on plasma lipoproteins and progression of atherosclerosis.

Main Objectives
The initial *objectives were*: to investigate whether beef-enriched in *n*-3 PUFA has beneficial effects that result in (1) slowed progression of atherosclerosis and (2) enhanced regression of atherosclerosis. However, following initial results of studies looking at the impact of modified beef on progression of atherosclerosis, the decision was taken to concentrate on establishing the type and amount of PUFA that would be required to overcome the potentially pro—atherosclerotic effects of SFA within beef and to evaluate whether n-3 PUFA had any specific benefits over n-6 PUFA. In total three feeding studies were carried out within this work package.

Results
Experiment 7 outcome: *The impact of beef from concentrate or forage-fed cattle on the development of atherosclerosis in ApoE*3 Leiden Transgenic Mice*
The fatty acid profile of beef is, at least partly, dependent on the nature of the diet fed to the cattle. Grass and clover both contain large amounts of α-linolenic acid while cereal crops are generally rich in linoleic acid. While a large proportion of unsaturated fatty acids are biohydrogenated in the rumen, significant amounts do escape this process and enter the tissues of the animal. These fatty acids can also be further elongated and desaturated to produce a range of other fatty acids. In the case of α-linolenic acid, this can include both EPA and DHA. The aim of this study was to investigate whether the difference in fatty acid composition of meat in grass/clover–fed cattle, compared to that from concentrate (cereal) fed animals is sufficient to influence plasma lipids and the development of atherosclerosis in a mouse model. The mouse model used was a transgenic strain that expresses the human apoE*3 Leiden gene, resulting in impaired lipoprotein clearance and a lipoprotein profile more similar to that of humans. Such mice readily develop diet-induced hyperlipidemia and atherosclerosis and are highly responsive to changes in the fatty acid profile of the diet.

The beef used in this experiment was collected from a study carried out at Aberystwyth University where thirty Charolais × Friesian steers (~22 weeks of age) were allocated to one of two dietary treatments; 1) forage (grass/white clover swards during grazing followed by grass silage in winter period), or 2) restricted barley straw and concentrate (40:60 on a DM basis). Animals were slaughtered and samples of *M. obliquus internus abdominis* were taken and frozen before being transported to The University of Nottingham. Samples were defrosted, minced, freeze dried and ground to a powder. Samples from each diet group were thoroughly mixed together prior to freeze drying to ensure the collective samples were homogeneous

Mice were randomly allocated to one of two treatments groups (*n*=10) with a mean age for each group of 12 weeks old. The two treatment groups were fed a semi-synthetic diet containing 36% (w/w) freeze dried beef from cattle fed either a forage based (FB) or concentrate based (CB) diet and both diets also contained 0.25% cholesterol. Diets were fed for a 12 week period and animals were given a fresh dish of food every morning with the previous days food weighed so daily food intake could be monitored throughout the study. At the end of the study mice were culled and the serum frozen for subsequent lipid/lipoprotein analysis. Livers were collected for determination of fatty acid composition and gene expression studies. Fatty acid composition of diets and liver was determined by gas chromatography of FAME. The heart and aorta were removed and frozen prior to sectioning of the aorta at the point at which it joins the heart. Every third section was subsequently stained with haematoxylin and oil-red O and the area of staining with the latter quantified to determine the extent of atherosclerosis. The section of aorta in which the 3 tricuspid valve leaflets first became visible was nominally designated and atherosclerosis determined in this and a further five sections.

Table 20 shows the fatty acid composition of the beef included in the diets and that of the total hepatic lipid in the mice. Both sources of beef contained similar amounts of SFA. FB was relatively enriched in oleic acid, α-linolenic acid and EPA compared to CB but contained significantly less linoleic acid. The livers of mice fed FB contained significantly more α-linolenic acid, EPA and DHA than those from

animals fed CB. The presence of higher concentration of DHA suggests significant elongation and desaturation of dietary α-linolenic acid.

Table 20. Fatty acid composition of beef and mouse liver lipids.

% total FAME	*Composition of Beef (n=6 replicates)*			*Composition of Mouse Liver*		
	Forage	Concentrate	*P*	Forage	Concentrate	*P*
16:0 palmitic	29.14 ± 0.75	30.71 ± 0.62	0.132	18.48 ± 0.41	16.47 ± 0.39	**0.011**
18:0 stearic	13.37 ± 1.16	12.71 ± 0.56	0.605	7.29 ± 0.34	5.30 ± 0.32	**<0.001**
18:1 *n-9* oleic	38.09 ± 0.89	34.95 ± 0.76	**0.021**	46.34 ± 1.20	47.79 ± 1.02	0.143
18:1 *trans*11	1.63 ± 0.11	2.29 ± 0.16	**0.007**	0.22 ± 0.10	0.58 ± 0.20	0.280
18:2 *n-6* linoleic	0.91 ± 0.08	2.55 ± 0.24	**<0.001**	6.45 ± 0.17	6.08 ± 0.18	0.148
CLA *c*9,*t*11	0.39 ± 0.03	0.24 ± 0.05	**0.018**	0.31 ± 0.07	0.09 ± 0.04	**0.019**
18:3*n-3* α-linolenic	0.74 ± 0.05	0.22 ± 0.06	**<0.001**	1.17 ± 0.05	0.98 ± 0.05	**0.013**
20:4*n-6* arachidonic	0.40 ± 0.05	1.03 ± 0.16	**0.004**	4.37 ± 0.28	4.54 ± 0.40	0.727
20:5*n-3* eicosapentaenoic	0.21 ± 0.03	0.06 ± 0.02	**<0.001**	0.24 ± 0.05	0.00 ± 0.00	**<0.001**
22:5*n-3* docosapentaenoic	0.39 ± 0.05	0.24 ± 0.03	**0.022**	0.39 ± 0.07	0.08 ± 0.03	**0.005**
22:6*n-3* docosahexaenoic	0.05 ± 0.01	0.03 ± 0.01	0.235	5.00 ± 0.27	2.52 ± 0.19	**<0.001**
Sum SFA	47.78 ± 1.06	48.70 ± 1.19	0.583	27.21 ± 0.66	22.60 ± 0.59	**<0.001**
Sum *n*-3	1.38 ± 0.12	0.55 ± 0.04	**0.001**	6.82 ± 0.23	3.57 ± 0.14	**<0.001**
Sum *n*-6	1.36 ± 0.13	3.96 ± 0.44	**0.001**	10.82 ± 0.42	10.62 ± 0.53	0.771

Table 21 shows the plasma lipids and lipoproteins from mice fed the two sources of beef. Those animals fed FB had significantly lower plasma triacylglycerol and HDL cholesterol than those fed CB.

Figure 2 shows a representative section through the aorta with the area stained red representing the deposit of lipid within an atherosclerotic lesion. As noted in Figure 3, in animals on both diets, the area of lesion was consistent along the length of the aorta examined and there was no significant impact of diet on lesion area.

Table 21. Plasma Lipids and Lipoproteins in mice fed two sources of beef.

mMol/l	FB	CB	*P*
Total Cholesterol	10.27 ± 0.62	11.28 ± 0.7	0.297
HDL Cholesterol	1.14 ± 0.06	1.41 ± 0.1	**0.038**
Non HDL Cholesterol	9.13 ± 0.57	9.87 ± 0.67	0.496
Non HDL/HDL ratio	8.04 ± 0.37	7.22 ± 0.55	0.233
Total Triacylglyerol	2.49 ± 0.22	3.16 ± 0.19	**0.035**

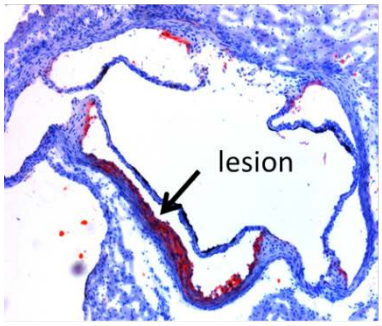

Figure 2. Example of athersclerotic lesion

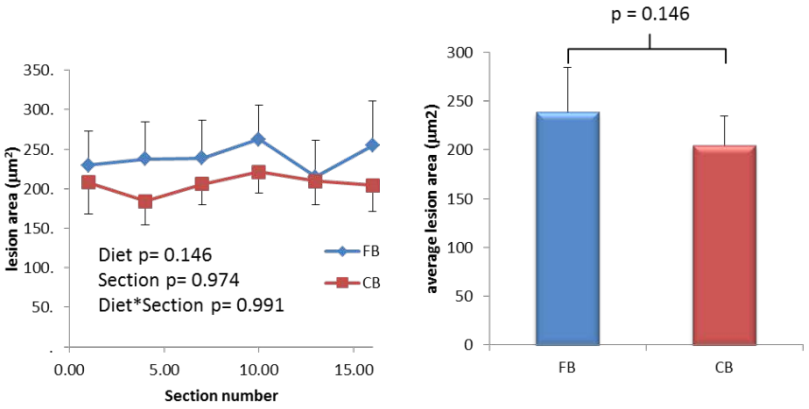

Figure 3. Lesion area in mice fed different souces of beef.

Message: Beef from animals fed forage-based diet showed significant differences in fatty acid composition than those fed a concentrate–based diet, with the former being relatively enriched in *n*-3 PUFA. This was reflected in the accumation of *n*-3 PUFA in the livers of FB fed mice and there was also evidence of the further elongation and desaturation of α-linolenic acid to produce increased DHA concentrations. FB–fed mice had lower serum triglyceride and HDL cholesterol compared to those fed CB. These may be predicted to have opposing effects on the development of atherosclerosis which may explain why no significant difference was seen in atherosclerosis between the two groups of mice.

Experiment 8 outcome: *A comparison of the impact of alpha-linolenic acid and linoleic acid on the development of atherosclerosis in ApoE*3 Leiden transgenic mice*

A number of differences were observed in the fatty acid composition of the beef including a marked change in the relative ratio of *n*-3:*n*-6 PUFA. This was largely due to a lower content of linoleic acid, and higher content of α-linolenic acid, in the beef from forage-fed cattle. A second experiment was designed to investigate whether the relative difference in proportion of these fatty acids may be responsible for the differences in plasma lipids and, whether larger changes in the amounts of these fatty acids might impact on the development of atherosclerosis.

Mice were randomly allocated to one of 3 treatments groups (*n*=10) with a mean age for each group of 12 weeks old. The three treatment groups were fed an "atherogenic diet" (chow supplemented with 15% cocoa butter, COB diet) or a similar diet in which a third of the cocoa butter was replaced with either flaxseed oil (rich in α-linolenic acid, FLO diet) or sunflower oil (rich in linoleic acid, SO diet). All diets were supplemented with 0.25% cholesterol. The feeding trial was performed as described for experiment 7.

Table 22 shows that replacing a proportion of the COB with either FLO or SO reduced saturated fatty acid (16:0 and 18:0) content of the diet by similar amounts. As expected the FLO diet was relatively enriched in α-linolenic acid and the SO-diet enriched in linoleic acid. This difference was reflected in the fatty acid composition of livers from mice fed these diets with those from FLO-fed mice showing an increased proportion of α-linolenic acid and those fed SO and increased proportion of linoleic acid.

The FLO-diet significantly reduced plasma cholesterol compared to both the COB and SO–diets (Table 23). Plasma triacylglycerol was also lower in FLO-fed animals but this was only statistically significant compared to the COB group. No significant differences were seen in plasma HDL.

Table 22. Fatty acid composition of diets and liver lipids from mice fed different sources of PUFA.

% total FAME	***Composition of Diet***			***Composition of Mouse Liver***		
	COB	FLO	SO	COB	FLO	SO
C16:0 palmitic	24.36	18.58	18.67	16.82±0.05^{a}	14.35±0.29^{b}	14.14±0.56^{b}
C18:0 stearic	30.34	21.41	21.21	8.15±0.4^{a}	6.83±0.37^{b}	6.47±0.49^{b}
C18:1 *n-9* oleic	31.4	27.40	28.96	51.06±1.29^{a}	44.08±0.89^{b}	44.2±0.98^{b}
C18:2 *n-6* linoleic	10.97	15.04	28.17	9.75±0.20^{a}	13.85±0.33^{b}	21.48±0.41^{c}
C18:3*n-3* α-linolenic	1.10	15.93	1.15	0.87±0.14^{a}	6.74±0.17^{b}	0.78±0.11^{a}
C20:4*n-6* arachidonic	ND	ND	ND	5.78±0.35^{a}	2.16±0.14^{b}	5.55±0.55^{a}
C20:5*n-3* eicosapentaenoic	ND	ND	ND	ND.	2.01±0.10	ND
C22:5*n-3* docosapentaenoic	ND	ND	ND	ND	0.98±0.04^{a}	0.03±0.03^{b}
C22:6*n-3* docosahexaenoic	ND	ND	ND	2.56±0.18^{a}	4.10±0.26^{b}	1.61±0.15^{c}
Sum SFA	55.96	41.08	41.13	25.76±0.91^{a}	21.65±0.55^{b}	21.19±0.10^{b}
Sum *n*-3 FA	1.1	15.93	1.15	3.43±0.11^{a}	13.84±0.32^{b}	2.42±0.10^{c}
Sum *n*-6 FA	10.97	15.04	28.17	16.5±0.54^{a}	16.73±0.49^{a}	28.43±0.92^{b}

Values within a row with different superscripts are signicantly different; ND = non detectable.

Table 23. Plasma lipids and lipoproteins in mice fed different sources of PUFA.

mMol/l	COB	FLO	SO	*P*
Total Cholesterol	9.6^{a} ± 0.31	8.79^{b} ± 0.19	9.76^{a} ± 0.33	**0.046**
HDL Cholesterol	2.00 ± 0.11	1.97 ± 0.11	1.90 ± 0.13	0.847
NonHDL Cholesterol	7.8 ± 0.24^{a}	6.83 ± 0.17^{b}	8.07 ± 0.31^{a}	**0.003**
NonHDL/HDL ratio	3.95 ± 0.22	3.6 ± 0.28	4.37 ± 0.39	0.230
Total Triacylglycerol	1.7^{a} ± 0.1	1.31^{b} ± 0.07	1.6^{ab} ± 0.15	**0.041**

Values within a row with different superscripts are signicantly different.

As noted in Figure 4 there was a significant effect of diet on lesion area but no effects of section number nor an interaction between the two. Posthoc anaysis indicated a significant lower average lesion area in the SO group compared the the COB group.

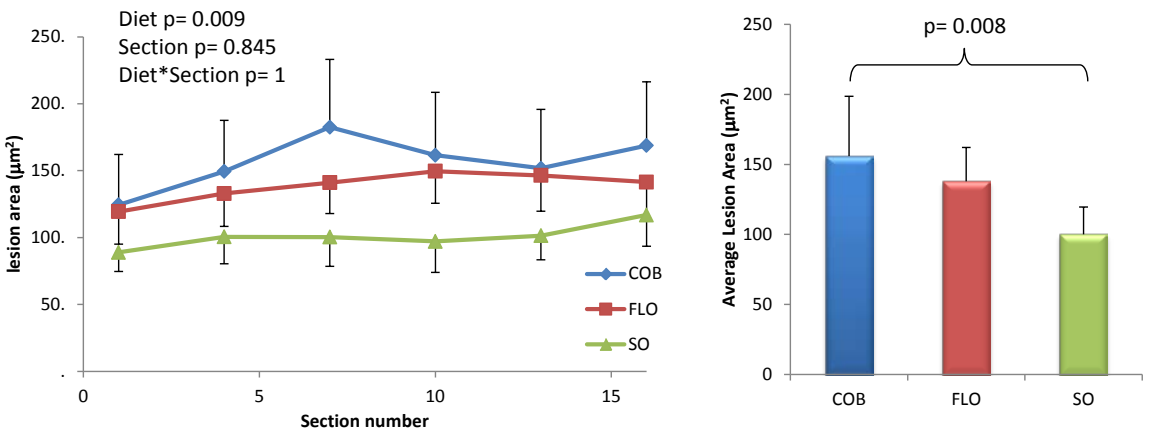

Figure 4. Analysis of atherosclerosis in mice fed different sources of PUFA.

Message: The impact of α-linolenic acid-rich FLO on plasma lipids (decreasing both cholesterol and triacylglycerol) would suggest that such a diet may be more protective against atherosclerosis than linoleic acid-rich SO. However, this is not supported by the direct determination of extent of atherosclerosis, where the SO-diet, but not the FLO-diet, significantly reduced the lesion area. This suggests that these oils are influencing factors other than plasma lipids that may impact on the development of atherosclerosis.

Experiment 9 outcome: *The impact of beef supplemented with different n-3 PUFA on the development of atherosclerosis in female ApoE*3 Leiden transgenic mice*
Experiment 7 demonstrated that the fatty acid composition of beef can be altered through grass/silage feeding sufficiently to alter plasma lipids (decreased triacylglycerol and HDL cholesterol), although it had no effect on the atherosclerotic lesion area. Experiment 8 demonstrated that α-linolenic acid can reduce plasma triacylglycerol (and cholesterol) but that this again was not sufficient to reduce the development of atherosclerosis. These findings raised the question as to how much, and which, *n*-3 PUFA are needed in beef to counteract the SFA content and reduce atherosclerotic lesion size.

In order to assess the quantities of long chain *n*-3 PUFA potentially needed in beef to have a beneficial effect on atherosclerosis development, a high DHA tuna oil powder was supplemented into diets containing freeze dried beef. The fish oil powder (Product name - Driphorm® HiDHA® ThermoMAX® 50) is a fine light tan, spray dried powder which contains 50% high DHA-rich tuna oil in a stable and protected microencapsulated form, which was kindly donated to this study from NuMega (Cambridge, UK). Supplementation was carried out to give a diet containing either 5 or 10 times the long chain *n*-3 PUFA that was originally found in the beef from grass/forage fed cattle.

There is considerable evidence to suggest that the long chain *n*-3 PUFAs are effective in reducing cardiovascular disease risk in humans. The conversion of α-linolenic acid to EPA is considered poor in both animals and humans since the first step in the pathway is the conversion to 18:4*n*-3 (stearidonic acid), which is catalysed by the enzyme Δ6-desaturase, and is both slow and rate limiting. Therefore a possible approach to increase concentrations of EPA/DHA is to bypass this initial step by increasing dietary 18:4*n*-3. Echium oil, which comes from the seeds of *Echium plantagineum* has been identified as a natural source of 18:4*n*-3, which accounts for approximately 13% of total fatty acids in the oil. In this study Echium oil was supplemented into diets containing beef to determine if it has cardioprotective effects and whether it is more efficiently converted into the longer chain fatty acids than α-linolenic acid.

Mice were randomly allocated to one of 5 treatments groups (*n*=10) with a mean age for each group of 13 weeks old. The 5 treatment groups were fed a semi-synthetic diet containing 36% (w/w) freeze dried beef purchased from a local butcher and a range of oils and powders as follows; (1) 5% rapeseed Oil (RO- control); (2) 1.4% Fish Oil Powder + 3.6% Rapeseed Oil (LFO); (3) 2.8% Fish Oil Powder + 2.2% Rapeseed Oil (HFO); (4) 5% Echium Oil (EO) and (5) 5% Flaxseed Oil (FXO)

All diets were supplemented with 0.25% cholesterol. The feeding trial was performed as described for experiment 7. Table 24 shows the fatty acid composition of the diets with significant differences from the RO diet highlighted in grey.

Table 24. Dietary fatty acid composition.

% total FAME	RO	LFO	HFO	EO	FXO
C16:0 palmitic	18.07	19.75	20.59	17.82	19.05
C18:0 stearic	12.47	13.87	14.2	11.77	13.95
C18:1 *trans*11	1.99	2.44	2.67	2.6	2.62
C18:1 *cis*9 oleic	41.36	37.84	35.52	29.54	28.71
C18:2*n-6* linoleic	12.32	10.32	9.56	12.07	10.11
CLA *c*9,*t*11	0.21	0.2	0.26	0.22	0.25
C18:3*n-3* α-linolenic	4.25	2.92	2.45	10.23	14.32
C18:3*n-6* g-linolenic	ND	ND	ND	3.00	ND
C18:4*n-6* stearidonic	0.09	0.16	0.16	3.93	0.16
C20:4*n-6* arachidonic	0.24	0.32	0.39	0.24	0.23
C20:5n-3 eicosapentaenoic acid	0.04	0.25	0.39	0.04	0.03
C22:5*n-3* docosapentaenoic	0.12	0.18	0.21	0.12	0.14
C22:6*n-3* docosahexaenoic	ND	1.17	1.86	ND	ND
Sum SFA	33.84	37.37	38.79	32.87	36.63
Sum *n*-3 FA	4.5	4.68	5.07	14.32	14.65
Sum *n*-6 FA	12.56	10.64	9.95	15.3	10.34

ND = non-detectable

Table 25. *Mouse liver lipid fatty acid composition.*

% total FAME	RO	LFO	HFO	EO	FXO	*P*
C16:0 palmitic	16.56^{a} ± 0.55	15.15^{ab} ± 0.31	15.7^{ab} ± 0.3	15.24^{ab} ± 0.29	14.67^{b} ± 0.26	**0.01**
C18:0 stearic	7.73 ± 0.39	6.86 ± 0.36	6.66 ± 0.49	8.07 ± 0.64	6.78 ± 0.39	0.116
C18:1 *cis*9 oleic	42.72^{a} ± 1.35	44.66^{a} ± 0.82	43.1^{a} ± 1.25	37.65^{b} ± 1.51	40.44^{ab} ± 0.97	**0.001**
C18:2*n-6* linoleic	9.73^{a} ± 1.23	9.19^{b} ± 0.13	8.43^{c} ± 0.16	10.43^{a} ± 0.16	10.62^{a} ± 0.18	**<0.001**
C18:3*n-3* α-linolenic	1.48^{a} ± 0.04	1.33^{ab} ± 0.03	1.09^{b} ± 0.03	3.22^{c} ± 0.15	5.85^{d} ± 0.11	**<0.001**
C18:4*n-6* stearidonic	ND	ND	ND	0.38 ± 0.09	0.2 ± 0.06	**<0.001**
C20:4*n-6* arachidonic	5.51^{a} ± 0.38	3.78^{b} ± 0.28	3.51^{b} ± 0.34	5.2^{a} ± 0.48	3.38^{b} ± 0.24	**0.001**
C20:5*n-3* eicosapentaenoic	0.79^{a} ± 0.05	0.96^{a} ± 0.04	1.29^{b} ± 0.07	1.76^{c} ± 0.07	2.13^{d} ± 0.06	**<0.001**
C22:5*n-3* docosapentaenoic	0.72^{a} ± 0.03	0.86^{b} ± 0.02	0.99^{c} ± 0.03	1.71^{d} ± 0.05	1.76^{d} ± 0.06	**<0.001**
C22:6*n-3* docosahexaenoic	6.3^{a} ± 0.25	9.46^{b} ± 0.3	11.43^{c} ± 0.4	7.01^{a} ± 0.5	6.18^{a} ± 0.32	**<0.001**
Sum SFA	25.29 ± 0.76	23.08 ± 0.55	23.63 ± 0.63	23.88 ± 0.82	22.25 ± 0.57	0.055
Sum *n*-3 FA	9.28^{a} ± 0.24	12.6^{b} ± 0.3	14.79^{cd} ± 0.42	14.09^{c} ± 0.44	16.11^{d} ± 0.18	**<0.001**
Sum *n*-6 FA	16.18^{a} ± 1.27	13.77^{bc} ± 0.38	12.65^{b} ± 0.52	17.76^{a} ± 0.65	14.7^{c} ± 0.44	**<0.001**

Values within a row with different superscripts are signicantly different.

Feeding DHA-rich fish oil produced the expected dose-dependent increase in hepatic lipid DHA content (Table 25). As noted in experiment 8 FXO increased hepatic α-linolenic acid, EPA, DPA and DHA concentrations (Table 25). EO feeding failed to increase hepatic 18:4*n*-3 but increased α-linolenic acid, EPA, DPA and DHA to similar levels as FXO. Thus, we were unable to demonstrate that bypassing the initial desaturation increased the efficiency of synthesis of these longer-chain, highly unsaturated fatty acids.

Table 26. Plasma lipids and lipoproteins in mice fed beef supplemented with different oils

mMol/l	RO	LFO	HFO	EO	FXO	*P*
Total Cholesterol	7.14 ± 0.32	7.81 ± 0.31	7.11 ± 0.28	7.93 ± 0.5	7.65 ± 0.35	0.374
HDL Cholesterol	1.39^{a}± 0.1	1.19^{ab} ± 0.06	1.09^{b} ± 0.05	0.95^{b} ± 0.08	1.21^{ab} ± 0.07	**0.007**
NonHDL Cholesterol	5.75 ± 0.26	6.62 ± 0.33	6.02 ± 0.29	6.98 ± 0.47	6.44 ± 0.37	0.129
NonHDL/HDL ratio	4.32^{a} ± 0.31	5.81^{ab} ± 0.61	5.66^{ab} ± 0.42	7.69^{b} ± 0.68	5.53^{ab} ± 0.56	**0.001**
Total Triacylglyerol	2.04 ± 0.21	1.81 ± 0.15	1.67 ± 0.18	1.61 ± 0.12	1.66 ± 0.16	0.390

Values within a row with different superscripts are signicantly different.

There was no significant difference in total plasma cholesterol or triacylglycerol between the diet groups. However, it is of note that plasma cholesterol in each of these groups was 25-30% lower than in animals fed un-supplemented beef in experiment 7. Compared to the RO-supplemented diet, HFO and EO both significantly reduced HDL cholesterol (Table 26). The reduction in total plasma cholesterol in each of these UFA-supplemented diets was associated with a significant reduction in the development of atherosclerosis with average lesion area being 60-90% lower than described in animals fed un-supplemented beef (Figure 5). However in the two groups in which a significant reduction in HDL cholesterol was observed (HFO and EO) lesion area was significantly higher than in

the RO-supplemented group. This experiment appears to demonstrate that the pro-atherosclerotic effects of SFA-rich beef fat can be reduced by supplementing diets with unsaturated fatty acid rich oils (decreasing the relative proportion of SFA by 10-15% of total fatty acids). However, we have failed to demonstrate that *n*-3 PUFA (of any chain length) is more protective than the combination of oleic and linoleic acid found in RO. In fact, both EO and HFO produced more atherosclerosis than RO, perhaps through a specific effect of lowering HDL cholesterol

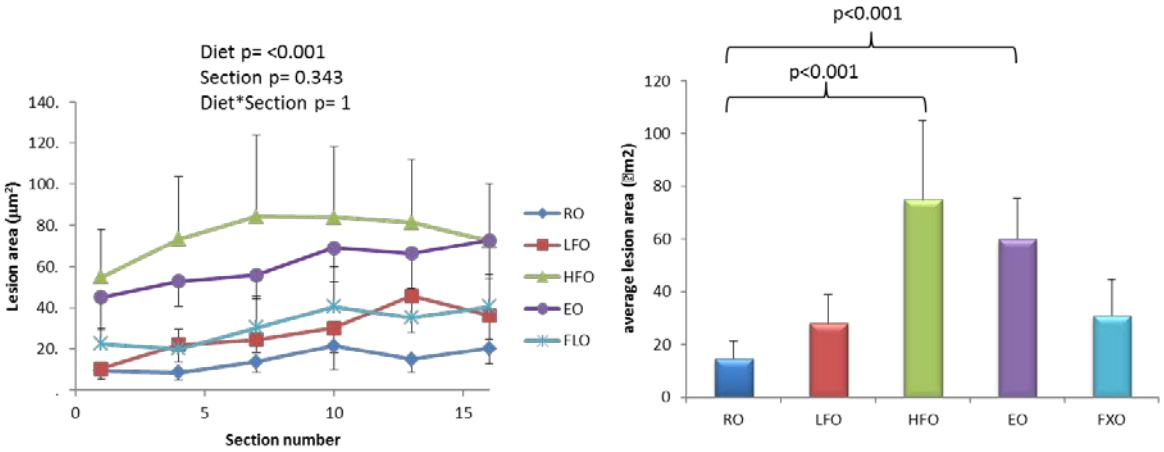

Figure 5. Analysis of atherosclerosis in mice fed beef supplemented with different oils.

Message: Overall, this work-package shows that in the highly artificial situation of beef providing virtually all of the fatty acids in the diet it does induce a significant level of atherosclerosis in this mouse model. The modest difference in fatty acid composition of beef from forage vs. concentrate fed cattle were enough to induce some significant differences in mouse tissue fatty acid but had no overall effect on the development of atherosclerosis. This may be because reductions in plasma triacylglycerol and HDL have opposing effects on atherogenesis. Supplementing beef with relatively modest amounts of unsaturated fatty acid markedly reduced plasma cholesterol and development of atherosclerosis. This effect was seen with all of the oils studied, but surprisingly, RO (relatively rich in oleic and linoleic acid) was more potent than any of the n-3 PUFA rich oils studied. Indeed high concentrations of EPA (HFO diet) or 18:4*n*-3 (EO diet) appeared to increase atherosclerosis compared to RO, perhaps by reducing the protective HDL cholesterol fraction. The direct comparison of the effect of α-linolenic acid and linoleic acid in experiment 8 appeared to show that *n*-6 PUFA is more protective against atherosclerosis than the *n*-3 PUFA and that this effect was independent of plasma lipids or lipoproteins. The impact of these oils on other atherosclerosis risk factors remains to be established.

While extrapolation of results in this animal model to humans should be done with caution, the results suggest that reducing the proportion of SFA in beef may be fundamentally more important that the type of fatty acid they are replaced with. They also suggest that at high concentrations *n*-3 PUFA may be more detrimental than *n*-6 PUFA, with reduction of HDL cholesterol being one possible underlying mechanism.

On-going work: Using a specific array, targeted at genes associated with lipid and lipoprotein metabolism, we have recently completed analysis of expression of 96 genes in the livers of animals in each of the diet groups described above. These data are currently undergoing appropriate bioinformatics analysis.

European Food Safety Authority (EFSA) and Food Labelling

The European Food Safety Authority (EFSA) recently concluded on the level of long chain PUFA that a product must contain in order for it to be labelled as 'a source of' or 'high in' *n*-3 PUFA. They concluded that it should be based around the requirement for 250mg per day of EPA plus DHA or 2g per day of 18:3*n*-3, and would require 40 or 80mg EPA plus DHA per 100g to be labelled as 'a source of' or 'high in' *n*-3 PUFA respectively (European Food Safety Authority, 2009). We recently conducted a review of a range of studies on the fatty acid composition of beef (Morgan *et al.* 2012), from animals

fed on a range of diets, different forages, oil supplementation etc. It was concluded beef from the forage-based systems summarised may provide ~ 0.71 - 34.4 mg/d EPA and ~ 0.15 - 6.3 mg/d DHA. The highest levels were for veal calves with a total EPA + DHA of ~ 41 mg/d (Costa *et al*., 2010). Thus, this is higher than the ~15% of the daily recommended intake for long chain PUFA required to be able to make a claim (250 mg/d, as described above). For comparison, Dunne *et al*. (2011) when feeding heifers ruminally protected fish oil supplement compared to a control achieved levels of 52.3 *v*. 13.0 and 15.4 *v*. 3.4, for EPA and DHA, respectively. The maximal levels of EPA + DHA delivered in beef from the studies reported would be ~ 67 mg/100 g muscle (Dunne *et al*., 2011). This is also higher than the ~ 15% of the daily recommended intake for long chain PUFA and as such this beef may be noted as a "source" of long chain PUFA.
All the forage treatments reported by Morgan *et al*. (2012), fall well below the 15% of recommended daily intake for EPA and DHA. These aspects present considerable challenges to approach levels of PUFA for which claims may be made. In this project, Probeef, levels of EPA and DHA ranged between 11-25 mg/100g muscle, with higher values noted on grass-fed beef supplemented with oils. Similarly, all the treatments fall below the level of 2g 18:3*n*-3 per 100 g product.

Conclusions

Nutritional quality is an increasingly important factor contributing to food product quality. Much attention is given to increasing the content of *n*-3 PUFA in beef and other foods as increased consumption of long chain *n*-3 PUFA would be beneficial in improving health and well-being and reducing disease in man. Green forage rich in the 18:3*n*-3 is an important tool to increasing delivery of *n*-3 PUFA through the ruminant animal into meat (and milk). As the 18:3*n*-3 is the building block of the long chain *n*-3 PUFA (EPA and DHA) feeding forage can increase these beneficial PUFA in meat. However, the levels of *n*-3 PUFA, 18:3*n*-3, EPA and DHA achieved by forage feeding fall below the level required to be able to claim that beef is either a "source" or "rich-in" *n*-3 PUFA. Hence, it is essential that the two main factors influencing the levels of *n*-3 PUFA in beef lipids are further addressed, namely (1) strategies to enhance levels of 18:3*n*-3 in forage and subsequent delivery into the animal and (2) increased ability to reduce lipolysis and/or biohydrogenation in the rumen. Recent progress in genetic control of lipids in perennial ryegrasses is likely to help significantly. Increased knowledge of the fate of the lipid rich chloroplast in the rumen represents a very exciting opportunity to deliver more beneficial *n*-3 PUFA from rumen through to the small intestine and hence to meat lipids.

Research Publication Outputs from the project:

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