

Forms of n-3 (ALA, C18:3n-3 or DHA, C22:6n-3) Fatty Acids Affect Carcass Yield, Blood Lipids, Muscle n-3 Fatty Acids and Liver Gene Expression in Lambs

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Abstract The effects of supplementing diets with n-3 alpha-linolenic acid (ALA) and docosahexaenoic acid (DHA) on plasma metabolites, carcass yield, muscle n-3 fatty acids and liver messenger RNA (mRNA) in lambs were investigated. Lambs ($n = 120$) were stratified to 12 groups based on body weight (35 ± 3.1 kg), and within groups randomly allocated to four dietary treatments: basal diet (BAS), BAS with 10.7 % flaxseed supplement (Flax), BAS with 1.8 % algae supplement (DHA), BAS with Flax and DHA (FlaxDHA). Lambs were fed for 56 days. Blood samples were collected on day 0 and day 56, and plasma analysed for insulin and lipids. Lambs were slaughtered, and carcass traits measured. At 30 min and 24 h, liver and muscle samples, respectively, were collected for determination of mRNA (FADS1, FADS2, CPT1A, ACOX1) and fatty acid composition. Lambs fed Flax had higher plasma triacylglycerol, body weight, body fat and carcass yield compared with the BAS group ($P < 0.001$). DHA supplementation increased carcass yield and muscle DHA while lowering plasma insulin compared with the BAS diet ($P < 0.01$). Flax treatment increased ($P < 0.001$) muscle ALA concentration, while DHA treatment

increased ($P < 0.001$) muscle DHA concentration. Liver mRNA FADS2 was higher and CPT1A lower in the DHA group ($P < 0.05$). The FlaxDHA diet had additive effects, including higher FADS1 and ACOX1 mRNA than for the Flax or DHA diet. In summary, supplementation with ALA or DHA modulated plasma metabolites, muscle DHA, body fat and liver gene expression differently.

Keywords Flaxseed · Algae · Blood lipids · Blood insulin · Carcass yield · Muscle fatty acids · Liver gene expression

Abbreviations

ALA	Alpha-linolenic acid
ACOX1	Acyl-CoA oxidase 1
ANOVA	Analysis of variance
cDNA	Complementary DNA
CPT1A	Carnitine palmitoyltransferase 1A
CT	Critical threshold
CP	Crude protein
DHA	Docosahexaenoic acid
DEGS2	Delta 4 desaturase
FADS1	Delta 5 desaturase
FADS2	Delta 6 desaturase
FID	Flame ionisation detector
DM	Dry matter
HOMA	Homeostatic model assessment
LDL	Low density lipoprotein
LL	Longissimus lumborum
ME	Metabolisable energy
mRNA	Messenger RNA
NEFA	Non-esterified fatty acid
n-3	Omega-3
n-6	Omega-6

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PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
QUICKI	Quantitative insulin-sensitivity check index
SED	Standard error of difference
TAG	Triacylglycerol
USDA	United States Department of Agriculture

Introduction

Human hunter–gatherer ancestors are thought to have consumed an array of muscle foods (meat) from range (wild) animals, which is in contrast to modern humans who generally derive meat from intensively farmed animals. The former consumed meat with higher levels of antioxidants, trace minerals and essential n-3 fatty acids but lower in n-6 and *trans* fatty acids [1]. Over the past 100 years, the consumption of n-3 essential fatty acids in Western human diets has decreased due to changes in agricultural practices, production systems and food processing technologies. It is reasonable to state that the shift in human diets away from meat derived from free-ranging animals to those cultivated in modern food production systems has contributed to the reduced concentrations of n-3 fatty acids in modern diets [2, 3].

Farm animal production consists of enterprises that bring not only economic benefits to primary producers but also social benefits to the public through wellness, and environmental benefits to global government agencies through sustainable food production systems. Ideally, there should be a balance between all these three components. However, producers often focus their efforts towards the economic benefits, trying to maximise these, and in turn sometimes ignore the credence values that the public and government seek. With an increasing human population, the demand for grains for human consumption and fuel production is expected to increase. As a result, producers are forced to look for alternative sources of feeds for farm animal production that provide suitable protein and energy (fat) [4]. One of the options is supplementing diets with lipids rich in n-3 fatty acids. Lipids rich in n-3 fatty acid can also cause a reduction in methane production accompanied by increased propionate in the rumen that improves the efficiency of growth [5]. Reports also indicate that inclusion of n-3 polyunsaturated fatty acid (PUFA) in the diets of rodents, humans and cattle altered muscle PUFA concentration, mRNA and protein expressions, lipid metabolism, and fat deposition in the body [6–10].

Recent literature suggests that producing foods with environmentally sustainable and healthy nutritive attributes for the consumer [11] is essential to maintaining market share. Replacing grain with sustainably derived aquatic algae as a source of protein and energy for farm animals may provide an opportunity to improve public health and decrease environmental impact. The current study investigated the effect of supplementing lamb with flax or algae on blood metabolites, carcass

yield, muscle long-chain n-3 fatty acids (alpha-linolenic acid, ALA and docosahexaenoic acid, DHA) content and liver gene expression. We hypothesise that supplementation of flax rich in ALA and algae rich in DHA to lambs consuming a basal hay diet can alter the muscle fatty acid concentration differently due to their specificity for cell membrane fluidity, membrane function and fat metabolism.

Materials and Methods

All procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2004). Approval to proceed was obtained from the DEPI Agricultural Research and Extension Animal Ethics Committee. One hundred and twenty (120) second-cross weaned ewe lambs (~9 months old) were stratified to 12 groups based on initial liveweight (35 ± 3.1 kg), and then within each group randomly allocated to four dietary treatments: basal diet (annual ryegrass hay/clover hay) = BAS, basal diet with flaxseed supplement [10.7 %, dry matter (DM) basis] = Flax, basal diet with algae supplement (1.8 %, DM basis) = DHA, basal diet with flaxseed (10.7 %, DM basis) and algae (1.8 %, DM basis) supplement = FlaxDHA. Table 1 presents the nutritional information of the ryegrass hay, clover hay, flaxseed and algae used for diet formulation in the current study. The experiment was conducted in two replicates with the six heaviest groups allocated to replicate one and the rest allocated to replicate two. The basal annual pasture hay pellet consisted of subterranean clover (*Trifolium subterranean* L.) and annual ryegrass (*Lolium rigidum* Gaud.) in the proportion of 40 and 60 %, respectively, mixed and then

Table 1 Nutritional information of ryegrass hay, clover hay, flaxseed and algae used for diet formulation

	Ryegrass hay	Clover hay	Flaxseed	Algae
Protein	7.9	15.0	20.0	16.7
Fat	0.5	0.5	41.0	55.6
Fibre ^a	53	35	27.0	4.5
Saturated fats	0.13	0.17	3	18.9
Polyunsaturated fats	0.16	0.20	32	36.7
Monounsaturated fats	0.03	0.03	8	–
Alpha-linolenic acid	0.09	0.16	25	<0.2
Eicosapentaenoic acid (EPA)	–	–	0	1.1
Docosahexaenoic acid (DHA)	–	–	0	23.5

Values given in g/100 g of diet and expressed on dry matter basis. Fat content of ryegrass hay/clover hay cubes was around 0.5 g of total FA/100 g of diet

^a For ryegrass hay and clover hay, neutral detergent fibre is reported, while for flaxseed and algae, crude fibre content is reported

pressed using heat (60–85 °C for <1 min) and extruded into a cube (3 cm³) using molasses as binder. All groups received a daily ration of dried molasses at 3.6 % mixed with the cubes. The flaxseed comprised whole flaxseed flakes (Melrose, Victoria, Australia), and the DHA algae product was DHA Gold™ (Aquadrow®, MARTEK, Columbia, USA). Table 2 presents the dietary ingredients used for each treatment in the current study.

All lambs were grazed on annual pasture for 3 weeks in the field prior to entering experimental housing, where they were introduced to the experimental diets for 7 days and then fed ad libitum for 56 days. The daily feed intake was recorded to measure the total daily intakes of crude protein (CP) and estimated metabolisable energy (ME). The CP and ME contents of the BAS, Flax, DHA and FlaxDHA diets were 118, 122, 118 and 122 g/kg DM and 10.2, 10.6, 10.6 and 10.8 MJ/kg DM of diet, respectively. Daily consumption of feed, total fat, ALA and DHA for treatment groups are given in Table 3.

Blood Collection, Hormone and Metabolite Analyses and Slaughter Procedure

Fasting blood samples were collected from all lambs at the commencement (day 0) and at the end (day 56) of the feeding study, with the plasma analysed for glucose, insulin, total cholesterol, triacylglycerol (TAG) and non-esterified fatty acid (NEFA) concentrations. Glucose and NEFA concentrations were determined as described previously [12].

Table 2 Dietary composition used for lambs fed basal ryegrass/clover hay (BAS), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets

	Basal	Flax	DHA	FlaxDHA
Ryegrass/clover (%)	96.4	85.7	94.6	83.9
Flaxseed (%)	0	10.7	0	10.7
Algae (%)	0	0	1.8	1.8
Dried molasses (%)	3.6	3.6	3.6	3.6

Values expressed on dry matter basis. Molasses was used as binder

Table 3 Mean daily intake (consumption) of feed, protein:energy, fat, alpha-linolenic acid (ALA) and docosahexaenoic acid (DHA) for lambs fed basal ryegrass/clover hay (BAS), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets

	Basal	Flax	DHA	FlaxDHA	SED Flax × DHA	P value Flax	P value DHA	P value Flax × DHA
Feed intake (g/day)	1313 ^b	1256 ^b	1106 ^a	1093 ^a	52.9	0.38	0.002	0.59
Protein:energy (intake/day) ^d	11.6	11.5	11.1	11.2	0.23	0.78	0.13	0.29
Fat intake (g/day)	6.6 ^a	60.4 ^c	15.3 ^b	63.1 ^c	3.0	0.001	0.02	0.15
ALA intake (g/day)	1.64 ^a	34.8 ^b	1.36 ^a	30.2 ^b	1.56	0.001	0.13	0.09
DHA intake (g/day)	0	0	4.18	4.19	–	–	–	–

SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED

SED standard error of difference

^{a,b,c} Mean values with different superscript letters within a row are significantly different ($P < 0.05$)

^d Protein:energy consumption/day = calculated as crude protein to metabolisable energy intake of diet consumed on dry matter basis

Insulin concentration was determined using a commercially available radioimmunoassay kit (Millipore Porcine Insulin RIA, USA Cat# MPPI12K). Total cholesterol (Procedure 401) and TAG (Procedure 343) concentrations were determined using kit reagents purchased from Sigma (Sigma-Aldrich Pty. Ltd., Sydney, Australia). The degree of insulin resistance was determined using the homeostatic model assessment (HOMA) and quantitative insulin-sensitivity check index (QUICKI) [13]. A decrease in HOMA is associated with reduced insulin resistance, and an increase in QUICKI is associated with increased insulin sensitivity. At the end of the feeding period, animals were transported to a commercial abattoir for slaughter and muscle and liver sample collection.

Muscle Sample Collection and Fatty Acid Determination

At 30 min post-slaughter, a 2 g liver sample was collected and immediately snap frozen in liquid nitrogen before storing at -80 °C for determination of gene expression. At 24 h post-mortem, the ultimate pH of the longissimus lumborum (LL) muscle was recorded. Body fat was assessed using GR knife by measuring the adipose and muscle tissue thickness at 11th/12th rib intersection 110 mm away from midline, which is a common procedure to express the fatness in the body or carcass. The LL muscle from the hind-quarter over the lumbar region was removed from the left side of the carcass, and duplicate samples (~25 g) were collected and stored at -20 °C for determination of fatty acids. The longissimus muscle is the major muscle present in the body of lambs as this muscle has been used as a common reference muscle for meat quality and nutritional studies. The deposition of individual fatty acids in different muscles in the carcass of lamb would be in a similar ratio or pattern, although the magnitude may differ slightly due to fibre type and muscle activity. A previous study conducted using different muscles showed that deposition of fatty acid occurs

in a similar pattern [14], therefore longissimus muscle was selected for determination of fatty acid composition in the current study.

One set of samples from all animals was freeze dried and homogenised, and approximately 0.5 g was used for fatty acid extraction using a modified procedure of [15]. One hundred microlitres of nonadecanoic acid methyl ester (C19:0, Sigma Aldrich Pty. Ltd., Castle Hill, NSW 2154, Australia) was added to muscle samples as internal standard dissolved in chloroform (10 mg C19:0/mL CHCl₃). The contents were then hydrolysed using 0.7 mL of 10 N KOH in water and 5.3 mL of methanol to form free fatty acids. After thorough mixing, the contents were incubated at 55 °C for 1.5 h, with vigorous mixing at 20 min intervals and then cooled to room temperature using tap water. Upon cooling, contents were mixed with 0.6 mL of 24 N sulphuric acid in water with the mixing, incubation and cooling process as above. After cooling the tubes to room temperature, the fatty acid methyl ester (FAME) was separated with 1 mL of hexane solvent by mixing for 5 min and centrifuging at 2000 rpm for 10 min.

Two hundred microlitres of hexane containing FAME was collected into a gas chromatograph (GC) vial, and fatty acid fractions were quantified by capillary GC Varian 3800 (HP INNOWAX 60 × 0.25 mm, 0.5 µm, Agilent J & W Scientific, Santa Clara, CA, USA) with a CP-8400 autosampler, split injector (25:1) maintained at 250 °C and flame ionisation detector (FID) maintained at 280 °C. The carrier gas was hydrogen at constant flow rate of 2.5 mL/min. The initial oven temperature of 120 °C was held for 1 min after injection and then increased at 20 °C/min to 250 °C and held for 19 min. The detector gas flows were 30 mL/min nitrogen make-up gas, 30 mL/min hydrogen, 300 mL/min air. Fatty acid peaks were identified using a reference standard (Supelco C4-C24 mix, Sigma Aldrich Pty. Ltd., NSW 2154, Australia) that was run in each batch. The concentrations of individual fatty acids including ALA, DHA and total fatty acid fractions in the muscles are reported as mg/100 g meat to be consistent with the nutritional information used to label foods according to Food Standards Australia & New Zealand (FSANZ). Total muscle fatty

acid content was calculated from the summation of individual fatty acids quantified by gas chromatography.

Liver (mRNA) Gene Expression

Total RNA was extracted from 50 mg of frozen liver samples using TRI reagent (Astral Scientific, Sydney, Australia) according to the manufacturer's specifications. The total RNA concentration was determined by the absorbance measured at 260 nm, and RNA purity was estimated by the ratio of the absorbance measured at 260 and 280 nm. One microgram of total RNA was reverse transcribed into complementary DNA (cDNA) using AMV reverse transcriptase first strand cDNA synthesis kit according to the manufacturer's protocol (Marligen Biosciences, Sydney, Australia). Real-Time polymerase chain reaction (PCR) was performed using a Bio-Rad IQ5 detection system, with reactions performed using SYBR Green Supermix (Bio-Rad, Sydney, Australia). Primers were designed using Primer 3, and obtained from GeneWorks (Hindmarsh, Australia; Table 4). Samples were normalized for the cDNA concentration determined with OliGreen (Invitrogen, Mulgrave, Australia) prior to PCR [16]. Amplification of cDNA samples was carried out using IQ SYBR Green™ following the manufacturer's protocols (BioRad, Sydney, Australia). Fluorescence emission data were captured, and mRNA levels were analysed using the critical threshold (CT) value [17]. Thermal cycling and fluorescence detection were conducted using the BioRad IQ5 sequence detection system (BioRad, Sydney, Australia).

Statistical Analysis

The data were analysed using analysis of variance (ANOVA) in the GenStat statistical package (GenStat Release 13.1, VSN International Ltd.). Analysis was by a 2 × 2 factorial design with two levels of flaxseed flakes (with and without) and two levels of DHA (with and without). The treatment structure was specified with fully factorial effects for the factors flaxseed (Flaxseed and Nil) by DHA (DHA and Nil). The blocking structure was specified

Table 4 PCR primers used for gene expression analysis

	Forward	Reverse
ACOX1	5'-TGGAGAGCCCTCAGCTATGG-3'	5'-CGTTTCACCGCCTCGTAAG-3'
CPT1A	5'-CTGTATCGTCGCACATTAGACCGT-3'	5'-CAGACCTTGAAGTACCGCCTCT-3'
FADS1	5'-AGGAACAGTGTGACCCCTTG-3'	5'-AAAAACAGTTCTCCAGCA-3'
FADS2	5'-ATCTGCCCTACAACCACAG-3'	5'-TGTGACCCACACAAACCAGT-3'
DEGS2	5'-CACCACCTCTGGGTGAAAGT-3'	5'-AACCTGGTCACTCCAACCTG-3'

ACOX1 acyl-CoA oxidase 1, *CPT1A* carnitine palmitoyltransferase 1A, *FADS1* fatty acid desaturase 1/delta 5 desaturase, *FADS2* fatty acid desaturase 2/delta 6 desaturase, *DEGS2* delta 4 desaturase/sphingolipid 2

Table 5 Body weight, feed consumed to weight gain (feed:weight gain), carcass yield (carcass weight/body weight = carcass dressing %), body fat (assessed as GR) and plasma hormones and metabolites of lambs fed basal ryegrass/clover hay (BAS), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets

	BAS	Flax	DHA	FlaxDHA	SED Flax × DHA	<i>P</i> value Flax	<i>P</i> value DHA	<i>P</i> value Flax × DHA
Body weight (kg) ^d	45.6 ^{ab}	49.2 ^c	45.1 ^a	46.9 ^b	0.71	0.002	0.02	0.11
Feed:weight gain	7.25 ^b	5.42 ^a	7.19 ^b	5.63 ^a	0.57	0.85	0.004	0.76
Carcass yield	43.2 ^a	45.6 ^b	44.9 ^b	45.3 ^b	0.26	0.001	0.007	0.001
Body fat (mm) ^e	10.7 ^a	15.1 ^c	11.4 ^a	13.3 ^b	0.85	0.001	0.51	0.03
Glucose (mmol/L)	3.73	3.97	3.84	3.81	0.19	0.44	0.83	0.30
Insulin (mU/mL)	7.69 ^b	7.97 ^b	5.46 ^a	6.57 ^a	0.51	0.23	0.002	0.05
HOMA (mmol.mU/L ²) ^f	1.31 ^b	1.41 ^b	0.93 ^a	1.15 ^{ab}	0.17	0.19	0.009	0.63
QUICKI ^g	0.37 ^a	0.37 ^a	0.40 ^b	0.39 ^{ab}	0.01	0.17	0.002	0.54
Cholesterol (μmol/L)	7.72 ^a	13.5 ^b	6.82 ^a	14.5 ^b	1.70	0.001	0.96	0.42
Triacylglycerol (mmol/L)	119 ^a	147 ^c	125 ^{ab}	132 ^b	5.2	0.001	0.22	0.01

SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED

SED standard error of difference

^{a,b,c} Mean values with different superscript letters within a row are significantly different ($P < 0.05$)

^d Body weight and body fat were adjusted to initial body weight of lambs at commencement (day 0) of experiment

^e Body fat was assessed using GR knife by measuring the adipose and muscle tissue thickness at 11th/12th rib intersection 110 mm away from midline, which is a common procedure to express the fatness in the body/carcass

^f Homeostatic model assessment (HOMA, see text for details)

^g Quantitative insulin-sensitivity check index (QUICKI, see text for details)

as Animal nested within Group nested within Run. The values for DHA (C22:6n-3) were transformed using a natural log transformation to establish normal distribution and constant variance. The means of treatment groups for blood lipids, glucose and insulin were determined using initial concentrations at day 0 as covariates. Gene data were tested for main and interactive effects of Flaxseed and DHA. *F* tests were used to determine the overall significant difference among the predicted means, whereas the difference between two predicted means was judged to be significant if it was at least two times the average standard error of difference (SED).

Results

Daily Energy and Protein Intakes

Lambs fed algae diets had lower daily intakes of protein ($P < 0.01$) and ME ($P < 0.01$) compared with lambs receiving no algae, which was associated with a lower daily feed intake (1.10 versus 1.29 kg, $P < 0.002$) (Table 3). The average daily protein and ME intakes of lambs fed BAS, Flax, DHA and FlaxDHA were 155, 153, 130 and 133 g and 13.3, 13.4, 11.8 and 11.8 MJ, respectively.

Blood Metabolites and Body Components

Body weight was increased by dietary Flax (45.4 versus 48.1 kg, $P < 0.001$) but was reduced by dietary DHA (47.5 versus 45.9 kg, $P < 0.02$) (Table 5). Carcass weights for BAS, Flax, DHA and FlaxDHA treatments were 19.8, 22.4, 20.1 and 21.2 kg, respectively. The ratio of feed consumed to liveweight gain (FCR) was improved by Flax (5.52 versus 7.22, $P < 0.004$, SED = 0.4) but not by dietary DHA or the interaction of Flax × DHA (Table 5). Carcass yield (carcass weight/body weight, which is also indicated by dressing percentage) was increased by both dietary Flax (44.0 versus 45.4 %, $P < 0.001$) and dietary DHA (44.4 versus 45.1 %, $P < 0.007$) (Table 5). However, there was an interaction ($P < 0.001$) indicating that the effects were not additive.

Back fat depth was increased by dietary Flax (11.1 versus 14.2 mm, $P < 0.001$). Although there was no main effect of dietary DHA on back fat, there was an interaction ($P < 0.03$) between dietary Flax and DHA such that the increase in back fat in response to Flax, although still present, was reduced when supplemental DHA was provided (Table 5). There were no effects of either dietary Flax or DHA on plasma glucose. Plasma insulin was reduced by dietary DHA (7.8 versus 6.0 mU/mL, $P < 0.002$). Although there was no main effect of dietary Flax on plasma insulin concentrations, there was an interaction ($P < 0.05$) between

Table 6 Muscle fatty acid composition of lambs fed basal ryegrass/clover hay (BAS), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets

	Basal	Flax	DHA	FlaxDHA	SED Flax × DHA	<i>P</i> value Flax	<i>P</i> value DHA	<i>P</i> value Flax × DHA
C8:0	0.36	0.38	0.47	0.42	0.05	0.65	0.04	0.26
C10:0	3.24	3.21	3.78	3.55	0.42	0.18	0.67	0.75
C12:0	2.55	2.49	2.80	2.86	0.31	0.99	0.20	0.79
C14:0	55.3	56.9	59.8	61.9	7.3	0.73	0.39	0.97
C14:1	1.48	1.38	1.54	1.44	0.25	0.56	0.78	0.99
C15:0	7.93	8.09	8.55	9.03	0.78	0.58	0.20	0.79
C16:0	615	587	663	623	60.2	0.45	0.36	0.90
C16:1	38.3	33.4	34.9	32.4	3.5	0.19	0.41	0.67
C17:0	31.4	26.4	32.1	27.7	2.4	0.03	0.56	0.86
C18:0	450 ^b	512 ^c	436 ^b	378 ^a	27.9	0.92	0.007	0.03
C18:1n-9cis-trans	1013	1005	929	1017	76.3	0.48	0.52	0.43
C18:2n-6cis	74.9	82.9	69.9	73.1	2.91	0.03	0.01	0.30
C18:2n-6trans	0.45 ^a	2.22 ^b	0.55 ^a	6.64 ^c	0.47	0.001	0.001	0.001
CLA	0.84 ^a	1.23 ^b	0.99 ^a	2.67 ^c	0.14	0.001	0.001	0.001
C18:3n-3	34.3 ^a	59.5 ^c	32.1 ^a	41.3 ^b	2.1	0.001	0.001	0.001
C18:3n-6	0.99	1.05	1.15	1.10	0.40	0.99	0.73	0.86
C20:0	2.92	2.90	3.08	2.74	0.23	0.31	0.99	0.39
C20:1n-9	9.9 ^a	18.2 ^b	10.7 ^a	26.8 ^c	1.71	0.001	0.01	0.02
C20:4n-6	29.6	22.7	41.8	37.4	0.76	0.001	0.001	0.09
C20:5n-3	17.6 ^a	18.1 ^a	21.8 ^b	17.2 ^a	0.82	0.01	0.02	0.01
C22:0	0.35	0.37	0.37	0.34	0.03	0.95	0.74	0.25
C22:4n-6	1.49 ^b	1.10 ^a	1.94 ^c	2.23 ^c	0.18	0.70	0.001	0.04
C22:5n-3	13.9 ^d	11.2 ^c	9.8 ^b	8.3 ^a	0.27	0.001	0.001	0.02
C22:6n-3 ^e	7.6 ^a	6.7 ^a	63.4 ^c	49.6 ^b	0.05	0.002	0.001	0.001
Total fatty acid	2549	2600	2564	2571	182	0.82	0.96	0.88

All values expressed in mg fatty acid/100 g of meat. SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED

SED standard error of difference

^{a,b,c,d} Mean values with different superscript letters within a row are significantly different ($P < 0.05$)

^e For C22:6n-3, the data were transformed to achieve normal distribution for statistical analysis, but untransformed means are presented

dietary Flax and DHA such that the decrease in plasma insulin concentrations in response to DHA, although still present, was not as pronounced when supplemental Flax was provided (Table 5). The HOMA was decreased by dietary DHA (1.36 versus 1.04, $P = 0.009$) but was not significantly altered by dietary Flax (1.12 versus 1.28, $P = 0.19$) (Table 5). The plasma QUICKI was increased by dietary DHA (0.375 versus 0.396, $P = 0.002$) but was not significantly altered by dietary Flax (0.390 versus 0.381, $P = 0.17$) (Table 5). Total cholesterol (14.0 versus 7.27 $\mu\text{mol/L}$, $P < 0.001$) and TAG (140 versus 122 mmol/L, $P < 0.001$) concentrations were higher in lambs supplemented with Flax but were not affected by dietary DHA. However, there was an interaction ($P < 0.01$) between dietary Flax and DHA such that the increase in plasma TAG in response to Flax, although still present, was not as pronounced when supplemental DHA was provided.

Muscle Fatty Acid Composition

Table 6 presents the fatty acid profile of muscle (meat) including ALA and DHA concentrations. The changes in other long-chain fatty acid concentrations such as C20:5n-3, C22:5n-3 and C22:4n-6 due to treatments were small, and the main focus is given to the concentrations of ALA and DHA in muscle associated with the dietary ALA and DHA supplied by flaxseed and algae, respectively. The changes observed in muscle ALA (C18:3n-3) and DHA (C22:6n-3) concentrations in relation to their dietary supply were substantial. The amount of ALA provided by both flaxseed diets (Flax and FlaxDHA) was greater ($P < 0.001$) than the amount of ALA provided by the basal hay diet (BAS) or algae (DHA) supplemented diet (Table 3). DHA was absent in both BAS and Flax treatment diets (Table 3). Flax supplementation increased ($P < 0.001$) the muscle

ALA concentration by 75 %, while algae supplementation increased ($P < 0.001$) the muscle DHA concentration by 800 % compared with the BAS group. When the supplements were combined (FlaxDHA diet), the ALA and DHA concentrations in muscle were increased by 20 and 614 %, respectively, compared with the control diet (Table 6). There were significant increases in C18:2n-6 *trans* and conjugated linoleic acid (CLA) concentrations with Flax and FlaxDHA. An additive effect was observed when DHA was added to Flax (FlaxDHA treatment) such that the C18:2n-6 *trans* and CLA concentrations were more than double those observed with Flax alone. There were small but significant changes observed in muscle eicosapentaenoic acid (EPA, C20:5n-3) and docosapentaenoic acid (DPA, C22:5n-3) concentrations. Algae-supplemented lambs had greater concentration of EPA than all other groups. DPA concentration was lowest for FlaxDHA and highest for BAS, while the other two groups had intermediate values but significantly differed between them. Dietary supplementation had no effect on total muscle fatty acid content (Table 6).

Gene Expression

Lambs fed algae had higher liver gene expression of delta 6 desaturase (FADS2; $P < 0.05$) and lower carnitine palmitoyltransferase 1A (CPT1A; $P < 0.02$) mRNA levels compared with the basal forage diet (Figs. 1, 2). Feeding the combination diet of flax and algae (FlaxDHA) resulted in higher liver gene expression of delta 5 desaturase (FADS1) compared with basal forage, and higher acyl-CoA oxidase 1 (ACOX1) mRNA compared with the basal forage and algae diets ($P < 0.05$, Figs. 3, 4). The liver gene expression of delta 4 desaturase (DEGS2) did not change with any of the diets.

Discussion

The current study clearly demonstrated that adding aquatic Algae or terrestrial Flax as dietary supplements significantly altered carcass yield, blood lipid parameters, muscle long-chain n-3 fatty acid (ALA and DHA) content and liver gene expression compared with lambs fed the basal diet. When compared with a basal hay diet, the ALA content in muscle was increased by 75 % with the Flax supplementation and DHA content in muscle was increased by 800 % with algae supplementation. However, when Flax and DHA were combined (FlaxDHA group), the increase in ALA and DHA concentration was by 20 and 614 %, respectively, suggesting that ALA and DHA compete for absorption at the peripheral tissue level. Alternatively, there could also be competition for absorption from the gut enterocyte level.

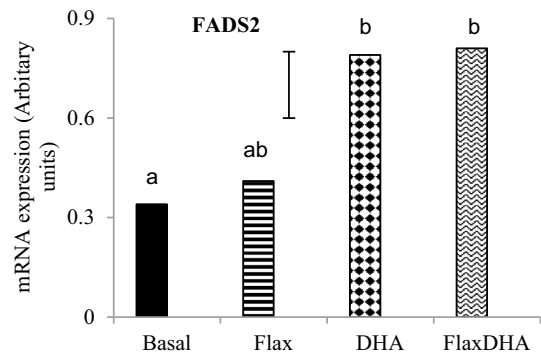


Fig. 1 FADS2: fatty acid desaturase 2/delta 6 desaturase. Gene expression in liver of lambs fed basal (ryegrass/clover), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets. Error bar indicates standard error of difference (SED) between treatment means. SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED. SED for FADS2 was 0.21. ^{a,b}Mean values with different superscript letters are significantly different ($P < 0.05$)

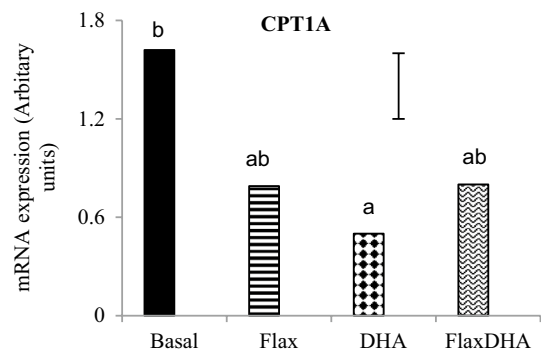


Fig. 2 CPT1A: carnitine palmitoyltransferase 1A. Gene expression in liver of lambs fed basal (ryegrass/clover), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets. Error bar indicates standard error of difference (SED) between treatment means. SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED. SED for CPT1A was 0.40. ^{a,b}Mean values with different superscript letters are significantly different ($P < 0.05$)

In the last two decades, there have been a number of studies focussed on enhancing the content of essential n-3 PUFA, particularly EPA and DHA, in beef, lamb, chicken and pork to increase the nutritional value of meat [8, 18–20]. In the present study, meat from lambs fed 1.8 % algae (DHA and FlaxDHA treatment groups) met the Australian and New Zealand standards [21] as a good source of n-3 (>60 mg/135 g serve) or the European standard [22] for a source (40 mg/100 g) and good source (80 mg/100 g) of EPA + DHA. In the context of

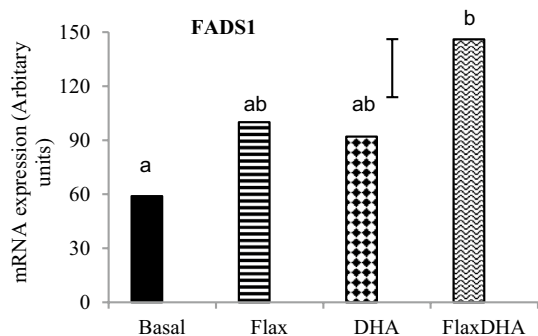


Fig. 3 FADS1: fatty acid desaturase 1/delta 5 desaturase. Gene expression in liver of lambs fed basal (ryegrass/clover), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets. Error bar indicates standard error of difference (SED) between treatment means. SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED. SED for FADS1 was 32.3. ^{a,b}Mean values with different superscript letters are significantly different ($P < 0.05$)

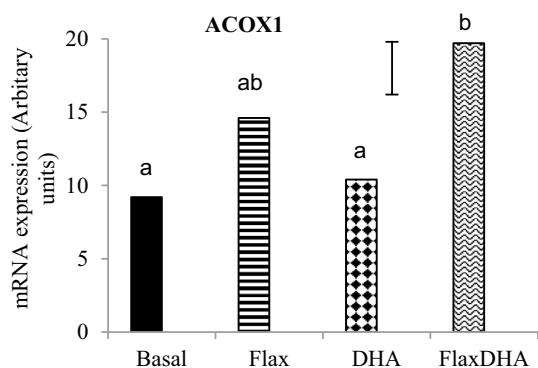


Fig. 4 ACOX1: acyl-CoA oxidase 1. Gene expression in liver of lambs fed basal (ryegrass/clover), flaxseed (Flax), algae (DHA) and flaxseed plus algae (FlaxDHA) diets. Error bar indicates standard error of difference (SED) between treatment means. SED was used to identify differences between treatment means at $P < 0.05$ level. The difference between two predicted means was judged to be significant if it was at least two times the SED. SED for ACOX1 was 3.6. ^{a,b}Mean values with different superscript letters are significantly different ($P < 0.05$)

Australia [23] and perhaps in other developed countries such as the USA, the contribution of red meat to the overall consumption of very long-chain n-3 PUFA is high because the consumption of fish rich in n-3 fatty acid among the population is very low. Accordingly, 20 % of the very long-chain n-3 PUFA intake in the Australian population is derived from meat and meat products [23]. The current study shows that inclusion of algae in a small quantity in lamb, or by extrapolation cattle, diets could possibly increase the n-3 PUFA intake of the population via consumption of red meat and meat products.

Dietary lipid supplementation can increase carcass yield (carcass weight/body weight) by increasing the dietary energy available to be partitioned into body components. Methane production can be high in animals grazing low-quality highly fibrous forage diets, with such diets requiring more time for microbial degradation and fermentation in the rumen [24], and this methane represents a loss of dietary energy that could be diverted towards animal production. Dietary fat supplementation to sheep and cattle, for example as oil seeds or algae, may be a mechanism to reduce methane production [25] and divert the energy loss as gas into body gain. It was noted that, when DHA from algae was added to the diet, the daily feed intake was reduced with both DHA and FlaxDHA treatments compared with the BAS group. A reduction in feed intake was also observed when DHA was offered to dairy cattle [26]. Even though the feed intake of the supplemented lambs was lower than the lambs fed the BAS diet, the carcass yield was greater for DHA and FlaxDHA supplemented lambs. These findings suggest that, when algae were supplemented to both DHA and FlaxDHA supplemented lambs, the conversion of dietary energy (or other nutrients) to carcass tissues was better even though these animals had a lower feed intake. This might have been due to some dietary energy loss being captured via a reduction in methane (gas) production with DHA diets that was diverted to body tissue (fat and muscle) gain. While this is speculative, since methane emission was not measured in this study, others [5] have reported that n-3 fatty acid from fish oil (DHA) increased rumen propionate and reduced rumen methane production, further supporting our findings.

At similar feed intakes and protein:energy ratio of diet, the lambs fed the Flax diet had greater carcass yield than the lambs fed the BAS diet. Previous study [9] conducted in beef heifers showed that supplementation of whole flax or rolled flax at 8 % DM basis in a feedlot ration during 56 day growing and then finishing stage significantly increased body weight and United States Department of Agriculture (USDA) yield grade compared with a control feedlot ration without flax, which is similar to the current findings. The increase in carcass yield with supplemental Flax was also associated with an increase in body fat, as indicated by GR fat measure (a common commercial measure of carcass fatness). The increase in carcass yield and body fat (GR) was most likely due to an increase in dietary fat intake (6.6 versus 60.4 g/day for BAS and Flax groups), an energy source that was provided by the flaxseed supplementation. Also, the lambs fed the Flax diet showed the greatest increase in plasma TAG concentrations compared with all other treatments. Others [8] have also noticed, with flaxseed supplementation to Angus steers at 907 g/day for 107 days before slaughter, increased muscle TAG concentration compared with unsupplemented animals. Elevated

blood TAG is one indicator for fat accumulation in the body as a result of impaired insulin action in peripheral tissue. Studies conducted in humans and rats showed inverse relationships between circulating TAG concentrations and insulin action in muscle, indicative of TAG accumulation in tissues and therefore an increase in body fat [27–30] as seen in the present study with flaxseed supplementation.

With similar amounts of dietary fat intake between Flax and FlaxDHA groups, it could be expected that the body fat (assessed by GR) and plasma TAG of both groups would be the same. However, body fat and plasma TAG with FlaxDHA treatment were lower than for the Flax group, although still significantly higher than in lambs fed the BAS and DHA diets. It is noteworthy that Flax supplementation provided 18:3n-3 as the main n-3 PUFA while algae supplementation provided 22:6n-3 as the main n-3 PUFA. It was also found that Flax supplementation increased 18:3n-3 and DHA algae supplementation increased 22:6n-3 fatty acids in skeletal muscle. When DHA was added to Flax in the diet (FlaxDHA group), the fat accumulation was influenced by a 13 % reduction in body fat (GR) and 11 % reduction in plasma TAG concentration compared with the Flax group. The latter supports the reports from others [2, 7] that long-chain n-3 PUFA such as EPA (20:5n-3) or DHA (22:5n-3) exerts its beneficial effects on human health (e.g. cardiovascular function, anti-inflammation, anti-obesity) through decreasing plasma TAG concentrations and inhibiting deposition of fatness in the body.

Previous studies have reported that increased DHA concentrations in muscle improved insulin-stimulated glucose utilisation in skeletal muscles [29]. The lower plasma insulin concentration in the DHA and FlaxDHA groups might be associated with a significant increase in muscle DHA concentration. However, the plasma glucose concentrations in all groups were similar to the levels reported earlier [12] in lambs with the same genetics and similar body weight. The HOMA and QUICKI that were calculated from both plasma glucose and insulin clearly indicated that dietary DHA reduced insulin resistance (improved insulin sensitivity) in these lambs. The lambs that had elevated blood TAG levels (i.e. those fed Flax and FlaxDHA) also had higher plasma cholesterol concentrations compared with the other lambs. Others have reported that flaxseed supplementation reduced circulating low-density lipoprotein (LDL) cholesterol [31]. However, in the current study, only total cholesterol was measured, and so it is not known whether the increase in total cholesterol was due to an increase in LDL cholesterol.

The conversion of linoleic acid to arachidonic acid and alpha-linolenic acid to EPA and DHA shares the same delta-5 (FADS1) and delta-6 (FADS2) desaturase enzymes [32–34]. The current study showed that there was an association between liver (a major organ responsible for fatty

acid metabolism) gene expression of FADS2 and muscle DHA concentration in animals fed DHA and FlaxDHA diets. In the case of FADS1 and ACOX1 gene expression, an additive effect was observed where FlaxDHA feeding further increased the expression over Flax or DHA alone. The differences in gene expression (FADS1 or ACOX1) with FlaxDHA might not be due to differences in fat intake between DHA or Flax or FlaxDHA treatments because daily consumption of fat for Flax treatment (60.4 g/day) and FlaxDHA treatment (63.1 g/day) was almost similar. Previous studies [8, 10] have shown that flaxseed supplementation at 907 g/day for 107 days in cattle weighing 454 kg significantly increased the levels of ALA, EPA and DHA in longissimus dorsi muscle. The latter in turn increased expression of peroxisome proliferator activated receptor (PPAR γ) gene expression that regulates lipid metabolism [8] and adipocyte fatty acid binding protein (A-FABP) expression involved in lipid trafficking [10] compared with unsupplemented animals.

However, when grass rich in ALA was fed to cattle [33], the mRNA gene expression of FADS1, FADS2, ACOX1 and PPAR γ was not affected compared with those fed a maize silage control diet. It should be noted that, in the latter study [33], the increases in the amounts of ALA, EPA and DHA in rectus abdominis and semimembranosus muscles were lower than the levels found in LL muscle in the current study. When flax oil (2.5 % linseed oil in the diet) rich in ALA was fed to chickens [34], the mRNA gene expression of FADS1, FADS2, ACOX1 and CPT1 were not affected compared with those fed a control diet having 4 % soybean oil. From previous [8, 33–35] and current research findings, we can state that the effects of dietary ALA and DHA consumption on tissue mRNA expressions (e.g. FADS1, FADS2, ACOX1, PPAR γ or CPT1) are dependent on the type of fatty acids or oils used in the experimental diet, the level at which they are added to the diet, the type of feed ingredients used in the control diet and how long the diet is fed to the animals.

Furthermore, the degree to which FADS1, FADS2 or ACOX1 expression is modulated by dietary DHA can be affected by the ratio of DHA to other fatty acids in the experimental diet provided [35]. For example, when DHA (algae) was added with ALA (flaxseed) to lambs consuming FlaxDHA treatment in the current study, there was a cumulative or synergistic effect found on the gene expression levels of FADS1 and ACOX1 compared with lambs fed DHA or Flax diet. The differential effects we have observed with ALA or DHA alone or in combination might be due to their specificity for particular action in the body such as physiological function (fatty acid and cholesterol metabolism), heat production through oxidation (thermogenesis), signal transduction through protein/gene expression or disease prevention through immunological function.

The concept of different n-3 fatty acids having divergent metabolic fates (storage, oxidation or excretion) is supported by investigations carried out by others [36]. The lower hepatic CPT1A mRNA expression in the DHA fed lambs is indicative of lower fatty acid β -oxidation [37], as these animals might be using glycogen as an energy source in the liver. Alternatively, the DHA fed lambs might be using TAG as an energy source in skeletal muscle through improved insulin action as evidenced by elevated muscle long-chain n-3 fatty acid (DHA) and lower plasma TAG concentration compared with lambs fed Flax.

There have been reports that feeding diets rich in fish oil increased long-chain n-3 fatty acid in muscle tissues [29], resulting in up-regulation of the expression of lipid catabolism genes [6, 38]. Others [39] have reported that long-chain n-3 fatty acids exert their effects on lipid metabolism and thermogenesis by up-regulating the expression of genes encoding proteins involved with fatty acid oxidation (e.g. carnitine palmitoyltransferase and acyl-CoA oxidase) while simultaneously down-regulating the transcription of genes encoding proteins involved in lipid synthesis (e.g. fatty acid synthase). Some reports indicated that increasing the intake of long-chain n-3 PUFA may improve body composition, most likely through altered gene expression favouring increased fat oxidation in adipose, liver, cardiac, intestinal and skeletal muscle tissue and reduced fat deposition in adipose tissue [2, 6, 7]. This was the case in the present study with lambs fed the DHA and FlaxDHA diets having lower fat deposition in the body compared with those consuming the Flax diet. The latter might be through altered nutrient partitioning to different tissues, blood parameters (TAG and insulin), muscle fatty acid metabolism (DHA) and liver gene expression.

In summary, dietary fat supplementation with flaxseed (ALA) significantly increased blood TAG, body fat and carcass yield compared with control animals fed a forage-based diet. Supplementation of DHA from algae significantly increased carcass yield and muscle DHA concentration while lowering feed consumption and blood insulin concentration. Flax supplementation increased muscle ALA concentration, while algae supplementation increased muscle DHA concentration. Liver gene expression of FADS2 was higher and CPT1A was lower with DHA supplementation compared with control group. When Flax and DHA were fed simultaneously, there was an additive effect observed such that FADS1 and ACOX1 gene expression was higher than either Flax or DHA treatment alone. Supplementation of ALA (18:3n-3) as long-chain n-3 and DHA (22:6n-3) as very long-chain n-3 act differently in modifying carcass yield, body fat deposition, muscle ALA and DHA concentrations and liver gene expression.

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Compliance with Ethical Standards

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Conflict of interest The authors declare no conflicts of interest.

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