Transcriptomic analyses of intestinal gene expression of juvenile Atlantic cod (*Gadus morhua*) fed diets with Camelina oil as replacement for fish oil

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Abstract

For aquaculture of marine species to continue to expand, dietary fish oil (FO) must be replaced with more sustainable vegetable oil (VO) alternatives. Most VO are rich in n-6 polyunsaturated fatty acids (PUFA) and few are rich in n-3 PUFA but Camelina oil (CO) is unique in that, besides high 18:3n-3 and n-3/n-6 PUFA ratio, it also contains substantial long-chain monoenes, commonly found in FO. Cod (initial weight ~1.4 g) were fed for 12 weeks diets in which FO was replaced with CO. Growth performance, feed efficiency and biometric indices were not affected but lipid levels in liver and intestine tended to increase and those of flesh, decrease, with increasing dietary CO although only significantly for intestine. Reflecting diet, tissue n-3 long-chain PUFA levels decreased whereas 18:3n-3 and 18:2n-6 increased with inclusion of dietary CO. Dietary replacement of FO by CO did not induce major metabolic changes in intestine, but

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affected genes with potential to alter cellular proliferation and death as well as change structural properties of intestinal muscle. Although the biological effects of these changes are unclear, given the important role of intestine in nutrient absorption and health, further attention should be given to this organ in future.

Keywords: Alternative diets; *Camelina sativa*; fish oil replacement; *Gadus morhua*; intestine; microarrays

1. Introduction

Sustainable development of aquaculture of carnivorous marine finfish species requires feed formulations to be based more on terrestrial plant products, plant meals and vegetable oils (VO), rather than the traditional marine resources, fishmeal and fish oil (FO) (Naylor et al., 2009; Hardy, 2010). Recent research has demonstrated that marine fish can be grown on feeds containing high levels of replacement (up to 70%) of FO with VO without compromising growth performance (Turchini et al., 2010). However, VO lack long-chain polyunsaturated fatty acids (≥C20; LC-PUFA) and most are rich in linoleic acid (18:2n-6; LOA), and their inclusion in feeds reduces the n-3/n-6 PUFA ratio of the fish flesh potentially compromising its nutritional quality to human consumers (Turchini et al., 2010). In contrast there are few oils rich in n-3 PUFA and they are only produced in relatively small volumes (Gunstone, 2010).

A potentially new source of n-3 PUFA is the (re)emerging Brassicaceae species *Camelina sativa* (commonly known as gold-of-pleasure, or false flax). Camelina used to be a major European oilseed crop prior to the early 20th century, and has recently been re-established as a potential bioenergy crop. This is on account of Camelina's modest input requirements and also its ability to thrive in semi-arid conditions (Tocher et al., 2010) and, in the USA, it is being actively grown as a biofuels crop (Murphy, 2011). In addition to these desirable agronomic traits, Camelina also accumulates high levels of α-linolenic acid (18:3n-3; ALA). Thus, Camelina oil (CO) can contain up to 45 % ALA with an ALA/LOA ratio generally around 2.5. However, CO also has a high antioxidant content with approximately 800 mg total tocopherol per kg, predominantly the γ-isomer and, as a result, the crude oil exhibits strong oxidative stability despite the high ALA content (Tocher et al., 2010). Camelina oil is relatively low in saturated fatty acids and, almost uniquely among VO, it contains high levels of long-chain C20 and C22 monoenes (20:1 and 22:1) that are regarded as good energy sources in fish (Tocher, 2003), giving

63 CO a potential nutritional advantage over other ALA-rich oils such as linseed and perilla (Tocher
64 et al., 2010). Furthermore, CO has already been shown to be suitable for inclusion in feeds for
65 Atlantic salmon and appears to be low in anti-nutritional factors that could be detrimental to fish
66 growth (Petropoulos et al., 2009).
67 Until depletion of the commercial stocks in the 1990's, Atlantic cod (*Gadus morhua* L.) was
68 the most valued food fish obtained from the North Atlantic (Kurlansky, 1998). Significant
69 progress has been made in the culture of cod and the life cycle was closed some years ago,
70 allowing production independent of wild fisheries (Brown et al., 2003). The large and highly
71 developed market for cod, high market price and quotas set to preserve wild stocks, have
72 stimulated considerable interest in cod culture over the years (Morais et al., 2001; Hemre et al.,
73 2004; Hansen et al., 2011; Kortner et al., 2011). However, the establishment of large-scale,
74 sustainable cod culture will require solutions to several nutritional issues including broodstock
75 and larval nutrition and replacement of dietary FO (Lall and Nanton, 2002; Salze et al., 2005).
76 Recently the genome of Atlantic cod was released, providing an important resource for further
77 studies and development of cod as an aquaculture species (Star et al., 2011).
78 In addition to traditional measures of the effects of dietary formulations on fish performance
79 (growth and feed efficiency), tissue composition and nutritional quality, recent advances in
80 functional genomics, essentially the study of gene expression, has opened up new possibilities for
81 understanding the basic mechanisms involved in the adaptation to new diets or feeds (Leaver et
82 al., 2008a). Determining patterns of gene expression through study of tissue transcriptomes
83 (mRNA expression) or proteomes (protein expression) can provide large amounts of information
84 on individual molecular responses that, with detailed bioinformatic analyses, can provide great
85 insights into the biochemical and physiological responses underpinning nutritional adaptations
86 (Panserat et al., 2009; Taggart et al., 2008). In recent years, we have specifically determined the
87 effects of replacement of FO with VO on tissue gene expression in Atlantic salmon using both
88 quantitative real-time PCR (candidate genes) and transcriptomic/microarray (global gene
89 expression) approaches (Leaver et al., 2008b; Torstensen and Tocher, 2010; Morais et al.,
90 2011a,b).
91 Our overall objective is to determine the utility of CO as a replacement for dietary FO in feeds
92 for Atlantic cod and, to this aim, cod were fed CO in a nutritional trial with a regression design
93 and the effects on growth performance, feed efficiency, basic biometry and tissue lipid and fatty
94 acid composition determined. However, the primary focus of the present study was to determine

the effects of dietary CO on gene expression in cod in order to elucidate metabolic pathways of adaptation and provide an underpinning fundamental science base for similar nutritional studies. Therefore, transcriptome analysis of intestinal tissue was performed using a recently developed Atlantic cod 16k cDNA microarray (Edvardsen et al., 2011).

2. Materials and methods

2.1. Diets and animals

Four diets containing approximately 55 % crude protein and 15 % crude lipid were formulated to satisfy the nutritional requirements of marine fish (National Research Council, 2011). The control diet (C0) was formulated with 100% FO (capelin oil) and three other diets contained an increasing proportion of crude cold-pressed *Camelina sativa* oil (CO) replacing 33 % (C33), 66 % (C66) and 100 % (C100) of the added fish oil (Table 1). The dry ingredients were combined and mixed for 5 min using a Hobart commercial mixer (Model 200A, Hobart, Glasgow, UK), and the oils, including antioxidant, then added and mixed for 5 min before adding water (10% w/w) and mixing for a further 5 min. Pellets of 1 mm diameter were formed by extrusion through an appropriate die using a California pellet mill (model CL3, California Pellet Mill Inc., San Francisco, CA). After pelleting the feeds were dried overnight in a heated cabinet at 25^{o}C. The fatty acid compositions of the diets are shown in Table 2.

Four hundred and twenty juveniles of Atlantic cod (*Gadus morhua* L.), obtained from Marine Farms Ltd, Machrihanish, UK, of initial mean weight 1.4 ± 0.1 g were randomly distributed among 12 circular fibreglass tanks of 0.4 m^3 with 35 fish/tank and supplied with UV treated, filtered flow-through seawater at a rate of 1 L/min at the Marine Environmental Research Laboratory, Machrihanish, Scotland. Triplicate tanks of fish were fed one of the four feeds for 12 weeks between February and May when ambient water temperature was 9.0 ± 1.2 ^{o}C (range, 7 – 11). Fish were fed a fixed ration of tank biomass ranging from 5.6 – 2.6 % as the experiment progressed with fish in each tank bulk weighed every 14 days and ration adjusted accordingly. At the end of the trial, 6 fish per tank (18 fish per dietary treatment) were anaesthetized with metacaine sulphonate (MS222; 50 mg/L) and killed by a blow to the head. Samples of liver and intestine (midgut) for analysis of gene expression were frozen in liquid nitrogen and subsequently stored at – 80 ^{o}C prior to RNA extraction. Flesh (muscle), liver and intestine for lipid analyses were also frozen in liquid nitrogen and stored at -20 ^{o}C prior to analysis. Fish were not starved prior to sampling and so any gut contents were gently extruded prior to freezing.

2.2. Proximate composition

Diets were ground prior to determination of proximate composition according to standard procedures (AOAC, 2000). Moisture contents were obtained after drying in an oven at 110 ^{o}C for 24 h and ash content determined after incineration at 600 ^{o}C for 16 h. Crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, U.K), and crude lipid content determined after acid hydrolysis followed by Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus, Foss, Warrington, U.K).

2.3. Lipid content and fatty acid analysis

Samples of liver, intestine and skinned and deboned flesh (white muscle) from three fish per tank (9 per treatment) were utilized for lipid analysis, with samples from the three fish per tank prepared as pooled tissue homogenates and so there were three replicate pools of three fish per treatment (n = 3). Total lipid was extracted according to the method of Folch et al. (1957). Approximately 1 g samples of pooled tissues were homogenized in 20 ml of ice-cold chloroform/methanol (2:1, by vol) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by addition of 5 ml of 0.88 % (w/v) KCl and allowed to separate on ice for 1 h. The upper non-lipid layer was aspirated and the lower lipid layer dried under oxygen-free nitrogen. The lipid content was determined gravimetrically after drying overnight in a vacuum desiccator. Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50 ^{o}C for 16 h according to the method of Christie (1993). Extraction and purification of FAME was carried out as described by Tocher and Harvie (1988). The FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, ThermoFisher Scientific, Hemel Hempsted, UK) using a 30m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50 ^{o}C. Hydrogen was used as carrier gas and temperature programming was from 50 ^{o}C to 150 ^{o}C at 40 ^{o}C min^{-1} and then to 230 ^{o}C at 2.0 ^{o}C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19).

2.4. RNA extraction

Liver and intestinal tissue (0.2 g) from six individuals per experimental group (2 per tank) were homogenized in 2mL of TRI Reagent (Ambion, Applied Biosystems, Warrington, U.K.) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, U.K.). The individuals were selected from the group of randomly sampled fish to represent the global average weight of fish in the experiment. Total RNA was isolated following manufacturer's instructions, and RNA quality (integrity and purity) and quantity was assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.).

2.5. Transcriptome analysis

A 16 k cDNA microarray from Atlantic cod was used in this experiment. Details of the microarray construction are given in Edvardsen et al. (2011). Only intestinal samples from the control (C0) and one of the CO dietary treatments (C66) were hybridized. The C66 diet was chosen rather than C100, as this would be the more likely commercial formulation. The low yield and quality of RNA obtained from liver, possibly due to high co-precipitation with glycogen, precluded the use of liver samples for the microarray experiment. Total RNA was purified using the Invitrogen iPrep Trizol® Plus RNA Kit on the iPrepT Purification Instrument following the manufacturer's recommendations. Samples were randomly labelled in a single batch and hybridized in 2 batches of 6 slides each, containing 3 samples from each treatment in each batch. Briefly, 10 µg total RNA was used for cDNA synthesis and labelling using Fair Play® Microarray Labeling Kit (Stratagene, USA) according to manufacturer's instructions. Samples were labelled with Cy5 and a common reference standard (obtained by pooling 40% intestinal RNA from all samples in the microarray experiment plus 60% RNA from a mixture of different tissues) was labelled with Cy3. Labelling efficiency and quantity of labelled cDNA were determined using the NanoDrop spectrophotometer. Slides were pre-hybridized in 20× SSC (Calbiochem, USA), 10% SDS (Sigma-Aldrich Chemie, Steinheim, Germany) and 1% BSA (Sigma-Aldrich Chemie) for 45 min at 65 °C followed by washing twice in water and once in isopropanol, and then drying by centrifugation. Sample and reference labelled cDNA were pooled and diluted in Tris buffer pH 8.0. After sample denaturation (100 °C, 2 min), hybridization was performed at 60 °C overnight with rotation using Agilent 2× hybridization buffer (250 µL) in Agilent hybridization chambers. The slides were put in 2× SSC/0.1% SDS at 65 °C to remove gasket slide and then washed for 5 min in 1×SSC at 65 °C, for 5 min in 0.2× SSC at RT, for 45 s in 0.05× SSC at RT, and centrifuged dry. Slides were immediately scanned

188 using an Agilent scanner (G2505 B Microarray Scanner, Agilent Technologies, Santa Clara,
189 USA) at a resolution of 10 μm with default settings.
190 The scanned microarray images were analyzed using the GenePix Pro 6.0 software package
191 and exported as image quantitation files (gpr- and jpg-files). The data files were quality
192 controlled using R (R Development Core Team, 2005, http://www.r-project.org), and analyzed
193 using J-Express Pro v.2.7 (Dysvik and Jonassen, 2001, http://www.molmine.com). Control
194 probes, empty spots and probes marked as bad quality were removed from the analysis. Genes
195 with more than 30% missing values were removed from the analysis and the remaining missing
196 values were estimated using LSimpute Adaptive (Bø et al., 2004). Each array was normalized by
197 Lowess (Cleveland and Devlin, 1988). Log 2 transformed ratios of foreground signals were used
198 in the final gene expression matrix. MIAME-compliant (Brazma et al., 2001) descriptions of the
199 microarray study and results are available in the EBI ArrayExpress database
200 (http://www.ebi.ac.uk/arrayexpress) under accession number E-TABM-1178.
201 Statistical analysis of the microarray hybridization data was performed in GeneSpring GX
202 version 11 (Agilent Technologies, Wokingham, Berkshire, U.K.) using a Welch (unpaired
203 unequal variance) t-test, at 0.05 significance and 1.2 fold change cut-off level. No multiple test
204 correction was employed as previous analyses, confirmed by RT-qPCR, indicate that such
205 corrections are over-conservative for this type of nutritional data (Leaver et al., 2008b; Morais et
206 al., 2011a). Gene Ontology (GO) enrichment analysis was performed using the same software, at
207 $p < 0.05$.

208 *2.6. Reverse transcription real-time quantitative PCR (RT-qPCR)*

209 Expression of candidate genes of interest (fatty acyl elongase, *elovl5*, and desaturase, *Δ6fad*)
210 in intestine and liver from fish fed all four treatments, as well as genes for microarray validation
211 in intestinal samples of fish fed C0 and C66, was determined by reverse transcription quantitative
212 real time PCR (RT-qPCR). Details on the target qPCR primer sequences and on the reference
213 sequences used to design them are given in Table 3. Primers were designed using Primer3
214 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi; Rozen and Skaletsky, 2000). Results
215 were normalized by amplification of three reference genes, β-actin (*bact*), ubiquitin (*ubq*) and
216 glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), which in our experimental conditions
217 presented M values, generated by the gene stability analysis software package geNorm
218 (Vandesompele et al., 2002), of 0.292, 0.268 and 0.326, respectively.

For RT-qPCR, 1 µg of total RNA per sample was reverse transcribed into cDNA using the VersoTM cDNA kit (ABgene, Surrey, U.K.), following manufacturer's instructions, using a mixture of random hexamers (400ng/µL) and anchored oligo-dT (500ng/µL) at 3:1 (v/v). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A similar amount of cDNA was pooled from all samples and the remaining cDNA was then diluted 20-fold with water. RT-qPCR analysis used relative quantification with the amplification efficiency of the primer pairs being assessed by serial dilutions of the cDNA pool. qPCR amplifications were carried out in duplicate (Quantica, Techne, Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL or 2 µL diluted (1/20) cDNA, 0.5 µM of each primer and 10 µL AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control (NTC-non template control). The qPCR profiles contained an initial activation step at 95 °C for 15 min, followed by 30 to 35 cycles: 15 s at 95 °C, 15 s at the specific primer pair annealing temperature (Ta; Table 3) and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C increments from 75 ºC to 90 °C was performed, enabling confirmation of the amplification of a single product in each reaction. RT-qPCR product sizes were checked by agarose gel electrophoresis and the identity of amplicons was confirmed by sequencing. PCR efficiency was above 90% for all primer pairs.

Gene expression results assessed by RT-qPCR were analyzed by the ΔΔCt method using the relative expression software tool (REST 2008, http://www.gene-quantification.info/), employing a pair wise fixed reallocation randomization test (10,000 randomisations) with efficiency correction (Pfaffl et al., 2002), to determine the statistical significance of expression ratios between two treatments.

2.7. Statistical analysis

All data are presented as means ± SD (n value as stated). The effects of dietary treatment on growth performance were analyzed by one-way analysis of variance (ANOVA) followed, where appropriate, by Tukey's post hoc test. The relationship between dietary treatment and chemical composition was analyzed by regression analysis. Percentage data and data identified as non-homogeneous (Levene's test) or non-normality (Shapiro-Wilks's test) were subjected to arcsine transformation before analysis. ANOVA and regression analysis were performed using a SPSS Statistical Software System version 14 (SPSS inc, Chicago IL, USA). Differences were regarded as significant when $P < 0.05$ (Zar, 1999).

3. Results

3.1. Growth and biometry

The juvenile cod showed a 5-fold increase in weight over the course of the feeding experiment (Table 4). There were no significant differences between treatments for any of the growth and feeding performance parameters although there was a trend for growth performance to increase with CO inclusion up to 66% with fish fed diet C66 showing highest final weight, SGR and TGC and lowest FCR. In general, very high and variable lipid content was observed in liver, as is typical of farmed cod which normally present enlarged fatty livers (Morais et al., 2001). There were also trends for increased HSI and VSI, as well as liver lipid contents with CO inclusion but only intestinal lipid content was significantly higher in fish fed diet C66 compared to fish fed diet C0 (Table 4).

3.2. Diet and cod tissue fatty acid compositions

The fatty acid compositions of the feeds reflected the increasing content of CO, with decreasing proportions of total saturated fatty acids, mainly 14:0 and 16:0, and total monoenes, specifically 16:1 and 22:1, although 18:0, and 18:1n-9 increased (Table 2). It was noteworthy that the level of 20:1 was constant across the feeds indicating that the 20:1 content of the FO (a northern hemisphere oil) was balanced by the 20:1 content of CO. In contrast, LOA (18:2n-6) and ALA (18:3n-3) and their immediate elongation products, 20:2n-6 and 20:3n-3 respectively, increased with dietary CO inclusion whereas the LC-PUFA, including arachidonic acid (20:4n-6; ARA), eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) all decreased. The overall effect on dietary PUFA levels was increased total n-6 PUFA, n-3 PUFA and total PUFA, but decreasing n-3/n-6 ratio (Table 2). The fatty acid compositions of the cod tissues reflected those of the feeds with liver (Table 5), intestine (Table 6) and muscle (Table 7) all characterized by generally decreasing proportions of saturated and monounsaturated fatty acids, and increasing proportions of PUFA as dietary CO inclusion increased. These effects were due to decreasing proportions of 16:0, 16:1n-7, 18:1n-7, 22:1, ARA, EPA and DHA, and increased proportions of 18:1n-9, LOA, ALA, 20:2n-6 and 20:3n-3, which together resulted in decreased tissue n-3/n-6 PUFA ratios as CO inclusion increased (Tables 5-7).

3.3. Expression of fatty acyl elongase and desaturase in liver and intestine

The expression of a fatty acyl elongase (*elovl5*) and desaturase (*Δ6des*) in the liver and intestine of Atlantic cod fed increasing levels of CO was assessed by RT-qPCR (Fig. 1). Changes in the

relative expression of both genes in fish fed the three diets containing CO, in relation to the C0 diet, were not significantly different. In the case of *elovl5*, expression ratios were around 1 in both intestine and liver in fish fed all diets, denoting no change in transcript levels. In liver, in spite of a high biological variability, a trend for higher expression of *Δ6des* in fish fed C66, followed by C100, in comparison to fish fed C0 (2.4- and 1.8-fold up-regulated, respectively), was observed, whereas such a trend was not evident (only 1.2-fold up-regulated in C66) in the intestine.

3.4. Transcriptomic analysis of intestinal tissue

Statistical analysis of the microarray data returned a list of 289 features differentially expressed in the intestine between cod fed C0 and C66. Genes in this list were categorized according to their biological function, by determining the main biological processes in which they are involved in mammalian counterparts. Distribution of the EST's, after removing non-annotated genes (36%) and features representing the same gene product (Fig. 2), revealed that the most affected biological processes or categories were translation (18% of all genes), cell proliferation, differentiation and apoptosis (14%) and genes with a structural molecule activity (12%), followed by transporter activity (9%) and immune response (7%). In contrast, metabolism appeared to be less affected, with 6% of the genes involved in proteolysis, 5% involved in energy metabolism or generation of precursor metabolites and 4% in lipid metabolism. Other minor categories represented were regulation of transcription (4%), signalling (4%) and protein folding (3%). A more detailed analysis of the list was restricted to the top 100 most significant hits, which presented a broadly similar distribution of genes by biological categories, with translation (25%) and cell proliferation, differentiation and apoptosis (20%) predominating, followed by structural molecules (9%) and immune response (9%) (Table 8).

Gene ontology (GO) enrichment analysis was performed on the entire significant dataset. This enabled identification of GO terms significantly enriched in the input entity list compared to the whole array dataset, providing evidence for which biological processes may be particularly altered in the experimental conditions being compared. The analysis returned 208 significant GO terms at $p<0.05$. However, most of the GO terms were interrelated and many of them were significantly enriched largely due to the repetition of multiple features for the same gene, either apolipoprotein A-IV (apoA-IV) or creatine kinase (CK) (Table 8). Nonetheless, some GO terms could be considered as significantly enriched by the presence of multiple genes. With respect to molecular function, the term "actin binding" was significantly enriched (adjusted p-value:

1.825E-4), containing features corresponding to myosin heavy chain, tropomyosin alpha-1 chain, actin alpha cardiac muscle 1, cofilin 1 (non-muscle), ectodermal-neural cortex (with BTB-like domain) and transgelin (Table 9). On the other hand, a few related categories of biological process, namely "multicellular organismal development" (adjusted p-value: 0.002), "anatomical structural development" (adjusted p-value: 0.003), "developmental process" (adjusted p-value: 0.006), "epidermal cell differentiation" (adjusted p-value: 0.031), and "regulation of cytoskeleton organization" (adjusted p-value: 0.01), were significantly enriched due to the presence of the following genes: CK, zinc finger protein 313, P-cadherin, collagen type II alpha 1, caspase 3, myosin light polypeptide 6B, thioredoxin interacting protein, phytanoyl-CoA 2-hydroxylase, cofilin 1 (non-muscle), NAD(P) dependent steroid dehydrogenase-like, ectodermal-neural cortex (with BTB-like domain), dihydrolipoamide dehydrogenase, ribosomal protein L35, thioredoxin interacting protein, peripheral myelin protein 22, tropomyosin 1 (alpha), intraflagellar transport 172 homolog, tumor susceptibility gene 101, transgelin, heat shock protein 90kDa alpha (cytosolic) class B member 4, stress-associated endoplasmic reticulum protein 2 and guanine nucleotide binding protein (G protein) (changes in expression between treatments can be found in supplementary material).

3.5. RT-qPCR validation of microarray results

The expression of selected genes was measured by RT-qPCR in order to validate the microarray results (Table 10). These genes were selected from within the categories that were more highly represented in the top100 most-significant list. In general, changes in expression levels were subtle, as in the microarray analysis, which explains why differences in transcript levels between fish fed diets C0 and C66 determined by RT-qPCR were not always statistically significant. However, a good match between the fold changes measured by microarray and RT-qPCR results was found for most genes related to cell proliferation and apoptosis, such as tumor protein p63 regulated 1-like (*tprg1l*), retinoic acid receptor responder protein 3 (*rarres3*), translationally-controlled tumour protein homolog (*tctp*) and caspase 3 (*casp3*), even if differences assessed by RT-qPCR were only significant for *rarres3* and *casp3*, these being 7.8- and 1.9-fold up-regulated, respectively, in the intestine of fish fed diet C66. In contrast, no agreement was found between the fold changes measured by microarray and RT-qPCR in genes related to translation, namely, elongation factor 1-alpha (*elf1a*), 60S ribosomal protein L13 (*rpl13*) and eukaryotic translation initiation factor 3 subunit F (*etf3f*). However, good agreement was found for the gene coding for the structural protein tropomyosin alpha-1 chain (*tpm*), which

346 was 1.5-fold up-regulated in diet C66 in both the microarray and RT-qPCR analysis, although
347 RT-qPCR did not confirm the microarray results for other structural genes, tektin-4 (*tekt4*) and
348 cadherin 3 type 1 (*cdh3*).
349
350 **4. Discussion**
351 There was no detrimental effect, and even a tendency for positive effects, of dietary CO on
352 growth of cod up to 66 % inclusion in the fishmeal-based feeds used in the present trial. As a
353 result the microarray gene expression data compared fish fed diet C0 (control) with fish fed diet
354 C66, which showed good adaptation to the alternative formulation. The effects on growth were
355 similar to those previously reported for replacement of dietary FO with the most common ALA-
356 rich oil, linseed, in marine fish including gilthead sea bream (*Sparus aurata*), sharpsnout sea
357 bream (*Diplodus puntazzo*), European sea bass (*Dicentrarchus labrax*) and turbot (*Psetta*
358 *maxima*) (Tocher et al., 2010). In these studies, up to 60 % of dietary FO could be replaced by
359 linseed oil without affecting growth, but higher levels of replacement could reduce growth
360 dependent upon species, fish size and duration of feeding (Izquierdo et al., 2003; Regost et al.,
361 2003; Menoyo et al., 2004; Mourente et al., 2005; Piedecausa et al., 2007). There are no previous
362 studies reporting the effects of CO in marine fish but, in the only study reported to date, growth
363 of Atlantic salmon smolts was not affected by feeding a diet with 100% of FO replaced with a
364 VO blend containing rapeseed, palm and Camelina oils in a 5:3:2 ratio (Bell et al., 2010).
365 The effects of dietary CO on tissue fatty acid composition in cod were as expected
366 considering the extensive data in the literature describing the effects of VO in fish, with increased
367 C18 fatty acids, including 18:0, 18:1n-9, LOA and ALA, and decreased levels of EPA and DHA
368 (Turchini et al., 2010). In the only trials to date utilizing CO, the fatty acid composition of liver in
369 Atlantic salmon fed a VO blend containing CO was characterized by increased levels of ALA
370 and its elongation product 20:3n-3, but decreased levels of all other n-3 fatty acids (Petropoulos
371 et al., 2009; Bell et al., 2010). The data in the present study with cod and CO are comparable with
372 the data obtained in other marine fish species fed ALA-rich oils (Tocher et al., 2010). Studies
373 feeding linseed oil to sea bass, turbot, gilthead and sharpsnout sea bream all showed increased
374 percentages of ALA, LOA and their elongation products, 20:3n-3 and 20:2n-6, and decreased
375 proportions of EPA, DHA and ARA and no increased percentages of any desaturated
376 intermediates such as 18:4n-3, 20:4n-3, 18:3n-6 or 20:3n-6 in tissue lipids (Izquierdo et al., 2003;
377 Regost et al., 2003; Menoyo et al., 2004; Mourente et al., 2005; Piedecausa et al., 2007).

Therefore, provision of ALA substrate was not able to compensate for the lack of LC-PUFA in the diet of marine fish and there was little evidence of any significant desaturation of dietary ALA. Although feeding high levels of CO did not prevent reductions in EPA and DHA compared to fish fed FO, the increased levels of ALA in flesh are useful as it is also recognized as an essential fatty acid in human nutrition (Burdge, 2006; Brenna et al., 2009). The human diet is imbalanced with overly high n-6/n-3 ratios, particularly in the developed countries (Simopoulos, 2006) and, although production of fish containing high levels of EPA and DHA should be a major goal in aquaculture, a product with a high n-3/n-6 ratio is still desirable even if some of the n-3 PUFA is ALA rather than EPA and DHA. In comparison to EPA and DHA, ALA is inferior as a component of fish flesh, but it is preferable to LOA (Whelan, 2008).

In order to directly address the question of whether cod have the capacity to up-regulate the LC-PUFA biosynthesis pathway in response to increasing levels of CO, the expression of fatty acyl desaturase (*Δ6fad*) and elongase (*elovl5*) genes, which have been shown to be transcriptionally regulated, and correlated with enzymatic activity, in Atlantic salmon (Zheng et al., 2004, 2005), was measured by RT-qPCR. Results in both liver and intestine showed no significant nutritional regulation of *elovl5* in cod fed CO. In liver, a trend for higher *Δ6fad* expression in the two treatments with higher levels of FO replacement, particularly C66, in comparison to the C0 diet was noticeable, but biological variability of the data was high and the results were not significant. A previous study on the nutritional regulation of these genes in cod showed that dietary FO replacement by VO tended to increase the expression of both *Δ6fad* and *elovl5* in hepatocytes and enterocytes, but there was high biological variability and so only *elovl5* in enterocytes was significant, although there was no significant effect on LC-PUFA biosynthesis enzyme activities in either tissue (Tocher et al., 2006). In the case of *Δ6fad*, this is likely due to lower basal expression of this gene, compared to salmon, perhaps associated with lower activity of the cod *Δ6fad* promoter (Zheng et al., 2009).

Recent studies have begun to investigate the molecular effects of alternative diet formulations, and hepatic responses to feeds containing high levels of replacement of FO by VOs are being characterized (Panserat et al., 2009; Morais et al., 2011a,b). However, studies on the intestinal transcriptome in fish have mainly focused on replacement of fishmeal by plant proteins, in particular soybean meal, given its potential to cause enteritis in some species (Murray et al., 2010; Skugor et al., 2011). Furthermore, the majority of these transcriptomic studies have been conducted on salmonid species, which can tolerate high levels of fishmeal and FO replacement

(Turchini et al., 2010; Hardy, 2010), whereas few data exist for marine species. Here we examined the effects of CO on the intestinal transcriptome of cod. This knowledge is important as it is well established that the intestine is more than simply the site of nutrient uptake, and that enterocytes are also sites of significant lipid metabolism (Tocher et al., 2002; Bell et al., 2003). For example, despite LC-PUFA biosynthesis ability being low in cod, the activity of this pathway was around 7-fold higher in enterocytes than in hepatocytes (Tocher et al., 2006). In addition, the intestine has a vital role in protecting against the entry of pathogens and is one of the largest immune organs in the body. Hence, its proper function is essential for maintaining optimal balance and health and can be significantly affected by replacing dietary FO by VO, accompanied by reductions in n-3/n-6 LC-PUFA ratios (Teitelbaum and Walker, 2001).

The microarray analysis identified translation, cell proliferation or differentiation and apoptosis, as well as structural molecules, as the biological categories in cod intestine most highly affected by dietary CO. Surprisingly, lipid metabolism and metabolism in general were little affected by changes in diet formulation, changes that were exclusively in lipid composition. The main observed effect was up-regulation of apoA-IV in the intestine of cod fed the C66 diet. In humans and rodents this protein is mainly found free in plasma but is also a major component of chylomicrons and very low density lipoproteins (VLDL) and is synthesized by enterocytes in the small intestine (Green and Glickman, 1981). In mammals, apoA-IV is the only apolipoprotein that showed a marked response to intestinal absorption and transport of lipid, through increased expression, synthesis and secretion (Kalogeris et al., 1997; Tso et al., 2001). A commonly reported effect of the inclusion of VO in the diet of carnivorous fish is an accumulation of lipid droplets in the enterocytes, which might be explained by a reduction in LC-PUFA levels leading to changes in re-acylation mechanisms and phospholipid synthesis rates and consequent lower lipoprotein assembly and export from intestinal cells (Caballero et al., 2003; Olsen et al., 2003). However, apoA-IV is one of the least well characterized apolipoproteins in fish species and there is no information regarding its physiological function. Furthermore, even in mammals, apoA-IV has diverse roles, including the regulation of appetite and gastrointestinal function (enzyme secretion and gastric emptying), and displays anti-oxidant and anti-atherogenic properties in rodents (Kalogeris et al., 1997; Tso et al., 2001; Stan et al., 2003). The biochemical results showed a trend for higher lipid content in fish fed the diets containing CO, and intestinal lipid was significantly higher in fish fed C66 compared to C0. Hence, although other factors might

explain the changes in apoA-IV expression, they may be related to effects of dietary lipid composition on lipid absorption and transport.

A large number of ribosomal proteins were slightly, but consistently, down-regulated in the intestine of cod fed the CO diet. Ribosomal proteins typically show a highly coordinated response in terms of expression changes in salmon microarrays and Skugor et al. (2011) reported increased expression in a number of these in response to cellular stressors. However, RT-qPCR did not confirm the microarray data, preventing firm conclusions on potential effects of diet on translation. In contrast, good agreement was obtained between the microarray and RT-qPCR results for some genes involved in cell proliferation or differentiation and apoptosis with *rarres3* and *casp* being 7.8- and 1.9-fold up-regulated, respectively, in the intestine of cod fed the C66 diet. Retinoic acid receptor responder protein 3 (*rarres3*), also known as retinoid-inducible gene 1 protein or tazarotene-induced gene 3, is a nuclear receptor and transcriptional regulator that is thought to act as a tumour suppressor, regulating growth and differentiation of many cell types, mediated via inhibition of cellular growth (G0/G1 arrest) or induction of apoptosis (DiSepio et al., 1998; Huang et al., 2000). The strong up-regulation of this transcript in the intestine of cod fed CO is thus likely reflected in lower cellular proliferation. Furthermore, up-regulation of *casp* in cod fed CO indicates increased apoptosis and hence possibly also lower intestinal tissue growth.

Other transcripts that were up-regulated (in the top100 most significant list) in the CO treatment were tumor protein p63 regulated 1 (*tprg1l*), 40S ribosomal protein SA (*rpsa*), origin recognition complex subunit 2-like and voltage-dependent anion-selective channel protein 1. The p63 tumor protein is a transcription factor that is highly expressed in basal cells of epithelial layers in mammals, and at least some of its variants have effects on induction of apoptosis and arresting growth (Yang et al., 1998; Wu et al., 2003). The origin recognition complex is essential for the initiation of DNA replication and heterochromatin assembly in eukaryotic cells, ensuring tight regulation of the cell cycle and preventing re-replication of DNA during a single cell division cycle. This may suggest tighter control of replication in the intestine of cod fed the CO diet, although this complex has other non-replication roles (Chesnokov, 2007; Sasaki and Gilbert, 2007). Voltage-dependent anion-selective channel protein 1 is also associated with regulation of cell growth and death, interacting with several pre- or anti-apoptotic proteins to allow the formation of pores in outer mitochondrial and plasma membranes, affecting their permeability. When the channel adopts an open conformation it enables cytochrome c release into the cytosol,

473 which triggers caspase activation and apoptosis (Shoshan-Barmatz et al., 2006). In addition,
474 down-regulation of proline-rich protein BCA3-like and translationally-controlled tumor protein
475 homolog (*tctp*) might indicate lower cell proliferation in intestine of cod fed CO. Proline-rich
476 protein BCA3-like is over-expressed tumor cell lines while normal tissues have low levels of
477 expression (Kitching et al., 2003). Several features coding for *tctp* were down-regulated in the
478 microarray results, confirmed by RT-qPCR. This protein is important for microtubule
479 stabilization and was up-regulated in several tumours (Hsu et al., 2007), and reduction of its level
480 can induce tumour reversion, and spatial distribution of *tctp* expression in *Hydra* correlated with
481 regions of active cell proliferation (Yan et al., 2000), all indicating an important role in cell
482 growth and proliferation (Tuynder et al., 2004).
483 The data therefore suggest a hypothesis of lower cell proliferation and/or higher apoptosis in
484 intestine of cod fed diet C66. Gastrointestinal cell proliferation and apoptosis were previously
485 found to be significantly reduced in Atlantic salmon when VO replaced dietary FO (Olsvik et al.,
486 2007). Although the cell's normal renewal capacity could have been impaired after a period of
487 potentially adverse intestinal conditions when feeding VO, given that lower transcription was
488 also measured for genes related to cellular and oxidative stress, the authors attributed their results
489 to reduced oxidative stress associated with lower levels of LC-PUFA in intestinal membranes of
490 fish fed VO (Olsvik et al., 2007). This would result in lower potential for lipid peroxidation and
491 production of reactive oxygen species (ROS), which are important signalling molecules in
492 apoptotic processes (Ramachandran et al., 2000). In the present study the evidence, albeit
493 circumstantial, collectively supports the concept of reduced cellular proliferation and increased
494 apoptosis in the intestine of fish fed CO, compared to those fed the FO diet. Furthermore, results
495 from GO analysis identified several categories of biological processes broadly related to
496 development, cell differentiation and cytoskeleton organization, which indicate potential effects
497 of VO on intestinal regenerative processes or repair, but this requires further investigation.
498 In addition to a high preponderance of structural proteins in the list of genes showing
499 significant effects of diet, GO analysis identified actin-binding as one of the molecular function
500 categories that was over-represented. Genes in this functional category included tropomyosin
501 alpha-1 chain, myosin heavy chain, actin alpha cardiac muscle 1, cofilin 1, ectodermal-neural
502 cortex and transgelin, which were 1.4- to 1.8-fold up-regulated (except actin, which was 1.2-fold
503 down-regulated) in cod fed diet C66. Changes in the expression of these genes indicate likely
504 modifications in the structural composition of smooth muscle, possibly affecting contractile

activity or motility of the intestine. Genes with similar biological function, including tropomyosin and myosin, were down-regulated in Atlantic halibut intestine in response to partial soybean meal replacement of fishmeal (Murray et al., 2010). In that study, genes coding for structural proteins were regulated in a coordinated matter with others involved in muscle physiology, such as creatine kinase (CK), which was also the case here, even if opposite changes were observed, indicating potentially conflicting effects of fishmeal and FO replacement in these two species. Creatine kinase has a key role in muscle energetic metabolism and CK activity relates to the oxidative capacity as well as contractile characteristics of muscle (Clark, 1994). Changes in CK activity and expression level are diagnostic markers for conditions involving muscle damage in mammals, including intestinal infarction or ischemia (Fried et al., 1991; Block et al., 2011). Furthermore, skeletal muscle atrophy has been associated with variations in gene expression profiles similar to those observed here, namely up-regulation of M-type CK as well as of fast-type isoforms of myosin heavy chains (Cros et al., 1999). These effects could not have been anticipated and, unfortunately, no samples were collected for histological analysis preventing gene expression to be related to intestinal morphology.

In summary, CO may be a useful more sustainable alternative for the replacement of FO in the diets of Atlantic cod. Replacement of FO by CO did not induce major metabolic changes in intestinal tissue, as might have been expected, but rather potentially affected rates of cellular proliferation and death, and changes in the structural properties of the intestinal muscle, most likely leading to different rates of tissue regeneration and/or repair, as well as potential changes in contractile activity or mechanical characteristics. The underlying molecular mechanisms explaining these effects on gene expression cannot be determined conclusively but, considering the important role of the intestine in nutrient absorption and whole body balance and health, further attention should be given to this organ in future studies examining effects of FO replacement by VO.

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Supplementary data: Features present in the following categories of biological process, all found to be significantly enriched by GO analysis: “multicellular organismal development” (adjusted p-value: 0.002), “anatomical structural development” (adjusted p-value: 0.003), “developmental process” (adjusted p-value: 0.006), “epidermal cell differentiation” (adjusted p-value: 0.031), and “regulation of cytoskeleton organization” (adjusted p-value: 0.01). Shown are fold changes in cod intestine between diets C66 and C0.

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Figure Captions

Fig. 1. Expression, measured by RT-qPCR, of fatty acyl elongase (*elovl5*) and desaturase (*Δ6fad*) genes in Atlantic cod intestine (A) and liver (B) tissues. Results are normalised expression ratios (average of n=6 ± SE) of the expression of these genes in fish fed each one of the diets containing *Camelina* oil, in relation to those fed diet C0 (100% FO).

Fig. 2. Distribution by categories of biological function of genes found to be differentially expressed in the intestine between cod fed diets C0 and C66 (Welch t-test, $p<0.05$; 1.2 fold change cut-off). Non-annotated genes (36% of a total of 289 features) and features corresponding to the same gene are not represented.

Table 1

Formulations (g/Kg) and proximate composition (percentage of total weight) of diets containing increasing levels of Camelina oil.

Component	C0	C33	C66	C100
[1]Fish meal	610.6	610.6	610.6	610.6
[1]Soya meal	150	150	150	150
[1]Wheat	65	65	65	65
[2]Krill meal	50	50	50	50
[1]Northern Fish oil	80	53.6	27.2	0
[3]Camelina oil	0	26.4	52.8	80
[4]Vitamin mix comp	10	10	10	10
[5]Mineral mix (M_2)	24	24	24	24
[6]Carboxymethyl cellulose	10	10	10	10
[7]Antioxidant mix	0.4	0.4	0.4	0.4
Proximate composition				
Protein	54.9	55.5	55.0	53.3
Lipid	14.2	15.3	14.6	14.3
Moisture	9.3	7.2	8.1	6.8
Ash	10.6	10.7	10.4	10.6

C0, control diet containing 100% fish oil. C33, C66 and C100, diets containing 33%, 66% and 100% Camelina oil as a replacement for fish oil,

[1]Ewos Ltd, Bathgate, UK. [2]Aker Biomarine, Norway. [3]Technology Crops International Inc., USA. [4]Contains g/kg diet, Vitamin A, 2500 IU; Vitamin D, 2400 IU; Vitamin E, 100; Vitamin K, 10; Ascorbic acid 1000; Thiamin, 10; Riboflavin, 20; Pyridoxine, 12; Pantothenic acid, 44; Nicotinic acid, 150; Biotin, 1; Folic acid, 5; Vitamin B12, 0.02; myo-inositol, 400; Choline Chloride, 3. [5] Contains g/kg diet or as stated, KH_2PO_4, 22; $FeSO_4.7H_2O$; 1.0; $ZnSO_4.7H_2O$, 0.13; $CuSO_45H_2O$, 12 mg/kg; $MnSO_4.4H_2O$, 53 mg/kg; KI, 2 mg/kg; $CoSO_4.7H_2O$, 2 mg/kg; α-cellulose, 0.8; [6]Sigma-Aldrich Ltd, Gillingham, UK. [7] Contains g/L; Butylated hydroxyanisole, 60; Propyl gallate, 60; Citric acid, 40; in propylene glycol, added at 0.4g/kg diet.

Table 2

Fatty acid compositions (percentage of weight of total fatty acids) of experimental diets.

	C0	C33	C66	C100
14:0	5.5	4.6	3.6	2.5
16:0	13.6	12.8	11.9	10.9
18:0	1.9	2.1	2.5	2.7
Total saturates[1]	22.1	20.8	19.3	17.4
16:1n-7	6.3	5.4	4.2	2.1
18:1n-9	9.3	10.5	11.5	12.2
18:1n-7	2.5	2.3	2.0	1.6
20:1[2]	11.6	11.6	11.5	11.2
22:1[3]	16.3	13.3	10.0	6.6
24:1	0.9	0.9	0.8	0.8
Total monoenes[4]	49.2	45.6	41.3	36.4
18:2n-6	3.5	5.7	8.6	11.6
20:2n-6	0.3	0.5	0.9	1.2
20:4n-6	0.4	0.4	0.3	0.3
Total n-6[5]	4.4	6.9	9.9	13.1
18:3n-3	1.2	6.4	12.6	18.7
18:4n-3	3.3	2.8	2.2	1.6
20:3n-3	0.1	0.3	0.6	0.8
20:4n-3	0.5	0.4	0.3	0.3
20:5n-3	8.4	7.2	5.8	4.6
22:5n-3	0.7	0.6	0.5	0.4
22:6n-3	9.0	8.1	6.9	6.2
Total n-3	23.0	26.4	28.7	32.6
n-3/n-6 PUFA	5.2	3.8	2.9	2.5
Total PUFA[6]	28.7	33.7	39.4	46.2

[1]Includes 15:0, 17:0 and 20:0. [2]Includes both 20:1n-9 and 20:1n-7; [3]Includes both 22:1n-11 and 22:1n-9; [4]Includes 14:1n-5 and 16:1n-9; [5]Includes 18:3n-6, 20:3n-6, 22:4n-6 and 22:5n-6; [6]Includes C16 PUFA.

867
868 **Table 3**
869 Primers used for RT-qPCR analyses.
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Transcript	Primer sequence (5'-3')	Fragment	Ta	Accession No./Contig
Δ6fad	AGGCACAACTACCAGGTGCT CTTAAGCGACCGCACGAC	117 bp	60 ºC	DQ054840[1]
elovl5	ACTGCCAAGACACACACAGC CTGGCGTGATGGTAGATGTG	163 bp	56 ºC	GW848298[1]
elf1a	GTGATCAAGAGCGTCGACAA GATGCGCATTAACCAGTCCT	189 bp	60 ºC	CL5Contig2[2]
rpl13	TCAGGTGTCCAACCATCAGA CCTCAGTGCTGTCTCCCTTC	249 bp	60 ºC	CL81Contig1[2]
etf3f	GCCACCGTTCTCACCTACAT TTGGGTGAGGTTGGACAAAT	186 bp	60 ºC	CL224Contig1[2]
tprg1l	TTGTCGAGCCTCTCCTGTCT GCTCGCCGACTTAACAACTC	207 bp	60 ºC	CL553Contig1[2]
rarres3	ATCTACCATGGCCCCAACTT ACAACGGCACAGGAACTGTC	174 bp	60 ºC	CL2455Contig1[2]
rpsa	GCTGACCAGAGAGGTTCTGC CACTCAGCCTGGAACTCCTC	162 bp	60 ºC	CL97Contig1[2]
tctp	TTTTTCATCGAGGTGGAAGG CTCTCTGGCTTGGTCTCCTG	248 bp	60 ºC	CL18Contig1[2]
casp3	AGCTGATGCAGATCATGACG TTTCCTCAGCACCCGTAGTT	216 bp	60 ºC	CL1129Contig1[2]
tpm	CCGCTTAGCGTTCAGTTAGG GCTCTGTCCAAGGCATTCTC	158 bp	60 ºC	CL54Contig1[2]
tekt4	ATTACGCGATCCTCCAACAG CTGCAACTCCGACTTCAACA	176 bp	60 ºC	CL1914Contig1[2]
cdh3	CCTGCCAATCCTGAAGACAT GAGGAGTTGAGGGACGACAG	152 bp	60 ºC	CE2051-2005-02-15.ab1[2]
Reference genes				
bact	CTACGAGGGGTATGCTCTGC CTCTCAGCAGTGGTGGTGAA	123 bp	56 ºC	AJ555463[1]
ubq[3]	GGCCGCAAAGATGCAGAT CTGGGCTCGACCTCAAGAGT	69 bp	60 ºC	EX735613[1]
gapdh[3]	CCATGACAACTTTGGCATCGT AGGGTCCGTCCACTGTCTTCT	83 bp	60 ºC	EX725566[1]

871 [1] GenBank Accession No (http://www.ncbi.nlm.nih.gov/)

872 [2] Contig in microarray (based on an assembly of cod ESTs; ArrayExpress: E-TABM-1178)

873 [3] Olsvik et al. (2008)

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875

875 **Table 4**

876 Growth, feed efficiency and biometry of Atlantic cod fed diets containing increasing proportions

877 of Camelina oil.

	C0	C33	C66	C100
Initial weight (g)	1.4 ± 0.0	1.4 ± 0.1	1.4 ± 0.2	1.4 ± 0.0
Final weight (g)	6.4 ± 0.8	7.0 ± 1.0	8.8 ± 0.8	6.6 ± 1.4
SGR	1.8 ± 0.1	1.9 ± 0.1	2.1 ± 0.2	1.8 ± 0.2
TGC	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.2
FCR	2.2 ± 0.2	1.9 ± 0.1	1.8 ± 0.2	2.0 ± 0.1
HSI	4.8 ± 0.5	5.2 ± 0.5	5.4 ± 0.5	5.5 ± 0.3
VSI	12.5 ± 0.4	14.2 ± 0.6	14.0 ± 0.6	13.8 ± 1.1
Liver lipid (%)	45.2 ± 4.7	44.1 ± 7.5	52.7 ± 9.5	50.9 ± 6.2
Flesh lipid (%)	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.0
Intestine lipid (%)	2.8 ± 0.4 [b]	3.2 ± 0.0 [ab]	3.5 ± 0.1 [a]	3.2 ± 0.1 [ab]

878

879 Columns with different letters are significantly different ($P < 0.05$).

880

Table 5

Fatty acid compositions (percentage weight of total fatty acids) of cod liver after feeding diets containing increasing levels of Camelina oil.

	C0	C33	C66	C100
14:0	3.7 ± 0.1[a]	3.1 ± 0.1[b]	2.4 ± 0.1[c]	1.7 ± 0.0[d]
16:0	12.4 ± 0.3[a]	11.6 ± 0.3[ab]	10.4 ± 0.3[bc]	9.7 ± 0.9[c]
18:0	2.7 ± 0.1	3.0 ± 0.1	2.9 ± 0.3	2.9 ± 0.2
Total saturates[1]	19.6 ± 0.3[a]	18.3 ± 0.4[a]	16.4 ± 0.5[b]	15.0 ± 0.8[c]
16:1n-7	6.4 ± 0.2[a]	5.6 ± 0.1[b]	4.4 ± 0.1[c]	3.5 ± 0.4[d]
18:1n-9	15.9 ± 0.4[b]	16.5 ± 0.5[ab]	17.1 ± 0.6[ab]	17.4 ± 0.5[a]
18:1n-7	4.1 ± 0.1[a]	3.8 ± 0.1[a]	3.2 ± 0.1[b]	2.7 ± 0.3[c]
20:1[2]	12.0 ± 0.4[a]	11.3 ± 0.5[ab]	10.7 ± 0.2[bc]	10.1 ± 0.3[c]
22:1[3]	8.9 ± 0.1[a]	6.8 ± 0.4[b]	4.8 ± 0.2c	3.0 ± 0.2[d]
24:1	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.2
Total monoenes[4]	50.6 ± 0.4[a]	46.7 ± 0.4[b]	42.6 ± 0.3c	39.0 ± 1.0[d]
18:2n-6	3.9 ± 0.1[d]	6.3 ± 0.1[c]	9.3 ± 0.4[b]	12.0 ± 0.2[a]
20:2n-6	0.5 ± 0.0[d]	0.8 ± 0.1[c]	1.2 ± 0.1[b]	1.6 ± 0.1[a]
20:4n-6	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
Total n-6 PUFA[5]	5.2 ± 0.1[d]	7.8 ± 0.2[c]	11.1 ± 0.4[b]	14.2 ± 0.3[a]
18:3n-3	1.3 ± 0.1[d]	6.5 ± 0.2[c]	12.7 ± 0.6[b]	18.0 ± 0.7[a]
18:4n-3	3.0 ± 0.1[a]	2.7 ± 0.1[b]	2.2 ± 0.1[c]	1.6 ± 0.1[d]
20:3n-3	0.2 ± 0.0[d]	0.5 ± 0.0[c]	0.8 ± 0.0[b]	1.1 ± 0.0[a]
20:4n-3	0.8 ± 0.1[a]	0.6 ± 0.0[b]	0.5 ± 0.0[bc]	0.4 ± 0.0[c]
20:5n-3	8.7 ± 0.2[a]	7.5 ± 0.3[b]	6.1 ± 0.2[c]	4.6 ± 0.3[d]
22:5n-3	0.9 ± 0.0[a]	0.8 ± 0.0[a]	0.6 ± 0.0[b]	0.5 ± 0.0[b]
22:6n-3	8.9 ± 0.3[a]	7.7 ± 0.2[b]	6.3 ± 0.5[c]	5.2 ± 0.2[d]
Total n-3 PUFA	23.7 ± 0.6[d]	26.3 ± 0.6[c]	29.1 ± 0.6[b]	31.3 ± 1.1[a]
n-3/n-6 PUFA	4.4 ± 0.3[a]	3.3 ± 0.3[b]	2.6 ± 0.4[bc]	2.2 ± 0.6[c]
Total PUFA[6]	29.8 ± 0.7[d]	35.0 ± 0.7[c]	41.0 ± 0.7[b]	46.0 ± 1.2[a]

Values are means ± SD (n = 3). Columns with different letters are significantly different ($P < 0.05$). [1]Includes 15:0, 17:0 and 20:0. [2]Includes both 20:1n-9 and 20:1n-7; [3]Includes both 22:1n-11 and 22:1n-9; [4]Includes 14:1n-5 and 16:1n-9; [5]Includes 18:3n-6, 20:3n-6, 22:4n-6 and 22:5n-6; [6]Includes C16 PUFA.

Table 6

Fatty acid composition (percentage weight of total fatty acids) of cod intestine after feeding diets containing increasing levels of Camelina oil.

	C0	C33	C66	C100
14:0	2.1 ± 0.6	2.5 ± 0.2	2.1 ± 0.1	1.7 ± 0.1
16:0	14.6 ± 0.4^{a}	13.4 ± 0.1^{b}	12.5 ± 0.2^{bc}	12.0 ± 0.7^{c}
18:0	4.0 ± 0.5	3.4 ± 0.1	3.6 ± 0.2	3.7 ± 0.3
Total saturates[1]	21.6 ± 0.2^{a}	20.2 ± 0.2^{ab}	18.9 ± 0.3^{bc}	18.4 ± 1.3^{c}
16:1n-7	3.2 ± 0.5^{ab}	3.6 ± 0.2^{a}	3.1 ± 0.2^{ab}	2.5 ± 0.2^{b}
18:1n-9	11.2 ± 0.1^{b}	11.2 ± 0.6^{b}	12.2 ± 0.5^{ab}	12.7 ± 0.5^{a}
18:1n-7	2.8 ± 0.2^{a}	2.7 ± 0.1^{a}	2.3 ± 0.1^{b}	1.8 ± 0.1^{c}
20:1[2]	6.8 ± 0.7^{b}	7.8 ± 0.3^{ab}	8.1 ± 0.1^{a}	8.2 ± 0.5^{a}
22:1[3]	4.7 ± 1.9	4.9 ± 0.8	4.0 ± 0.2	3.3 ± 0.4
24:1	2.2 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	2.0 ± 0.5
Total monoenes[4]	31.2 ± 2.7	32.3 ± 0.8	31.7 ± 0.4	30.9 ± 1.4
18:2n-6	2.7 ± 1.0^{d}	4.5 ± 0.2^{c}	6.9 ± 0.2^{b}	9.4 ± 0.7^{a}
20:2n-6	0.5 ± 0.0^{d}	0.9 ± 0.1^{c}	1.3 ± 0.1^{b}	1.6 ± 0.0^{a}
20:4n-6	1.7 ± 0.2^{a}	1.2 ± 0.1^{b}	1.0 ± 0.1^{b}	0.9 ± 0.1^{b}
Total n-6 PUFA[5]	5.4 ± 0.8^{d}	7.0 ± 0.1^{c}	9.6 ± 0.1^{b}	12.3 ± 0.7^{a}
18:3n-3	0.8 ± 0.3^{d}	4.1 ± 0.3^{c}	8.5 ± 0.4^{b}	12.5 ± 1.3^{a}
18:4n-3	1.2 ± 0.4	1.5 ± 0.2	1.3 ± 0.1	1.0 ± 0.1
20:3n-3	0.1 ± 0.0^{d}	0.4 ± 0.0^{c}	0.7 ± 0.0^{b}	1.0 ± 0.2^{d}
20:4n-3	0.5 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.1^{a}	0.3 ± 0.1^{b}
20:5n-3	10.1 ± 0.3^{a}	9.9 ± 0.3^{a}	8.4 ± 0.2^{b}	6.8 ± 0.3^{c}
22:5n-3	1.2 ± 0.1^{a}	1.0 ± 0.1^{ab}	0.8 ± 0.1^{bc}	0.7 ± 0.0^{c}
22:6n-3	24.1 ± 3.9^{a}	18.9 ± 1.4^{ab}	16.3 ± 0.5^{b}	13.9 ± 0.9^{b}
Total n-3 PUFA	38.0 ± 3.7	36.5 ± 1.3	36.5 ± 0.3	36.3 ± 1.5
n-3/n-6 PUFA	7.2 ± 1.8^{a}	5.2 ± 0.2^{a}	3.8 ± 0.1^{b}	2.9 ± 0.1^{c}
Total PUFA[6]	44.1 ± 2.9^{b}	44.2 ± 1.3^{ab}	46.8 ± 0.3^{ab}	49.0 ± 2.1^{a}

Values are means ± SD (n = 3). Columns with different letters are significantly different ($P < 0.05$). [1]Includes 15:0, 17:0 and 20:0. [2]Includes both 20:1n-9 and 20:1n-7; [3]Includes both 22:1n-11 and 22:1n-9; [4]Includes 14:1n-5 and 16:1n-9; [5]Includes 18:3n-6, 20:3n-6, 22:4n-6 and 22:5n-6; [6]Includes C16 PUFA.

Table 7

Fatty acid compositions (percentage weight of total fatty acids) of cod muscle after feeding diets containing increasing levels of Camelina oil.

	C0	C33	C66	C100
14:0	1.6 ± 0.0[a]	1.7 ± 0.4[a]	1.5 ± 0.1[ab]	1.0 ± 0.2[b]
16:0	15.8 ± 0.5[a]	14.5 ± 1.2[ab]	14.9 ± 1.0[ab]	13.2 ± 0.4[b]
18:0	3.3 ± 0.1	3.3 ± 0.3	4.1 ± 0.8	3.6 ± 0.2
Total saturates[1]	21.7 ± 0.4[a]	19.8 ± 1.0[ab]	20.4 ± 1.5[ab]	18.2 ± 0.6[b]
16:1n-7	2.8 ± 0.0[a]	2.6 ± 0.3[a]	2.1 ± 0.1[b]	1.6 ± 0.2[c]
18:1n-9	11.4 ± 1.4	11.3 ± 0.9	11.3 ± 0.7	11.2 ± 0.7
18:1n-7	3.2 ± 0.2[a]	2.8 ± 0.1[b]	2.5 ± 0.1[bc]	2.2 ± 0.1[c]
20:1[2]	4.5 ± 0.1	5.1 ± 1.0	4.5 ± 0.2	4.5 ± 0.2
22:1[3]	2.3 ± 0.2[ab]	2.6 ± 0.7[a]	1.7 ± 0.5[ab]	1.4 ± 0.1[b]
24:1	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Total monoenes[4]	26.7 ± 2.2	27.0 ± 3.5	24.5 ± 1.3	22.7 ± 1.3
18:2n-6	3.0 ± 0.1[d]	4.8 ± 0.3[c]	6.6 ± 0.4[b]	8.8 ± 0.3[a]
20:2n-6	0.4 ± 0.0[d]	0.7 ± 0.0[c]	1.0 ± 0.1[b]	1.4 ± 0.0[a]
20:4n-6	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.0	1.1 ± 0.1
Total n-6 PUFA[5]	5.0 ± 0.2[d]	7.3 ± 0.2[c]	9.3 ± 0.4[b]	11.9 ± 0.3[a]
18:3n-3	0.9 ± 0.0[d]	4.3 ± 0.7[c]	7.5 ± 0.4[b]	11.0 ± 0.7[a]
18:4n-3	1.5 ± 0.2[a]	1.4 ± 0.4[ab]	1.1 ± 0.1[ab]	0.8 ± 0.1[b]
20:3n-3	0.1 ± 0.0[d]	0.4 ± 0.0[c]	0.7 ± 0.0[b]	1.0 ± 0.0[a]
20:4n-3	0.7 ± 0.0[a]	0.6 ± 0.0[ab]	0.5 ± 0.0[ab]	0.4 ± 0.0[b]
20:5n-3	14.5 ± 1.6[a]	13.5 ± 0.9[a]	11.9 ± 0.4[ab]	10.8 ± 0.7[b]
22:5n-3	1.6 ± 0.2	1.5 ± 0.2	1.3 ± 0.0	1.3 ± 0.1
22:6n-3	25.5 ± 3.8	23.4 ± 2.7	21.7 ± 1.0	21.3 ± 1.4
Total n-3 PUFA	43.1 ± 4.4	44.9 ± 3.3	44.7 ± 1.0	46.6 ± 1.8
n-3/n-6 PUFA	8.6 ± 2.1[a]	6.2 ± 1.1[ab]	4.8 ± 0.7[b]	3.9 ± 0.8[b]
Total PUFA[6]	50.3 ± 5.1	52.6 ± 3.2	54.3 ± 1.1	58.6 ± 1.8

Values are means ± SD (n = 3). Columns with different letters are significantly different ($P < 0.05$). [1]Includes 15:0, 17:0 and 20:0. [2]Includes both 20:1n-9 and 20:1n-7; [3]Includes both 22:1n-11 and 22:1n-9; [4]Includes 14:1n-5 and 16:1n-9; [5]Includes 18:3n-6, 20:3n-6, 22:4n-6 and 22:5n-6; [6]Includes C16 PUFA.

Table 8

Transcripts corresponding to the top 100 most significant features exhibiting differential expression in cod intestine between diets C0 and C66 (fold change given as C66 / C0). Annotated features (66%) are arranged by categories of biological function and, within these, by decreasing significance (p-value, assessed by Welch t-test). Indicated is also the percentage of gene distribution by functional categories, after removing features representing the same gene.

Probe No.	Accession No.	SwissProt Annotation	Fold change (C66/C0)	p-value
Lipid metabolism (5%)				
CE2148	GO377376	Apolipoprotein A-IV	1.48	0.0018
CPY1052	GO387010	Apolipoprotein A-IV	1.50	0.0034
CPY442	GO387753	Apolipoprotein A-IV	1.53	0.0048
CPY1606	GO387357	Apolipoprotein A-IV	1.51	0.0049
CPY1409	GO387232	Apolipoprotein A-IV	1.54	0.0057
CPY465	GW857638	Apolipoprotein A-IV	1.50	0.0057
CHY224	GO383431	Apolipoprotein A-IV	1.59	0.0057
CPY1627	GO387370	Apolipoprotein A-IV	1.45	0.0064
CLE718	GW849678	Lipovitellin-2	- 1.21	0.0098
CE1608	GW843824	Apolipoprotein A-IV	1.53	0.0075
CPY225	GO387561	Apolipoprotein A-IV	1.48	0.0104
CPY205	GO387527	Apolipoprotein A-IV	1.40	0.0141
CTA293	N/A	Apolipoprotein A-IV	1.51	0.0161
Energy metabolism (5%)				
CHO292	GO382345	Creatine kinase B-type	1.62	0.0104
CE1110	GO376622	Creatine kinase B-type	1.59	0.0138
CTA327	GO390332	Creatine kinase B-type	1.37	0.0146
CE2531	GW843201	Creatine kinase M-type	1.28	0.0152
CTE1039	GO390859	Creatine kinase B-type	1.51	0.0174
CTE1378	GW861048	Creatine kinase B-type	1.60	0.0178
Proteolysis (5%)				
CLE650	GW849656	Carboxypeptidase N polypeptide 1	- 1.84	0.0052
CE1303	GO376762	F-box and leucine-rich repeat protein 18	- 1.21	0.0082
Protein folding (5%)				
CSMI546	GO388637	Peptidyl-prolyl cis-trans isomerase FKBP5	- 1.24	0.0022
CPY173	GO387434	Heat shock cognate 70 kDa protein	- 1.24	0.0081
Regulation of transcription (5%)				
NM814	N/A	Transcription factor IIIA	- 1.49	0.0131
NM115	N/A	Protein strawberry notch homolog 1	1.23	0.0172
Translation (25%)				
CE2965	GO378001	40S ribosomal protein S15a	- 1.23	0.0006
CE2958	GW843393	60S ribosomal protein L13	- 1.33	0.0011
CE2753	GW843313	Elongation factor 1-alpha	- 1.22	0.0038
CE102	GO376549	60S ribosomal protein L27a	- 1.26	0.0055
CE916	GO378560	60S ribosomal protein L27	- 1.22	0.0068

CE1965	GW843877	60S ribosomal protein L38	- 1.23	0.0077
CHO1803	GO381721	Elongation factor 1-beta	- 1.20	0.0098
CE2064	GW842964	60S ribosomal protein L6	- 1.22	0.0111
CHY916	GO383943	Eukaryotic translation initiation factor 3 subunit F	- 1.27	0.0115
CLE430	GO384373	Polyadenylate-binding protein 1	- 1.24	0.0137
CSMI161	GO388318	60S ribosomal protein L35	- 1.22	0.0154
CE2959	GO377996	40S ribosomal protein S17	- 1.21	0.0166
Cell proliferation, differentiation and apoptosis (20%)				
CTE684	GW861483	Tumor protein p63 regulated 1	1.22	0.0007
CTE495	GO391915	40S ribosomal protein SA	2.03	0.0011
NM303	N/A	Tumor suppressor candidate 2	- 1.22	0.0017
NM253	N/A	Proline-rich protein BCA3-like	- 1.37	0.0032
CHJ1269	GO380651	Translationally-controlled tumor protein homolog	- 1.26	0.0040
NM449	N/A	origin recognition complex, subunit 2-like	2.32	0.0096
CHJ1272	GO380654	Translationally-controlled tumor protein homolog	- 1.23	0.0104
CHO1630	GW846809	Caspase 3	1.21	0.0106
CTA632	GO390541	Translationally-controlled tumor protein homolog	- 1.22	0.0107
CTA484	GO390436	Translationally-controlled tumor protein homolog	- 1.23	0.0108
CPY269	GO387613	Retinoic acid receptor responder protein 3	2.38	0.0110
CSMI714	GW860190	Translationally-controlled tumor protein homolog	- 1.24	0.0111
COV1041	GW855020	Voltage-dependent anion-selective channel protein 1	1.22	0.0135
CE1707	GO377048	Translationally-controlled tumor protein homolog	- 1.27	0.0161
CTE1915	GO391572	Caspase-3 subunit p12	1.38	0.0176
Structural molecule (9%)				
CTE1047	GO390866	Tektin-4	1.26	0.0067
CE2716	GO377798	Tropomyosin alpha-1 chain	1.53	0.0110
CE2051	GW842958	Cadherin 3, type 1	- 1.20	0.0120
COV757	GW855647	Claudin 4	- 1.33	0.0124
Immune response (9%)				
CPY1238	GO387126	Viperin	1.37	0.0159
CTE1043	GO390863	Barrier-to-autointegration factor	1.21	0.0159
CSMI679	GO388738	Interferon-induced protein 44	1.60	0.0166
CHO1910	GW846481	High affinity immunoglobulin epsilon receptor subunit gamma	- 1.38	0.0177
Miscellaneous or unknown function (14%)				
CE2960	GW843396	Autophagy-related protein 16-1	- 1.22	0.0052
CTE324	GO391798	Endoribonuclease	1.65	0.0124
CLE567	GO384459	Processed zona pellucida sperm-binding protein 3	- 1.37	0.0129
NM762	N/A	Omega-amidase NIT2-B	1.61	0.0140
CHO673	GO382599	Hemoglobin beta 1 chain	- 1.68	0.0153
CTE829	GW861558	Tetraspanin-14	1.21	0.0175

Table 9

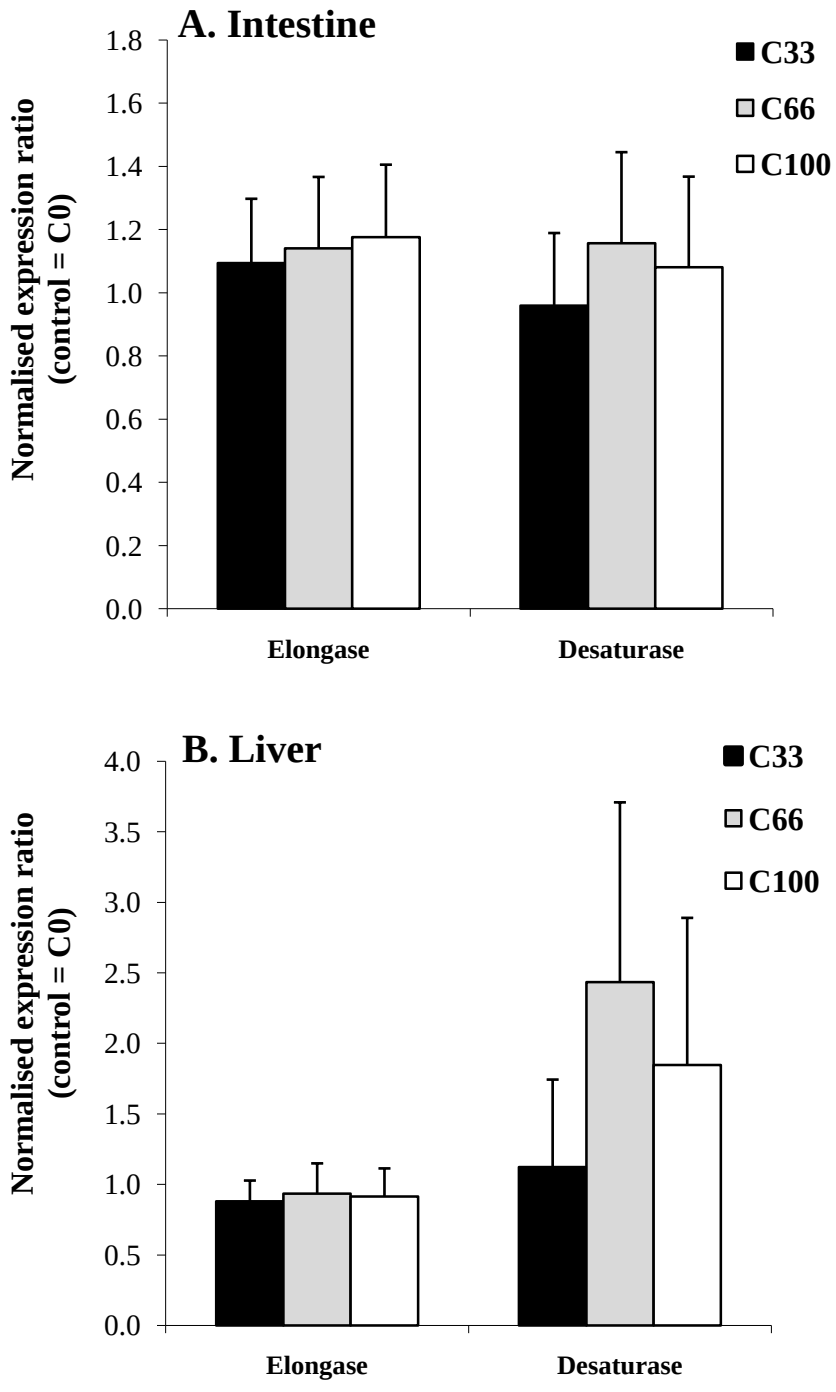
GO analysis results showing significant enrichment, at an adjusted p-value of 1.825E-4, of features for the molecular function category GO:0003779; actin binding (14.9% count in selection; 1.8% count in total).

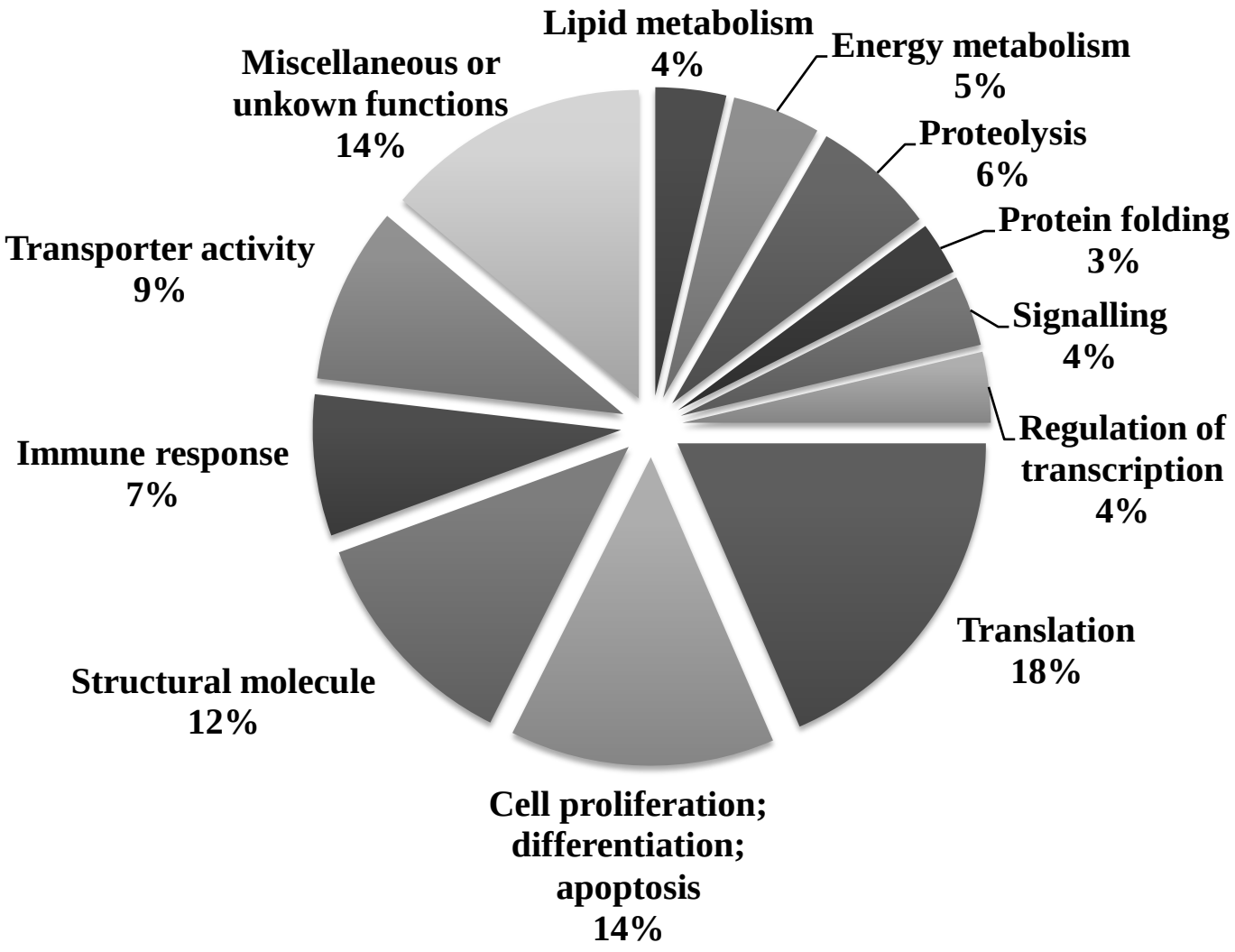
Probe No.	Accession No.	SwissProt Annotation	Fold change (C66/C0)
CE1462	GW842770	Myosin heavy chain	1.38
CE2112	GO377351	Tropomyosin 1 (alpha)	1.42
CE2636	GW843270	Actin, alpha cardiac muscle 1	- 1.21
CE2716	GO377798	Tropomyosin 1 (alpha)	1.53
CE457	GO378247	Tropomyosin 1 (alpha)	1.43
CE551	GO378302	Tropomyosin 1 (alpha)	1.46
CHJ685	GO381113	Actin, alpha cardiac muscle 1	- 1.26
CHO2151	GO382012	Actin, alpha cardiac muscle 1	- 1.21
CHY703	GO383785	Actin, alpha cardiac muscle 1	- 1.23
COV1757	GW855413	Cofilin 1 (non-muscle)	1.82
COV702	GW855592	Ectodermal-neural cortex (with BTB-like domain)	1.80
CSMI989	GW859865	Actin, alpha cardiac muscle 1	- 1.21
CTA516	GO390453	Tropomyosin 1 (alpha)	1.41
CTE369	GO391832	Transgelin	1.57
CTE807	GO392129	Transgelin	1.60

Table 10

Validation, by RT-qPCR, of selected genes found to be significantly different when expressed in the microarray analysis. Indicated is the fold change (FC) between expression levels in diets C66 and C0 and p-value (assessed by REST2008) of the RT-qPCR analysis. Asterisks indicate fold changes, assessed by RT-qPCR, that are statistically significant.

Genes	**Microarray**	**RT-qPCR**	
	FC (C66/C0)	**FC (C66/C0)**	**p-value**
Elongation factor 1-alpha (*elf1a*)	-1.22	1.18	0.095
60S ribosomal protein L13 (*rpl13*)	-1.33	-1.00	0.983
Eukaryotic translation initiation factor 3 subunit F (*etf3f*)	-1.27	-1.00	0.985
Tumor protein p63 regulated 1-Like (*tprg1l*)	1.22	1.14	0.256
Retinoic acid receptor responder protein 3 (*rarres3*)	2.38	*7.81	0.014
40S ribosomal protein SA (*rpsa*)	2.03	1.03	0.658
Translationally-controlled tumor protein homolog (*tctp*)	1.26	-1.23	0.136
Caspase 3 (*casp3*)	1.21	*1.89	0.000
Tropomyosin alpha-1 chain (*tpm*)	1.53	*1.55	0.036
Tektin-4 (*tekt4*)	1.26	-1.33	0.341
Cadherin 3 type 1 (*cdh3*)	-1.20	1.01	0.921


A. Intestine
Normalised expression ratio (control = C0)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C33
C66
C100
Elongase
Desaturase
B. Liver
Normalised expression ratio (control = C0)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C33
C66
C100
Elongase
Desaturase


Lipid metabolism
4%
Energy metabolism
5%
Proteolysis
6%
Protein folding
3%
Signalling
4%
Regulation of
transcription
4%
Translation
18%
Cell proliferation;
differentiation;
apoptosis
14%
Structural molecule
12%
Immune response
7%
Transporter activity
9%
Miscellaneous or
unkown functions
14%