# Omega-3 canola oil effectively replaces fish oil as a new safe dietary source of docosahexaenoic acid (DHA) in feed for juvenile Atlantic salmon

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**Abstract**

Limited availability of fish oils (FO), rich in omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (FA), is a major constraint for further growth of the aquaculture industry. Long-chain omega-3 rich oils from crops genetically modified with algal genes are promising new sources for the industry. This project studied the use of a newly developed omega-3 canola oil (DHA-CA) in diets of Atlantic salmon fingerlings in freshwater. The DHA-CA oil has high proportions of the omega-3 FA 18:3n-3 and DHA and lower proportions of omega-6 FA than conventional plant oils. Levels of phytosterols, vitamin E, and minerals in the DHA-CA were within the natural variation of commercial canola oils. Pesticides, mycotoxins, polyaromatic hydrocarbons, and heavy metals were below lowest qualifiable concentration. Two feeding trials were conducted to evaluate effects of two dietary levels of DHA-CA compared to two dietary levels of FO at two water temperatures. Fish increased their weight approximately 20-fold at 16 °C and 12-fold at 12 °C during the experimental periods, with equal growth in salmon fed the FO diets compared to DHA-CA diets. Salmon fed DHA-CA diets had approximately the same EPA+DHA content in whole body as salmon fed FO diets. Gene expression, lipid composition, and oxidative stress related enzyme activities showed only minor differences between the dietary groups and the effects were mostly a result of dietary oil level, rather than the oil source. The results demonstrated that DHA-CA is a safe and effective replacement for FO in diets of Atlantic salmon during the sensitive fingerling life-stage.

## 1. Introduction

Diets for farmed Atlantic salmon, *Salmo salar*, have changed considerably during the last decades. In Norway, while 90% of the ingredients were of marine origin in the 1990s, current diets only contain approximately 30% marine ingredients [1]. This situation is very similar in Chile, Canada, and Australia, whereas salmon diets used in Scotland in 2013/14 contained 60% plant ingredients and 40% marine ingredients on average [2]. The shift from marine to plant ingredients, or in some cases land animal-by products, has been driven by the need of the feed producers to reduce dietary reliance on the finite marine-derived ingredients fish oil (FO) and fish meal (FM) to secure global aquaculture production [3]. However, vegetable oils are naturally devoid of the nutritionally beneficial omega-3 (n-3) long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), and cannot be used as the sole oil source in fish diets with a low FM content, since EPA and DHA are essential FA in salmon diets [4; 5]. The physiological relevance of these fatty acids (FA) is well recognized, being involved in maintaining the structural and functional integrity of cellular membranes, acting as precursors of important metabolites, such as eicosanoids, and working as signalling molecules able to regulate the transcription of genes involved in lipid metabolism [6; 7].

The Norwegian, Scottish, and Australian salmon industries have reported a significant decrease in the absolute amounts of EPA and DHA in salmon fillets and a concomitant increase in FA typical of terrestrial-derived lipid sources, reflecting the changes in the feed formulation throughout the years [1; 2; 8; 9; 10]. In addition, the high inclusion levels of vegetable and/or animal oils rich in n-6 FA in salmon diets result in an associated increase in the n-6/n-3 ratio in fish organs and tissues, including fish fillets [11]. This is an important issue that might affect not only fish and human consumer health, but also influence consumer perspectives towards farmed fish. Alternative sources of n-3 LC-PUFA oils are being pursued, however, neither oils from traditional oilseeds such as linseed, nor the genetically modified SDA (stearidonic acid, 18:4n-3) or EPA rich oils have so far shown efficient conversion to DHA in Atlantic salmon [12].

In terrestrial genetically modified (GM) plant oils, enriched levels of DHA have been achieved, combined with lower n-6 PUFA levels than occurs in conventional canola oils [12; 13; 14; 15]. Today, close to 70% of the oil fraction in feeds for Atlantic salmon in Norway consists of canola oil, demonstrating that the fish already grow and perform well on canola oil rich

diets. In addition, several Atlantic salmon feeding studies using GM plant material from soybean and maize have shown that they are as safe as commercial varieties (reviewed in [16]). GM camelina oil rich in EPA, although containing a high level of n-6 FA, has been tested in diets of post smolt Atlantic salmon. This oil did not influence the fish performance relative to a FO diet; however liver transcriptome analyses revealed that the response in fish was closer to the expected response to plant oils than to that of FO [17]. Some studies have shown that Atlantic salmon have a more defined requirement for DHA than for EPA [18; 19; 20]. When replacing conventional feed ingredients with new ingredients, it is important to ensure that they are as safe as traditional products.

In the present study, two separate feeding trials with DHA-CA oil were performed, one in Australia at a water temperature of 16°C and one in Norway at a lower water temperature of 12°C. The overall study was undertaken to determine if the new DHA-CA oil containing enhanced levels of n-3 LC-PUFA and a lower level of n-6 LC-PUFA than conventional canola oils, would influence growth, performance, health, and n-3 LC-PUFA, content in different organs and tissues in Atlantic salmon fingerling.

## 2. Material and methods

### *2.1.Canola oils*

The DHA-CA tested in the experiment was obtained from Event NS-B50027-4, which is a modified canola (*Brassica napus*) line developed by Nuseed Pty. Ltd. (Australia). The transgenic seeds were modified to produce the long-chain polyunsaturated PUFA - EPA and DHA; construct details are provided in Petrie *et al.* [15]. The crop used in this trial was grown in Australia during 2015 and 2016. The control canola oil used in the diets was an Australian variety (Garnet) with an oil profile similar to that of the parent (untransformed) line from which NS-B50027-4 was derived; this oil is defined as Ctr-CA in this study. Oils were cold-pressed and filtered.

### *2.2.Analyses of elements and undesirables in the oils*

Multielement determination of the oils was done by ICP-MS (inductively coupled plasma mass spectrometry) [21] while analysis of selected undesirable compounds were conducted by Eurofins (GmbH, Hamburg) using accredited methods (accreditation number D-PL-14602-01-

00) Chlorinated pesticides (aldrin; chlordane, cis-; chlordane, oxy-; chlordane, trans-; dieldrin; endrin; gamma-HCH (lindane); HCH, alpha-; HCH, beta-; HCH, delta-; heptachlor; heptachlor epoxide, cis-; heptachlor epoxide, trans-; hexachlorobenzene (HCB); mirex; nonachlor, trans-; o,p'-DDD; o,p'-DDE; o,p'-DDT; octachlorstyrene; p,p'-DDD; p,p'-DDE; p,p'-DDT; pentachlorobenzene; toxaphene parlar 26; toxaphene parlar 50; toxaphene parlar 62, endosulfan (-alpha,-beta,-sulfat), toxaphene -26, -50, -60), organophosphate pesticides (119 compounds), 16 PAH-compounds (benzo(a)antracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(c)fluorene, benzo(ghi)perylene, benzo(j)fluoranthene benzo(k)fluoranthene, chrysene, cyclopenta(cd)pyrene, dibenz(ah)anthracene, indeno(1,2,3-cd)pyrene, 5-methylchrysene, dibenzo(a,e)pyrene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene and Dibenzo(a,l)pyrene) and five lipid-soluble mycotoxins beauvericin and enniatin (A, A1, B and B1) were analyzed in the oils.

### *2.3. Experimental diets used in the 16 °C and 12 °C trials*

Tables 1 and 2 show the formulation and chemical composition of the experimental diets. The four experimental diets for the 16 °C and 12 °C trials were isoproteic (i.e., 59% and 57%), isolipidic (≈17% and 20%), and isoenergetic (≈21 MJ/kg for both experiments) (Tables 1 and 2). The diets for the two trials were both formulated to contain similar amounts of all nutrients which satisfied the dietary requirement of small fingerling salmon. The ingredients FM and FO used in the two trials came from different sources and therefore there were some differences in the total quantity of EPA and DHA in their respective diets. The dietary treatments tested in both trials consisted of two diets containing low or high levels of FO, and two diets containing low or high levels of DHA-CA oil. Within each temperature study, the FO-based diets were supplemented with standard (control) canola oil (Crt-CA) to provide a similar content of EPA+DHA to the diets containing DHA-CA at 50% and 100% of total oil supplementation. The FA compositions of diets for the two temperature trials are presented in Table 3. All diets were produced in four pellet sizes and were used in accordance with increasing fish size. Due to the high FM inclusion (79%) and low supplemental oil inclusion (7.8%) in the fingerling diet formulations, phytosterols only contributed 8-16% of the total sterols (the rest being cholesterol) in the feeds (Table 2). The calculated levels of EPA+DHA provided by the ingredients used in the diets are provided in Tables 1 and 2.

### *2.4.Fish trials at 16 °C and 12 °C*

Atlantic salmon fingerlings with a mean weight of 0.83 gram (± 0.02) were the starting points for the 16 ℃ trial in Australia (CSIRO research facilities, Bribie Island). The Australian experimental fish fry was produced in September 2016 by the industry hatchery Salmon Enterprises of Tasmania Pty Ltd (Saltas). Atlantic salmon fingerlings with a mean weight of 2.08 gram (± 0.05) were the starting points for the 12 °C trial in Norway (Nofima research facilities Sunndalsøra). The Norwegian experimental fish fry was produced in January 2016 from the Broodstock population CrossBreed Stofnfiskur by the company SalmoBreed. The experimental fish were distributed in 12 tanks, 100 individuals per tank (300L) and 200 individuals per tank (150L) for the 16 °C and 12 °C trials, respectively. The fibreglass tanks were equipped with automatic belt feeders. Freshwater at a constant 16 ℃ (± 0.1) and 12 °C (± 0.1) was supplied at a flow rate of 3 and 5 l/min, respectively. As the fish grew and oxygen consumption increased, flow rate was increased stepwise. Fish were kept under constant light (24L:0D) and fed every 10 minutes. Feed was distributed according to the expected growth rate and a level of overfeeding that would allow all fish to feed *ad lib*. The four experimental feeds were fed to triplicate groups of fish. Temperature was measured daily in six random tanks.

### *2.5.Fish sampling in the 16 °C and 12 °C trials*

In the 16 °C temperature trial, three individual samples of 30 pooled fish were taken initially and 100 fish per tank were bulk-weighed (20-30 fish at a time) on the same day. Fish were thereafter bulk weighed on day 0, 34, 56, and 70. On day 70, ten fish per tank were sampled and pooled for whole body proximal composition and FA analyses and stored at -40°C until analyses.

In the 12 °C trial, fish weights were bulk weighed on day 0, 27, 41, and 83. In the final sampling, five fish from each tank were sampled for whole body analyses of total lipid and FA content and composition. Additionally, five fish per tank were sampled and used for other analyses. Blood samples were taken, livers and hearts were dissected out and weighed, and organ indices were calculated. Samples of intestine, liver, heart, and red blood cells were frozen in liquid nitrogen and stored at -70°C until analyses for FA composition, gene expression, and enzyme activities.

### *2.6.Chemical analysis in the 16 °C and 12 °C trials*

Ten fish per tank were used for analyses of carcass chemical composition. Whole fish and dry matter content of the feeds were determined by gravimetric analysis following drying at 105 $^{o}$C for 16 h. Ash content was determined based on mass change after combustion in a muffle furnace at 550 $^{o}$C for 16 h. Measurement of total nitrogen content was undertaken using an elemental analyser (Flash 2000 Thermo Fisher Scientific, USA) and data were used to calculate sample protein content based on N x 6.25. Gross energy was determined by isoperibolic bomb calorimetry in a Parr 6200 oxygen bomb calorimeter (Par Instrument Company, Moline, IL, USA). Carbohydrate was calculated by difference.

### *2.7.Fat content and fatty acid composition in the 16 °C and 12 °C trials*

Total lipids were extracted from the dietary oil, red blood cells, whole body, muscle, liver, intestine, and diets following the method described by Folch *et al.* [22]. For the 16 °C temperature trial only whole body and diets were analysed. In each dietary group, 15 fish were used for lipid analysis (five from each tank). The chloroform-methanol phase after Folch extraction was used for the analysis of the FA composition of total lipids using the method described by Mason *et al.* [23]. Briefly, the extract was dried under nitrogen gas, and residual lipid was trans-methylated overnight with 2′,2′-dimethoxypropane, methanolic-HCl, and benzene at room temperature. The methyl esters were separated and analysed using a gas chromatograph (Hewlett Packard 6890; HP, Wilmington, DE, USA) equipped with a split injector by using an SGE BPX70 capillary column (length, 60 m; internal diameter, 0.25 mm; and film thickness, 0.25 μm; SGE Analytical Science, Milton Keynes, UK), flame ionization detector, and HP Chem Station software. The carrier gas was helium, and the injector and detector temperatures were both set at 280 °C. The oven temperature was increased from 50 to 180 °C at the rate of 10 °C/min, and then increased to 240 °C at a rate of 0.7 °C/min. Individual FA methyl esters were identified by referring to well-characterised standards. The relative amount of each FA was expressed as a percentage of the total amount of FA in the analysed sample, and the absolute amount of FA per gram of tissue was calculated using C23:0 methyl ester as the internal standard.

Similar methods were used for the high temperature study and while most FA were the same, a few different FA methyl esters were measured in the two standard protocols. In brief, 3 mg of oil was esterified using methanol-HCl at 105 °C for 60 min then extracted with hexane for separation by gas chromatography (Agilent 6890N), using a DB-23 capillary column (length, 60 m; internal diameter, 0.25 mm; and film thickness, 0.15 μm). The carrier gas was hydrogen. Individual FA methyl esters were identified by referring to well-characterized standards and the Agilent RTL FAME Method, relative to C21:0 methyl ester as the internal standard.

### *2.8.Gene expression analysis in the mid-intestine of fish reared at 12 ℃*

Total RNA was isolated from mid-intestine using PureLink Pro 96 RNA Purification Kit (Invitrogen), according to manufacturer's instructions. RNA was treated with PureLink DNase1 (ThermoFisher, MA, USA) to remove any contaminating DNA. The RNA concentration was measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (900 ng) was reverse-transcribed into cDNA in a 20 μl reaction using a TaqMan® reverse transcription reagents kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative PCR was performed in a QuantStudio5 instrument (ThermoFisher, MA, USA), and the PCR master mix consisted of 0.5 μl forward and 0.5 μl reverse primer (Supplementary Table S1, final concentrations of 0.5 μM), 2 μl of a 1:10 dilution of cDNA and 2.5 μl PowerUp™ SYBR™ Green Master Mix (ThermoFisher, MA, USA). All samples were analysed in duplicate with a non-template and non-RT enzyme control for each gene. The reaction was performed by incubating the samples at 95 ºC for 20 seconds, forty cycles of 95 ºC for 1 second, and 60 ºC for 20 seconds. Primer efficiency was evaluated using 10-fold serial dilutions of cDNA for each primer pair. The specificity of PCR amplification was confirmed by melting curve analysis (95 ºC for 1 second and 60 ºC for 20 seconds, followed by an increase of 0.075º per second until 97 ºC). *Ef1a, rpol2,* and *etif3* were evaluated as reference genes, and *etif3* was identified as the most stable. Relative expressions of mRNA were calculated using the ΔΔCT method using *etif3* as a reference gene [(24)].

### *2.9.Histology of intestine of fish reared at 12 ℃*

Histological analysis of the mid-intestinal tissue was performed on 15 samples from the mid intestine of each dietary group from the low temperature trial, with salmples collected at the final sampling, and fixed in 10% phosphate buffered formalin and stored at 4 °C until analysis. The samples were dehydrated and processed according to standard protocols. Paraplast-embedded samples were cut using a microtome (5 µm) and stained with hematoxylin and eosin (Merck KGaA; Darmstadt, Germany). Stained slides were examined using a standard Nikon Optiphot light microscope (Nikon, Japan). Images were captured using a MicroPublisher 3.3 RTV camera and analysed using QCapture suite software (QImaging, Canada). The sections were evaluated in a blinded manner to identify any pathological or other systematic variations in tissue morphology.

### *2.10. Phytosterols, cholesterol, vitamin E, and vitamin K analyses in the 12 °C trial*

Phytosterols and cholesterol were analyzed in the oils, feeds, and in fish liver samples (six individual fish per tank with triplicate tanks per diet group), on a GC as described in detail by Sissener *et al.* [25], based on Laakso [26]. High-performance liquid chromatography (HPLC) was used for determination of tocopherols in the oils according to CEN [27], with two analytical parallels. Phylloquinone (vitamin K1), menaquinone (MK4-10, K2), and menadione (K3) were analyzed both in the oils and feed samples with four analytical parallels by HPLC as described by Graff *et al.* [28].

### *2.11. Enzyme activities in the liver from fish reared at 12 °C*

Livers were frozen separately in liquid nitrogen, and subsequently analysed for the activities of catalase, glutathione peroxidase, and superoxide dismutase (SOD). The activity of catalase was measured according to a method described in Baudhuin *et al.* [29]. SOD activity colorimetric assay kit (Biovision, California, USA) and glutathione peroxidase assay kit (Cayman Chemicals, Ann Arbor, MI, USA) were used to measure the activity of the two

enzymes following the manufacturers' protocol. The enzyme reactions were measured using a Spectrostar Nano plate reader from BMG LABTECH GmbH (Ortenberg).

### *2.12. Liver lipidomics in the 12 °C trial*

Liver lipids were extracted in the presence of authentic internal standards by the method of Folch *et al.* [22] using chloroform:methanol (2:1 v/v). Neutral lipid classes were separated on a solvent system consisting of petroleum ether/diethyl ether/acetic acid (80:20:1). Phospholipid classes were separated using the Agilent Technologies 1100 Series LC. Each lipid class was transesterified in 1% sulfuric acid in methanol in a sealed vial under a nitrogen atmosphere at 100 °C for 45 minutes. The resulting FA methyl esters were extracted from the mixture with hexane containing 0.05% butylated hydroxytoluene and prepared for GC by sealing the hexane extracts under nitrogen. FA methyl esters were separated and quantified by capillary GC (Agilent Technologies 6890 Series GC), equipped with a 30 m DB 88 capillary column (Agilent Technologies) and a flame ionization detector. For detection of ceramides, deuterium-labeled internal standards were added, and samples were solubilized in methanol followed by a crash extraction. A bilayer was formed with the addition of KCl in water, and the organic layer was removed and concentrated under nitrogen. The extract was spun, filtered, and split into two injections; one for ceramides and one for sphingosines. The extract was injected onto an Agilent C8 column connected to an Agilent 1290 Infinity LC and ABI 4000 QTRAP. The analytes were ionized via positive electrospray and the mass spectrometer was operated in the tandem MS mode. The absolute concentration of each sphingolipid was determined by comparing the peak to that of the relevant internal standard.

### *2.13. Liver metabolomics in the 12 °C trial*

The metabolomics work was performed by Metabolon (Durham, NC, USA) as previously described [30; 31] with liver samples sampled from 9 fish from each of the four diet groups. Several recovery standards were added for quality control, and samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was analyzed by four different methods; two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), analysis by RP/UPLC-MS/MS with negative ion mode ESI and analysis by hydrophilic-interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The

sample extract was dried before being reconstituted in solvents compatible to each of the methods, and each reconstitution solvent contained a series of standards at fixed concentrations. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution (further details can be found in the references above). Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra [32].

### *2.14. Microarray analysis of liver in the 12 °C trial*

Liver transcriptome was analysed with Nofima's 44 k microarray Salgeno containing oligonucleotide probes to all identified genes of Atlantic salmon. Analyses included all four experimental groups with five fish per group, and total 20 arrays were used. Custom microarrays were produced by Agilent Technologies and all reagents and equipment were purchased from the same provider. One-Color Quick Amp Labelling Kit was used for RNA amplification, labelling, and fragmentation of labelled RNA was performed with a Gene Expression Hybridization kit. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 0.01g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with the Agilent scanner. Nofima's bioinformatics pipeline STARS was used for data processing and mining of results. The high FO group was used as reference. Differentially expressed genes (DEG) were selected by lows stringency criteria that are commonly applied to feeding trials: $\log_2$-Expression Ratio > |0.6| (1.5-fold) and $p < 0.05$.

### *2.15. Calculations*

Fish growth rate was calculated as follows, based on mean weights:

Specific growth rate (SGR) = $(e^{(\ln W1 - \ln W0)/t} - 1)*100$

Thermal growth coefficient (TGC) = $(W1^{1/3} - W0^{1/3})*1000/d°$

W0 is start weight (g), W1 is final weight (g), t is number of days, and d° is sum day degrees.

Hepatosomatic index (HSI) = liver weight / body weight *100;

Cardiosomatic index (CSI) = heart weight / body weight *100;

### *2.16. Statistical analyses*

In the 16 °C trial study, tanks were used as experimental units and differences in performance were tested by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey-Kramer tests. Before all analyses, the ANOVA assumptions of normality of residuals and homogeneity of variances were tested using the Shapiro-Wilk and Levene tests, respectively. All analyses were performed using NCSS 11.

In the 12 °C trial temperature trial, tanks were used as experimental units and the chosen level of significance was $p < 0.05$. Changes in growth, FA compositions, and enzyme activities were analysed by one-way ANOVA and Duncan's multiple range test. The mRNA transcript abundance of metabolic relevant genes in the intestine was analysed by one-way ANOVA followed by the Tukey's honest significant difference post hoc test to detect differences within dietary groups. These statistical analyses were conducted using the software SAS (SAS Institute Inc., Cary, NC, 1989-2007).

Sterol levels in fish livers were analyzed by nested ANOVA, with tank as random factor and diet as fixed factor, conducted in Statistica (version 13.1, Statsoft, Tulsa, OK, USA). For statistical analyses of the metabolomics/lipidomics data, any missing values were assumed to be below the limits of detection and these values were imputed with the compound minimum (minimum value imputation). While the lipidomics data are quantitative, the other metabolites are given in relative quantities, and for these the raw data for each biochemical was re-scaled to have a median of 1. Statistical analysis of log-transformed data was performed using R (http://cran.r-project.org/), which is a freely available, open-source software package. A two-way ANOVA with contrasts was used to identify statistically significant ($p < 0.05$) effects of oil source (DHA-CA vs FO), effects of inclusion level (high vs low), and interaction between oil source and level. Multiple comparisons were accounted for by estimating the false discovery rate (q-value < 0.10). In the figures, the lipid metabolites and enzyme activities are displayed in Tukey boxplots, showing median, upper and lower quartiles, maximum and minimum of distributions, while outlier data points are indicated by a triangle.

## 3. Results

### *3.1.Characterization of DHA-CA and Ctr-CA*

There were major differences in the FA composition of the dietary oils DHA-CA vs Ctr-CA, with lower levels of 18:1n-9 (39% vs 64%) and 18:2n-6 (9% vs 13%) and higher levels of 18:3n-3 (21% vs 13%) and 22:6n-3 (8.2% vs 0%) occurring in the DHA-CA than in the Ctr-CA, respectively (Table 3). The DHA-CA also contained 0.4% 20:5n-3 and 0.8% 22:5n-3. In addition, the DHA-CA had a higher level of total vitamin E forms compared with the Ctr-CA, which was primarily due to an increased level of gamma-tocopherol, with values of 325 mg $kg^{-1}$ in the DHA-CA compared to 282 mg $kg^{-1}$ in the Ctr-CA.

A range of undesirables were analyzed in the two canola oils and of the 119 organophosphate pesticides, the 30 chlorinated pesticides, and heavy metals, all were below limit of quantification (LOQ) in both oils (data not shown). Only compounds often detected in commercial oils above the LOQ are shown in Table 3. In general, the undesirables normally detected in canola oils used in commercial fish feeds were not detected or were below LOQ in the two canola oils (DHA-CA and Ctr-CA) used in the current feeding trials. The profile of individual phytosterols differed, with a lower proportion of brassicasterol occurring in the phytosterols in the DHA-CA than in Ctr-CA.

The total content of vitamin K was higher in the DHA-CA with 3.6 mg kg-1, compared to 1.6 mg kg-1 in the Ctr-CA. In the oils, 0.0-0.1% of total vitamin K was K3, while the remainder was K1 (~95% trans-K1 and ~5% cis-K1).

### *3.2.Composition of fish feeds*

Table 4 shows the FA composition of the diets used in the two temperature trials. When exchanging FO with DHA-CA, the typical plant FA 18:1n-9 and 18:2n-6 remained relatively similar between the comparable diet groups, but the total saturated fatty acids (SFA), was reduced compared to the level of these FA in the corresponding FO diets, while the levels of 18:3n-3 increased. The sum of EPA+DHA was similar in the two comparable diets (Low FO vs Low DHA-CA and High FO vs High DHA-CA), but due to the fact that the DHA-CA oil mainly contains DHA and less EPA, the DHA-CA diets contained slightly more DHA and less EPA than the corresponding FO diets. There were some differences between the diets for the two temperature trials; in general the EPA+DHA and SFA were higher and the

monounsaturated fatty acids (MUFA) and n-6 PUFA were lower in the diets for the 16 °C trial compared to the level of these in the diets for the 12 °C trial due to different sources of the ingredients FM and FO.

### *3.3.Fish performance and whole-body composition*

The fish at 16 °C grew from an initial weight of 0.8 g to a final weight of approximately 15.5 g, while fish in the 12 °C trial grew from 2 g to 24 g, resulting in an ≈20-fold increase in weight during the course of the trial at 16 °C and an ≈12-fold increase at 12 °C (Table 5). The specific growth rate at 16 °C was 40% higher than at 12 °C. There was no difference in SGR or TGC between the dietary groups within each temperature trial. Survival was very high (≥ 94%) in all groups.

There were also no differences in proximate whole-body composition (protein, dry matter, and ash) and energy content of fish in the different experimental groups in either of the experiments (Table 6). However, when salmon were reared at 16 °C, the whole-body lipid content was significantly increased in fish fed diets containing the DHA-CA oil compared to those fed the FO-diets.

FA profiles in the whole body of fish from the two temperature experiments are presented in Table 7. In general, for both trials, the percentages of SFA were lower and the total n-3 FA were higher in both the DHA-CA groups than in the FO groups, reflecting the high 18:3n-3 content and the lower SFA contents of these diets. The two Low dietary groups (Low FO vs Low DHA-CA) and the two High dietary groups (High FO vs High DHA-CA) had similar percentages of EPA+DHA in whole body in both trials, although the actual level was higher in the low temperature trial due to the higher level occurring in their diets.

### *3.4. Fat content and fatty acid compositions of erythrocytes and muscle in fish in the 12 °C trial.*

There were no significant differences in total lipid content in muscle between the dietary groups, with the lipid content (% of wet weight) of approximately 5% in all dietary groups (Table 8). The total FA composition of the muscle showed only minor differences in the percentage of SFA (≈21% of total FA) between the groups. The percentages of MUFA and

total n-6 FA were mostly affected by the inclusion level of the oils and only moderately by the oil source. Thus, slightly lower percentages of MUFA (≈41% vs 43%) and of n-6 FA (≈5.4% vs 6.0%) were observed in the muscle from the High groups compared to those in the Low groups. The opposite result was found for the content of total n-3 FA, where the two High dietary groups had the highest percentages of n-3 FA (≈29% vs 27%). The percentage of EPA was lower in the muscle of the two groups fed the DHA-CA diets compared to the FO diets, while 18:3n-3 and DHA were higher. The two Low dietary groups (Low FO vs Low DHA-CA) had ≈22% and the two High dietary groups (High FO vs High DHA-CA) had ≈24% and 22% respectively of EPA+DHA in muscle, showing that the EPA+DHA from the DHA-CA oil was incorporated into muscle tissue to approximately the same extent as from FO.

Total lipid content of erythrocytes was similar in all dietary groups (Table 9). The percentages of SFA were in general higher in the FO groups than in the two DHA-CA groups, while the MUFA and total n-6 FA were similar in all diet groups. The total n-3 FA were lower in the FO groups relative to their corresponding DHA-CA groups (Low FO vs Low DHA-CA and High FO vs High DHA-CA). The increase in total n-3 FA was due to the higher content of 18:3n-3 in the erythrocytes from fish fed the DHA-CA diets. Erythrocytes EPA content was higher in the FO groups than the DHA-CA groups due to a higher content of EPA in the FO diets. However, there was no significant difference in DHA content in erythrocytes of the different groups.

### 3.5. *Liver responses in the fish reared at 12 °C: liver lipids, metabolomics, markers for oxidative stress, and transcriptome analysis*

Liver lipids were analyzed both by traditional methods for total lipid, total FA profile, and sterol content (Table 10), as well as by lipidomic analysis, the later providing quantitation of the total triacylglycerol (TAG) (Figure 1) and determination of how the dietary FA are influencing the FA composition of different polar and neutral lipid classes (Figure 2). Table 10 shows that there were no significant differences in total lipid content in liver between the dietary groups. The lipid content (% of wet weight) was 5.8% in the High DHA-CA and approximately 6.3% in the other dietary groups. The levels of TAG were affected by the inclusion level of the oils, but not by the oil source. Total TAG ($p = 0.01$, Figure 1) was significantly lower in High FO and High DHA-CA compared to Low FO and Low DHA-CA.

The total FA composition of the liver (Table 10) showed no difference in the percentage of SFA (25% of total FA) between the dietary groups. The percentages of MUFA and total n-6 FA showed slightly lower percentages of MUFA (≈27% vs 29%) and of n-6 FA (≈5.6% vs 6.5%) in the High groups compared to the Low groups. The opposite result was found regarding the content of total n-3 FA, where the two "High" diets had the highest percentages of n-3 FA (≈41% vs 37%). The percentage of EPA was lower in the liver of the two groups fed the DHA-CA diets compared to the FO diets, while 18:3n-3 and DHA were higher. The two Low dietary groups (Low FO vs Low DHA-CA, ≈34%) and the two High dietary groups (High FO vs High DHA-CA, ≈37%) had similar percentages of EPA+DHA in liver.

Lipidomic analyses did not separate between families of FA, but the FA compositions of different polar and neutral lipid classes in the liver were to different degrees affected by the dietary oils (Figure 2). The most pronounced effects were seen on the incorporation of 18:3 FA, with a 2.4-2.9-fold increase in High DHA-CA vs High FO in most lipid classes. The proportion of 20:5 FA was decreased in most polar lipids in the liver of fish fed High DHA-CA vs High FO, with differences from 1.2- to 1.7-fold. 20:4 FA increased in phosphatidyl choline (PC) (fold difference 1.3, *p-value 0.003*), and also in cholesterol ester (CE) and free fatty acids (FFA) in fish fed High DHA-CA vs High FO. 22:6 FA was significantly enriched in phosphatidylinositol (PI) of fish fed the High DHA-CA vs High FO, and also in CE (both 1.2-fold difference, p=0.02). Liver cholesterol was similar in all diet groups, while the DHA-CA dietary groups had increased liver content of phytosterols, primarily campesterol and sitostanol (Table 10).

There were no significant differences in the activities of the key enzymes, catalase, GPX, and SOD associated with oxidative stress between the dietary treatments (Figure 3). S-Adenosyl-L-methionine (SAMe) is also one of the major physiologic protectors against oxidative stress. For S-adenosylmethionine (SAM), there was both a diet effect (DHA-CA fed fish livers had higher marker levels than FO fed fish livers, *p=0.005, q=0.02*) and an effect of level (the High inclusion level groups had higher than the Low inclusion groups, *p=0.006, q=0.05*). Reduced glutathione (GSH) had a clear effect due to the oil inclusion level, with higher metabolite levels at Low inclusion compared to High inclusion (*p=0.003, q=0.04*), irrespective of oil source. The oxidized glutathione was constant (not shown). As oxidized glutathione was constant, the ratio of oxidized to reduced glutathione was increased at the high inclusion levels.

The gene expression profiles in livers of salmon fed High DHA-CA and High FO were nearly identical. Salmon fed Low FO and Low DHA-CA diets showed highly consistent stimulation of genes in lipid and cholesterol metabolism relative to the High groups. Twenty-two up-regulated DEG encoded 17 proteins with key roles in lipid and cholesterol metabolism (Table 11). Eighteen genes encode 12 enzymes of terpenoid backbone and steroid biosynthesis (diphosphomevalonate decarboxylase, farnesyl diphosphate synthase, 7-dehydrocholesterol reductase, isopentenyl-diphosphate delta-isomerase, lanosterol 14-alpha demethylase, lanosterol synthase, mevalonate kinase, squalene monooxygenase, squalene synthase, stearoyl-CoA desaturase b, sterol-4-alpha-carboxylate 3-dehydrogenase, and sterol-C5-desaturase). Fatty acid-binding protein is required for transport of highly lipophilic compounds, while insulin-induced gene 1 and lipin control steroid and fatty acid metabolism at different levels. The expression differences of genes involved in lipid and steroid metabolism were related to the levels of n-3 LC-PUFA in the diets, but not to the source of these FA as shown in Table 11. The microarray analysis revealed no significant differences in transcription of genes associated with stress, apoptosis, inflammation, xenobiotic metabolism, or any other processes that might indicate adverse effects of the diets (data not shown).

*3.6.Gene expression and histological analyses of the intestine.*

The histological analyses of intestinal morphology revealed no differences between the dietary groups (data not shown). Gene expression analysis of intestine tissue did not show any significant differences in expression of markers for fatty acid oxidation (*aco, cpt1*), fatty acid synthesis (*fas, scd1, scd2*), stress (*hsp70*), inflammation (*cox2*), or oxidative stress (*nrf2, cat, sod1s, gpx2*) between FO and DHA-CA for either the Low or the High groups. The High DHA-CA had a small, but significantly higher expression level of the apoptosis marker *bax* compared to the High FO group (Supplementary Table S2).

## 4. Discussion

The major overall aim of the two fish feeding trials was to rigorously investigate the suitability and possible fish health safety risk associated with the use of oil from a newly developed genetically modified canola crop (DHA-CA) as an omega-3 FA source in feed for Atlantic salmon. The experiments used different fish populations, water temperatures and

sources of raw ingredients to provide a robust assessment of DHA-CA in varying experimental conditions. Very small fingerlings were chosen as the target life stage as they undergo extremely rapid growth and are highly responsive to dietary changes due to their heightened sensitivity.

*Influence of DHA-CA on culture performance*

The experimental fish showed a 12- and 20-fold increase in body weights at 12 °C and 16 °C, respectively, over the course of both experiments. The SGR was higher in the high temperature experiment, with Australian strains adapted to warm water conditions. TGC indicated both cohorts grew similarly well at their respective temperatures. The DHA-CA groups, relative to the corresponding FO groups, grew equally well with high survival rates (≥94%) achieved in both experiments.

*Influence of DHA-CA on salmon health and safety*

Intestine and liver are particularly sensitive organs to harmful effects. The expression of genes associated with stress or toxicity, or enzyme activities related to oxidative stress, were not affected in fish fed DHA-CA. The moderate effects observed in these organs were primarily due to the inclusion level of the oil in the diet and not to the oil source, results which further strengthen the fact that our trial could not reveal any major fish health risk associated with inclusion of DHA-CA in salmon diets. This is in agreement with studies showing that both a high-EPA oil and an EPA+DHA oil from transgenic *Camelina sativa* included in feeds for post-smolt Atlantic salmon had no detrimental effects on fish performance, metabolic responses or the nutritional quality of fillet [17; 33].

The analysed composition of the DHA-CA oil in our trial compared to a conventional canola oil showed that it contained very low levels of undesirables, with levels measured far below LOQ (limit of quantification) of pesticides, mycotoxins, heavy metals, and PAH. Further, DHA-CA also had lower levels of the typical plant oil FA - 18:1n-9 and 18:2 n-6 – compared with a conventional canola oil and higher levels of ɣ-tocopherol and the health-beneficial n-3 FA - 18:3n-3 and DHA. Overall, these results show that the DHA-CA oil has a nutritionally beneficial composition compared to conventional canola oils in line with other n-3 rich oils derived from newly developed transgenic plant crops as reviewed by Tocher *et al.* [34]. The healthy profile of the DHA-CA oil investigated in the present study, can explain that no

negative effects on fish performance and health were found, even at high inclusion levels. The vitamin E (particularly ɣ-tocopherol) differed between DHA-CA and Ctr-CA, however, both oils were well within the normal range given for the different vitamin E isomers in commercial canola oil [35].

*Influence of DHA-CA on composition of whole body, muscle, and erythrocytes*

The study showed no major differences in total lipid, protein, or energy content of the whole body of fish in the various dietary groups, while the FA compositions were affected. Generally, for both temperature trials, lower percentages of both SFA and MUFA and higher percentages of 18:3n-3 and DHA were found in whole body of DHA-CA groups compared to the FO groups, reflecting the FA compositions of the diets. The level of EPA was moderately reduced with increased dietary inclusion of DHA-CA in the diet, due to the lower levels of this FA in DHA-CA oil. Overall, the results showed that the comparable Low FO vs Low DHA-CA and the High FO vs High DHA-CA diets resulted in equal percentages of EPA+DHA in the whole body, showing that the fish could equally utilise the EPA and DHA from traditional FO and from the DHA-CA oil. This is also in agreement with what has been observed with the EPA rich oil and the EPA+DHA rich oil from *Camelina sativa* [33; 36].

An additional benefit of DHA-CA compared to conventional canola oil is the lower levels of n-6 FA, which results in lower body levels of n-6 FA when included in the diet. The n-6 FA are linked to pro-inflammatory pathways and generally tend to bio-accumulate with the use of terrestrial plant oils to replace fish oil [37]. The muscle tissue showed similar trends to the fatty acid profiles of those of the whole body, except for lower relative percentages of MUFA and higher percentages of n-3 PUFA. This indicates the fish fillet FA profile is potentially of greater nutritional benefit to human nutrition as a result feeding DHA-CA rather than a more conventional mixture of canola oil and fish oil.

The different dietary groups had only minor differences in their FA composition of red blood cells, showing that the erythrocytes are less affected by the dietary FA than whole body and muscle. There were no significant differences in the EPA+DHA content or total n-6 FA content between any of the dietary groups in red blood cells, the most marked effect was increased 18:3n-3 and reduced 18:2 n-6 with increase in dietary DHA-CA level in the diet.

*Response of liver to DHA-CA*

Previous data have shown that genetic modification of canola can give large unintended effects on phytosterol composition (both increase and decrease) [38]. Results from our study show that dietary DHA-CA oil results in a 10-30% higher level of phytosterols in liver than occurs in the comparable FO-fed groups. However, a recent feeding trial with Atlantic salmon fed diets with added different phytosterol levels, showed no negative effect of phytosterols up to 3000 mg $kg^{-1}$ feed [25; 39]. In our study, the highest dietary level with the highest dose of DHA-CA was 739 $kg^{-1}$ feed, showing that no negative effects due to increased phytosterol in diet or in the liver would be expected. There was a moderately lower level of cholesterol in the diets containing the DHA-CA than in the two FO diets, however, the gene expression results from livers showed that a relatively large number of genes in the sterol biosynthesis, among them several genes with important roles in cholesterol synthesis, including lanosterol synthase, squalene synthase, and sterol-4-αcarboxylate-3 dehydrogenase, were up-regulated in both the Low FO and Low DHA-CA groups compared to the two High groups, indicating that it is not the cholesterol level in the diet that effects the expression of these genes in liver in our study, rather the n-3- LC PUFA levels. This is contradictory to studies showing that the cholesterol synthesis in Atlantic salmon is regulated by the dietary content of cholesterol, i.e. high dietary levels able to inhibit and low dietary levels able to stimulate cholesterol synthesis [40]. However, other factors may also influence cholesterol metabolism. In line with our findings, previous mammalian studies have reported that EPA and DHA have hypo-cholesterolemic effects by reducing hepatic cholesterol synthesis [41].

The FA that varied the most in the liver total lipids between the four experimental dietary groups was 18:3n-3 (from 0.8-12.4% of total FA), and this was also reflected in the different lipid classes, with 18:3 enriched in most liver lipid classes in fish fed the DHA-CA dietary groups compared to the FO groups. While EPA+DHA was well balanced between DHA-CA diets and their corresponding FO control groups, the DHA/EPA ratio was higher in the DHA-CA dietary groups. Hence, 20:5 FA decreased in the cell membrane PLs phosphatidyl choline (PC), phosphatidylethanolamine (PE), lyso phosphatidyl choline (LPC), cholesterol ester (CE) and TAG in the livers of fish fed the DHA-CA diets. DHA increased primarily in CE and PI. In the current diets, formulated for small fish, a high proportion of LC-PUFA was also provided from the fish meal fraction rather than the oils, resulting in fairly high EPA levels in

all feeds above requirements. The FA 20:3 increased in most lipid classes. Although the lipid class analyses do not separate between FA families, this is probably the elongation product from 18:3n-3 to 20:3n-3, since this FA also increases in the total lipid fraction.

There were no significant differences in the total lipid level in the liver, while total TAG was moderately lower in both the high inclusion level of LC-PUFA irrespective of source. This is opposite to earlier findings with EPA rich oil from *Camelina sativa* in salmon diets which resulted in a moderate increase in liver TAG [33]. However several studies have shown that a FO rich in EPA and DHA results in decreased TAG level in liver when compared to a diet rich in plant oil, in line with our findings here [5; 42]. A factor could be 18:1n-9, which was higher in the two feeds with low oil levels (Low FO and Low DHA-CA) compared to the two feeds with high oil levels (High FO and High DHA-CA) and has in previous trials/cell trials been implicated in liver lipid accumulation [43]. These data indicate a positive effect on fish liver health and metabolism of a high inclusion level of LC-PUFA, irrespective of whether this source is FO or DHA-CA.

## Conclusions

Our results show that the newly developed modified canola oil (DHA-CA) has a beneficial FA profile compared to conventional plant oils, with particularly high levels of the n-3 FAs - 18:3 n-3 and DHA. Levels of phytosterols, vitamin E, and minerals in the n-3 modified canola oil fall within the natural variation of commercial canola oils. Levels of undesirable compounds were undetectable.

Two feed trials have been carried out with salmon in fresh water, the purpose of which has been to evaluate the suitability and safety associated with the use of n-3 rich modified canola oil on fish performance, composition, and health at two water temperatures. The fish increased their weight approx. 20-fold at 16 °C and 12-fold at 12 ° C. Both experiments showed high survival across all dietary groups and corresponding growth in salmon fed with a FO diet to salmon fed n-3 modified canola oil. The salmon fed the n-3 modified canola oil had the same EPA+DHA content and proportional (%) level in the whole body as salmon fed FO. The level of oil in the experimental feeds was the main factor affecting differential expression of genes and not the source of the oil. The level of oil in the feeds was also the determining factor for the measured health biomarkers in liver, intestine, and muscle and not

the source of the oil. Based on the current studies it is concluded that the n-3 rich modified canola oil (DHA-CA) is as safe as other commercial canola oils and is suitable as an alternative oil source in salmon diets.

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**Conflict of interest**

None.

**Authorship**

Bente Ruyter has as project leader, contributed to the formulation of the research questions, design of the study and writing of article. Nini H. Sissener and Monica Sanden contributed to the design of the study, analyses of environmental pollutants, nutrients and lipidomic analyses and writing of article. Cedric J. Simon and Peter D. Nichols contributed to design and analyses of the 16°C water temperature trial and writing of article. Aleksei Krasnov and Esmail Lutfi contributed with the gene expression analyses and writing of article. Gerd M.

Berge, Tone-Kari Østbye and Marta Bou contributed with design and analyses of the 12°C water temperature trial and writing of article.

**Compliance with Ethical Standards:**

The cultivation and production of DHA-CA oil was conducted with approval from the CSIRO Animal Ethics Committee, approval number A6/2016. Approved methodology was in accordance with the 'Australia Code for the care and Use of Animals for Scientific Purposes' 8th edition, and all associated legislation and regulations regarding the welfare of animals.

The feeding trial with Atlantic salmon (project FOTS ID 8378) was conducted in compliance with the Norwegian national regulation for use of experimental animals (FOR-2015-06-18-761) and classified as not requiring a specific license (§2-f, corresponding to Directive 2010/63/EU Article 1, section 5f), since the experimental treatments were not expected to cause any distress or discomfort for the fish (FOTS decision ref. nr. 8378).

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**Figure legends**

Figure 1. Total triacylglycerol (TAG) in liver from salmon fed the four different diets (n = 9 per diet group). TAG is given as nmol/g liver tissue, and the figure is a Tukey boxplot, showing the median, the interquartile range and the min/max, excluding outliers (which is indicated by a triangle). Two-way ANOVA showed significant difference ($p = 0.01$, $q = 0.07$) between the two inclusion levels (High vs Low) of transgenic canola (DHA-CA) in the diet. Between the individual diet groups, there was a significant difference between High FO and Low DHA-CA (indicated by letters in the figure, $p = 0.005$, $q = 0.02$).

Figure 2. Overall differences in composition of polyunsaturated fatty acids in the different lipid classes in fish fed a diet containing high levels of an n-3 rich modified canola oil (High DHA-CA; blue = enriched in this group) versus in fish fed a diet containing high levels of fish oil (High FO; red = enriched in this group). Colour indicates a significant difference ($p < 0.05$) between the two diet groups. The color intensity shows the fold difference (white = not significant, grey = not detected).

Figure 3. Differences between the two inclusion levels (High vs Low) of transgenic canola (DHA-CA) in the diet. Upper panel shows the activities of three enzymes involved in the oxidative stress response in liver, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX). Lower panels show the level of two metabolites related to redox management - SAM and GSH and are given as relative quantities. The graphs are Tukey boxplots, showing the median, the interquartile range and the min/max, excluding outliers (which is indicated by a triangle), n =9 per diet group. For SAM, two-way ANOVA showed significant difference ($p = 0.006$, $q = 0.05$) between the two inclusion levels (High vs Low) of transgenic canola (DHA-CA) in the diet. Between the individual diet groups, there was a significant difference between Low FO and High DHA-CA (indicated by letters in the figure, $p = 0.0002$, $q = 0.004$). Similarly, for GSH, two-way ANOVA showed significant difference ($p = 0.003$, $q = 0.04$) between the two inclusion levels. Between the individual diet groups, there was a significant difference between Low FO and High DHA-CA (indicated by letters in the figure, $p = 0.006$, $q = 0.04$).

Table 1. Formulation and composition of the experimental diets used in the 16 °C water temperature experiment

| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA |
|---|---|---|---|---|
| **Formulation, %** | | | | |
| Fish meal | 79.00 | 79.00 | 79.00 | 79.00 |
| Wheat | 7.00 | 7.00 | 7.00 | 7.00 |
| Mineral mix | 0.59 | 0.59 | 0.59 | 0.59 |
| Vitamin mix | 1.92 | 1.92 | 1.92 | 1.92 |
| Carophyll Pink | 0.01 | 0.01 | 0.01 | 0.01 |
| Choline chloride | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin C | 0.14 | 0.14 | 0.14 | 0.14 |
| Vitamin E (50%) | 0.03 | 0.03 | 0.03 | 0.03 |
| $CaHPO_4$ | 1.00 | 1.00 | 1.00 | 1.00 |
| Betaine | 1.00 | 1.00 | 1.00 | 1.00 |
| Soy lecithin | 1.00 | 1.00 | 1.00 | 1.00 |
| Tuna oil (TO) | 1.6 | 3.2 | - | - |
| Control canola oil | 6.2 | 4.6 | 3.9 | 0.00 |
| DHA-CA | - | - | 3.9 | 7.8 |
| **Origin of EPA+DHA, % in diet** | | | | |
| Fish meal (FM) | 1.90 | 1.90 | 1.90 | 1.90 |
| TO | 0.47 | 0.95 | - | - |
| DHA-CA | - | - | 0.47 | 0.94 |
| **Chemical composition:** | | | | |
| Dry matter | 94.2 | 94.2 | 93.1 | 94.0 |
| Fat | 16.2 | 16.2 | 16.7 | 17.0 |
| Protein | 58.7 | 59.6 | 60.0 | 58.7 |
| Ash | 11.7 | 11.6 | 11.7 | 11.8 |
| Gross energy (MJ $kg^{-1}$) | 21.4 | 21.5 | 21.6 | 21.5 |

All ingredients were sourced from an Australian provider. The fishmeal used was commercial fishmeal from South America (Peru & Chile) provided by Ridley Aquafeeds. The source of fish oil was tuna oil in order to more closely match the balance of EPA:DHA in control and test feeds. Feed pellets (three class sizes; 0.5-1.0 mm, 1.0-1.4 mm, and 1.4-2.0 mm) were prepared at the CSIRO Bribie Island Research Centre. QLD. The composition is an average of all three pellet sizes.

Table 2. Formulation and composition of the experimental diets used in the 12 °C water temperature experiment

| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA |
|---|---|---|---|---|
| **Formulation, %** | | | | |
| Fish meal [1] | 79.00 | 79.00 | 79.00 | 79.00 |
| Wheat [2] | 7.50 | 7.50 | 7.50 | 7.50 |
| Mineral mix [3] | 0.52 | 0.52 | 0.52 | 0.52 |
| Vitamin mix [4] | 2.00 | 2.00 | 2.00 | 2.00 |
| Monoammonium phosphate [5] | 1.00 | 1.00 | 1.00 | 1.00 |
| Carophyll Pink [6] | 0.01 | 0.01 | 0.01 | 0.01 |
| Vitamin D3. IU/g [7] | 2.0 | 2.0 | 2.0 | 2.0 |
| Vitamin E [8] | 0.03 | 0.03 | 0.03 | 0.03 |
| Vitamin C [9] | 0.14 | 0.14 | 0.14 | 0.14 |
| Betafin [10] | 1.00 | 1.00 | 1.00 | 1.00 |
| Soy lecithin [11] | 1.00 | 1.00 | 1.00 | 1.00 |
| Fish oil [12] | 1.72 | 3.41 | 0.00 | 0.00 |
| Control canola oil [13] | 6.08 | 4.39 | 3.88 | 0.00 |
| DHA-CA | 0.00 | 0.00 | 3.92 | 7.79 |
| **Origin of EPA+DHA, % in diet** | | | | |
| Fish meal | 1.62 | 1.62 | 1.62 | 1.62 |
| Fish oil | 0.32 | 0.63 | 0.00 | 0.00 |
| DHA-CA | 0.00 | 0.00 | 0.32 | 0.63 |
| **Chemical composition** | | | | |
| Dry matter | 93.9 | 93.7 | 93.8 | 93.7 |
| Fat | 20.9 | 20.6 | 20.9 | 20.7 |
| Protein | 56.8 | 56.3 | 56.4 | 56.6 |
| Ash | 13.4 | 13.3 | 13.3 | 13.2 |
| Gross energy (MJ $kg^{-1}$) | 20.9 | 21.0 | 21.0 | 21.0 |
| **Sterol composition** | | | | |
| Cholesterol (mg $kg^{-1}$) | 4227 | 4469 | 4028 | 3950 |
| Sum phytosterols (mg $kg^{-1}$) | 497 | 378 | 671 | 739 |
| Campesterol (% of sum phyto.) | 32.8 | 31.0 | 33.7 | 35.4 |
| β-sitosterol (% of sum phyto.) | 48.6 | 48.6 | 48.1 | 45.5 |
| Brassicasterol (% of sum phyto.) | 3.3 | 4.4 | 2.3 | 2.0 |
| **Vitamin K isomers (mg $kg^{-1}$)** | | | | |
| Phylloquinone (vit $K_1$) | 0.05 | 0.04 | 0.10 | 0.14 |
| Menaquinone (vit $K_2$) | 0.07 | 0.07 | 0.06 | 0.06 |
| Menadione ($K_3$) | 5.91 | 5.79 | 5.84 | 5.94 |

[1]Nordsildmel.
[2]Norgesmøllene.
[3]Individual minerals purchased from Vilomix and mixed by Nofima.
[4-10] Vilomix.
[11]Denofa.
[12]Nordsildmel.
[13](Australia. var. Garnet)

Table 3. Selected undesirables, phytosterols, vitamin E isomers, and fatty acids in the n-3 rich modified canola oil (DHA-CA) and the control canola oil (Ctr-CA).

| | DHA-CA | Ctr-CA |
|---|---|---|
| **Heavy metals (mg kg$^{-1}$)** | | |
| Total arsenic | <0.009 | <0.009 |
| **Polyaromatic hydrocarbons (µg kg$^{-1}$)** | | |
| Benzo(a)antracene | <0.5 | <0.5 |
| Benzo(a)pyrene | <0.5 | <0.5 |
| Benzo(b)fluoranthene | <0.5 | <0.5 |
| Chrysene | <0.5 | <0.5 |
| **Organophsophate pesticides (µg kg$^{-1}$)** | | |
| Pirimiphos-methyl | <20 | <20 |
| **Lipid-soluble mycotoxins (µg kg$^{-1}$)** | | |
| Enniatin B | <10 | <10 |
| **Phytosterols** | | |
| Sum phytosterols (mg kg$^{-1}$) | 9659 | 7878 |
| Campesterol (% of sum) | 39.6 | 34.2 |
| β-sitosterol (% of sum) | 43.0 | 44.5 |
| Brassicasterol (% of sum) | 3.5 | 12.2 |
| **Vitamin E isomers (mg kg$^{-1}$)** | | |
| Alfa-tocopherol | 200 | 188 |
| Gamma-tocopherol | 330 | 283 |
| Delta-tocopherol | 6.7 | 6.9 |
| Beta-tocotrienol | 95 | 71 |
| **Vitamin K isomers (mg kg$^{-1}$)** | | |
| Phylloquinone (vit $K_1$) | 3.62 | 1.61 |
| Menaquinone (vit $K_2$) | 0.00 | 0.00 |
| Menadione ($K_3$) | 0.00 | 0.00 |
| **Fatty acid composition (%)** | | |
| 16:0 | 4.6 | 3.9 |
| 18:0 | 1.9 | 1.6 |
| Σ SFA[1] | 7.4 | 6.7 |
| 18:1n-9 | 38.7 | 63.6 |
| 18:1n-7 | 5.6 | 3.5 |
| Σ MUFA[2] | 49.3 | 69.0 |
| 18:2n-6 | 9.0 | 13.1 |
| 18:3n-3 | 20.9 | 10.3 |
| 20:5n-3 | 0.4 | 0.1 |
| 22:5n-3 | 0.8 | nd |
| 22:6n-3 | 8.2 | nd |
| Σ PUFA[3] | 41.1 | 23.9 |
| Σ n-6 | 10.0 | 13.3 |
| Σ n-3 | 30.6 | 10.4 |

nd, not detectable levels.
[1]Includes 14:0, 17:0, 20:0, 22:0, and 24:0.
[2]Includes 16:1n-9, 20:1n-9, 20:1n-11, 22:1n-7, and 24:1n-9.
[3]Includes 16:2n-3, 16:2n-6, 18:3n-6, 20:2n-6, 20:3n-3, 20:3n-6, and 22:2n-6.

Table 4. Fatty acid composition (% of total fatty acids) of the experimental feeds

| | 16 °C WATER TEMPERATURE TRIAL | | | | 12 °C WATER TEMPERATURE TRIAL | | | |
|---|---|---|---|---|---|---|---|---|
| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA |
| 14:0 | 4.1 | 4.5 | 3.7 | 3.7 | 3.7 | 4.3 | 3.1 | 3.2 |
| 16:0 | 16.4 | 18.4 | 14.8 | 15.0 | 11.2 | 12.2 | 10.4 | 10.6 |
| 18:0 | 3.4 | 3.9 | 3.2 | 3.3 | 1.9 | 2.1 | 2.0 | 2.0 |
| 20:0 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 | 0.4 | 0.4 |
| **Σ SFA[1]** | **27.2** | **30.5** | **23.9** | **23.5** | **17.7** | **19.7** | **16.6** | **16.8** |
| 16:1n-7 | 4.3 | 4.8 | 3.8 | 3.9 | 2.6 | 3.1 | 2.0 | 2.1 |
| 18:1n-7 | 3.0 | 3.0 | 3.0 | 3.0 | 2.7 | 2.5 | 3.0 | 3.2 |
| 18:1n-9 | 34.2 | 28.8 | 32.9 | 26.7 | 29.8 | 23.6 | 29.7 | 24.2 |
| 20:1n-9 | 1.6 | 1.6 | 1.6 | 1.6 | 5.8 | 6.3 | 5.0 | 5.1 |
| 22:1n-11 | nd | nd | nd | nd | 9.2 | 10.3 | 7.7 | 7.8 |
| **Σ MUFA[2]** | **44.0** | **39.0** | **42.1** | **35.9** | **52.2** | **49.1** | **49.8** | **44.8** |
| 18:2n-6 | 7.7 | 6.7 | 7.8 | 7 | 6.5 | 5.2 | 6.6 | 5.7 |
| 20:4n-6 | 0.5 | 0.6 | 0.3 | 0.3 | 0.3 | 0.4 | 0.3 | 0.3 |
| **Σ n-6[3]** | **8.5** | **8.0** | **8.5** | **8.0** | **7.1** | **5.9** | **7.4** | **6.6** |
| 18:3n-3 | 4.0 | 3.3 | 7.8 | 10.9 | 4.4 | 3.4 | 7.8 | 10.2 |
| 18:4n-3 | 1.1 | 1.2 | 1.6 | 2.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| 20:4n-3 | 0.3 | 0.3 | 0.5 | 0.9 | 1.4 | 1.8 | 1.4 | 1.8 |
| 20:5n-3 | 7.0 | 7.5 | 6.7 | 6.8 | 5.0 | 6.2 | 3.9 | 4.0 |
| 22:5n-3 | 0.2 | 0.2 | 1.0 | 1.7 | 0.6 | 0.7 | 0.7 | 0.8 |
| 22:6n-3 | 7.5 | 9.9 | 7.4 | 9.6 | 8.1 | 8.7 | 9.0 | 11.0 |
| **Σ n-3[4]** | **22.4** | **25.0** | **27.7** | **34.8** | **19.7** | **20.9** | **23.2** | **28.4** |
| **EPA+DHA** | **17.0** | **20.5** | **17.0** | **19.4** | **13.1** | **14.9** | **13.0** | **15.0** |

nd, not determined

[1]Includes 15:0, 17:0, 19:0, 22:0, and 24:0.

[2]Includes 14:1n-5, 16:1n-5, 17:1-n7, 18:1n-11, 22:1n-7, 22:1n-9, and 24:1n-9.

[3]Includes 16:2n-6, 18:3n-6, 22:4n-6, and 22:5n-6.[4]Includes 16:2n-3 and 20:3n-3.

Table 5. Growth and survival over the experimental period. Data are shown as mean values using tank as a statistical unit (n = 3) with their standard errors.

| | 16 °C WATER TEMPERATURE TRIAL | | | | | 12 °C WATER TEMPERATURE TRIAL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value |
| **Body weight, g** | | | | | | | | | | |
| Day 0 | 0.83 ± 0.01 | 0.84 ± 0.02 | 0.86 ± 0.03 | 0.83 ± 0.02 | 0.74 | 2.04 ± 0.09 | 2.16 ± 0.04 | 2.08 ± 0.04 | 2.05 ± 0.01 | 0.46 |
| Period 1 | 4.63 ± 0.12 | 4.49 ± 0.06 | 4.49 ± 0.14 | 4.44±0.13 | 0.68 | 4.47 ± 0.19 | 4.79 ± 0.07 | 4.52 ± 0.10 | 4.38 ± 0.03 | 0.14 |
| Period 2 | 10.35 ± 0.08 | 10.14 ± 0.28 | 10.17 ± 0.09 | 9.94 ± 0.20 | 0.48 | 6.82 ± 0.29 | 7.33 ± 0.10 | 6.81 ± 0.16 | 6.67 ± 0.03 | 0.11 |
| Period 3 | 15.49 ± 0.47 | 15.29 ± 0.31 | 16.22 ± 0.77 | 15.25 ± 0.22 | 0.47 | 23.82 ± 0.94 | 25.41 ± 0.80 | 23.28 ± 0.62 | 23.74 ± 1.02 | 0.38 |
| **SGR (% $d^{-1}$)** | | | | | | | | | | |
| Period 1 | 5.04 ± 0.09 | 4.94 ± 0.02 | 4.90 ± 0.04 | 4.94 ± 0.03 | 0.18 | 2.95 ± 0.03 | 3.01 ± 0.12 | 2.93 ± 0.07 | 2.87 ± 0.03 | 0.64 |
| Period 2 | 3.66 ± 0.08 | 3.72 ± 0.05 | 3.73 ± 0.10 | 3.67 ± 0.06 | 0.90 | 3.05 ± 0.03 | 3.08 ± 0.03 | 2.97 ± 0.07 | 3.03 ± 0.08 | 0.57 |
| Period 3 | 2.87 ± 0.26 | 2.91 ± 0.12 | 3.18 ± 0.20 | 3.06 ± 0.04 | 0.65 | 3.02 ± 0.03 | 3.00 ± 0.05 | 2.97 ± 0.01 | 3.07 ± 0.11 | 0.73 |
| SGR total | 4.17 ± 0.04 | 4.20 ± 0.02 | 4.20 ± 0.02 | 4.16 ± 0.02 | 0.72 | 3.01 ± 0.01 | 3.02 ± 0.07 | 2.96 ± 0.02 | 3.00 ± 0.04 | 0.76 |
| TGC total | 1.36 ± 0.02 | 1.35 ± 0.01 | 1.38 ± 0.027 | 1.35 ± 0.00 | 0.73 | 1.59 ± 0.02 | 1.63 ± 0.04 | 1.57 ± 0.02 | 1.59 ± 0.04 | 0.50 |
| Survival (%) | 99.7 ± 0.40 | 99.0 ± 0.40 | 98.0 ± 0.49 | 99.3 ± 0.40 | 0.14 | 97.2 ± 1.2 | 97.0 ± 1.5 | 96.7 ± 1.4 | 93.7 ± 3.0 | 0.55 |

The period from 0 to 34 days is denoted Period 1, from 34 to 56 days is denoted Period 2, and from day 56 to 70 days is denoted Period 3 in the 16 °C trial. The period from 0 to 27 days is denoted Period 1, from 27 to 41 days is denoted Period 2, and from day 41 to 83 days is denoted Period 3 in the 12 °C trial.

Table 6. Proximate composition and energy content of whole fish at the end of the experiment. Data are shown as mean values using tank as a statistical unit (n = 3, being each sample represented by a pool of five fish) with their standard errors. Different superscript letters indicate statistically significant differences ($p<0.05$).

| | 16 °C WATER TEMPERATURE TRIAL | | | | | 12 °C WATER TEMPERATURE TRIAL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value |
| Dry matter | 25.9 ± 0.55 | 25.8 ±0.66 | 27.3 ± 0.3 | 27.3 ± 0.20 | 0.09 | 27.2 ± 0.36 | 27.6 ±0.15 | 27.1 ± 0.06 | 27.2 ± 0.18 | 0.43 |
| Protein | 15.3 ± 0.36 | 15.6 ± 0.61 | 16.2 ± 0.22 | 15.8 ± 0.17 | 0.6 | 16.1 ± 0.05 | 16.2 ±0.07 | 16.4 ± 0.19 | 16.1 ± 0.07 | 0.16 |
| Lipid | 9.6 ± 0.31$^{a}$ | 9.5 ± 0.13$^{a}$ | 10.6 ± 0.28$^{b}$ | 10.9 ± 0.40$^{b}$ | 0.03 | 10.7 ± 0.53 | 11.0 ± 0.15 | 10.4 ± 0.07 | 10.4 ± 0.09 | 0.44 |
| Ash | 1.6 ± 0.07 | 1.6 ± 0.09 | 1.5 ± 0.08 | 1.6 ± 0.07 | 0.87 | 2.4 ± 0.06 | 2.4 ± 0.06 | 2.4 ± 0.15 | 2.3 ± 0.06 | 0.74 |
| Energy (MJ kg$^{-1}$) | 7.18 ± 0.15 | 7.16 ± 0.19 | 7.56 ± 0.19 | 7.76 ± 0.16 | 0.10 | 7.27 ± 0.12 | 7.52 ± 0.03 | 7.21 ± 0.05 | 7.25 ± 0.05 | 0.06 |

Table 7. Fatty acid composition (% of total) in the whole body of Atlantic salmon fed the experimental diets for 70 days at high (16°C) and low (12°C) water temperature. Data are shown as mean values using tank as a statistical unit (n = 3, being each sample represented by a pool of five fish) with their standard errors. Different superscript letters indicate statistically significant differences ($p<0.05$).

| | 16 °C WATER TEMPERATURE TRIAL | | | | | 12 °C WATER TEMPERATURE TRIAL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value |
| 14:0 | 3.7 ± 0.02$^{a}$ | 4.05 ± 0.03$^{b}$ | 3.4 ± 0.03$^{c}$ | 3.5 ± 0.02$^{c}$ | < .001 | 3.4 ± 0.03$^{b}$ | 3.9 ± 0.09$^{a}$ | 3.0 ± 0.03$^{c}$ | 3.0 ± 0.03$^{c}$ | < .0001 |
| 16:0 | 17.0 ± 0.13$^{a}$ | 18.5 ± 0.17$^{b}$ | 15.8 ± 0.10$^{c}$ | 16.0 ± 0.12$^{c}$ | < .0001 | 13.0 ± 0.03$^{b}$ | 13.7 ± 0.03$^{a}$ | 12.2 ± 0.23$^{c}$ | 12.1 ± 0.03$^{c}$ | < .0001 |
| 18:0 | 4.3 ± 0.05$^{a}$ | 4.7 ± 0.01$^{b}$ | 4.2 ± 0.01$^{a}$ | 4.3 ± 0.05$^{a}$ | < .001 | 2.9 ± 0.03 | 2.9 ± 0.03 | 2.9 ± 0.03 | 3.0 ± 0.03 | 0.44 |
| 20:0 | 0.3 ± 0.00$^{a}$ | 0.3 ± 0.00$^{b}$ | 0.3 ± 0.00$^{a}$ | 0.3 ± 0.00$^{a}$ | < .001 | 0.3 ± 0.00 | 0.3 ± 0.03 | 0.3 ± 0.03 | 0.3 ± 0.00 | 0.33 |
| **Σ SFA[1]** | **26.3 ± 0.18$^{a}$** | **28.8 ± 0.22$^{b}$** | **24.8 ± 0.20$^{c}$** | **25.1 ± 0.13$^{c}$** | **< .0001** | **20.0 ± 0.06$^{b}$** | **21.3 ± 0.13$^{a}$** | **18.9 ± 0.30$^{c}$** | **19.0 ± 0.07$^{c}$** | **< .0001** |
| 16:1n-7 | 4.0 ± 0.05$^{a}$ | 4.5 ± 0.03$^{b}$ | 3.7 ± 0.03$^{c}$ | 3.7 ± 0.01$^{c}$ | < .0001 | 3.2 ± 0.15$^{b}$ | 3.6 ± 0.10$^{a}$ | 2.3 ± 0.09$^{c}$ | 2.2 ± 0.00$^{c}$ | < .0001 |
| 18:1n-7 | 3.4 ± 0.01$^{a}$ | 3.4 ± 0.01$^{a}$ | 3.3 ± 0.02$^{b}$ | 3.3 ± 0.01$^{b}$ | < .01 | 3.7 ± 0.07$^{c}$ | 3.6 ± 0.00$^{c}$ | 4.0 ± 0.03$^{b}$ | 4.3 ± 0.03$^{a}$ | < .0001 |
| 18:1n-9 | 34.9 ± 0.16$^{a}$ | 30.3 ± 0.20$^{b}$ | 34.0 ± 0.07$^{c}$ | 28.2 ± 0.09$^{d}$ | < .0001 | 31.6 ± 0.20$^{a}$ | 26.7 ± 0.20$^{b}$ | 31.3 ± 0.12$^{a}$ | 26.2 ± 0.07$^{b}$ | < .0001 |
| 20:1n-9 | 2.4 ± 0.01$^{a}$ | 2.2 ± 0.03$^{b}$ | 2.3 ± 0.03$^{c}$ | 2.1 ± 0.00$^{d}$ | <.0001 | 6.3 ± 0.06$^{b}$ | 6.8 ± 0.03$^{a}$ | 5.6 ± 0.03$^{c}$ | 5.4 ± 0.00$^{d}$ | < .0001 |
| 22:1n-11 | nd | nd | nd | nd | | 6.1 ± 0.06$^{a}$ | 6.5 ± 0.24$^{a}$ | 4.8 ± 0.24$^{b}$ | 5.3 ± 0.06$^{b}$ | 0.0005 |
| **Σ MUFA[2]** | **45.7 ± 0.17$^{a}$** | **41.4 ± 0.252$^{b}$** | **44.2 ± 0.11$^{c}$** | **38.1 ± 0.09$^{d}$** | **< .0001** | **54.0 ± 0.23$^{a}$** | **51.3 ± 0.26$^{b}$** | **51.9 ± 0.18$^{b}$** | **47.8 ± 0.09$^{c}$** | **< .0001** |
| 18:2n-6 | 6.4 ± 0.05$^{a}$ | 5.7 ± 0.03$^{b}$ | 6.4 ± 0.03$^{a}$ | 5.9 ± 0.07$^{c}$ | < .0001 | 5.4 ± 0.06$^{a}$ | 4.5 ± 0.00$^{c}$ | 5.5 ± 0.10$^{a}$ | 4.9 ± 0.00$^{b}$ | < .0001 |
| 20:4n-6 | 0.5 ± 0.01$^{a}$ | 0.6 ± 0.01$^{b}$ | 0.3 ± 0.01$^{c}$ | 0.3 ± 0.01$^{c}$ | < .0001 | 0.3 ± 0.00 | 0.3 ± 0.00 | 0.3 ± 0.00 | 0.3 ± 0.00 | . |
| **Σ n-6[3]** | **7.8 ± 0.06$^{a}$** | **7.3 ± 0.11$^{b}$** | **7.6 ± 0.03$^{a}$** | **7.1 ± 0.11$^{b}$** | **< .0001** | **6.8 ± 0.03$^{a}$** | **5.9 ± 0.09$^{c}$** | **6.9 ± 0.09$^{a}$** | **6.4 ± 0.03$^{b}$** | **< .0001** |
| 18:3n-3 | 3.1 ± 0.05$^{a}$ | 2.6 ± 0.06$^{b}$ | 5.9 ± 0.02$^{c}$ | 8.5 ± 0.12$^{d}$ | < .0001 | 3.2 ± 0.06$^{c}$ | 2.6 ± 0.00$^{d}$ | 5.7 ± 0.10$^{b}$ | 7.8 ± 0.03$^{a}$ | < .0001 |
| 18:4n-3 | 0.9 ± 0.01$^{a}$ | 0.9 ± 0.01$^{a}$ | 1.3 ± 0.01$^{b}$ | 1.8 ± 0.03$^{c}$ | < .0001 | nd | nd | nd | nd | |
| 20:3n-3 | 0.2 ± 0.00$^{a}$ | 0.2 ± 0.00$^{b}$ | 0.5 ± 0.00$^{c}$ | 0.7 ± 0.00$^{d}$ | < .0001 | 0.2 ± 0.00$^{b}$ | 0.2 ± 0.00$^{b}$ | 0.5 ± 0.03$^{a}$ | 0.6 ± 0.12$^{a}$ | 0.003 |
| 20:4n-3 | 0.5 ± 0.00$^{a}$ | 0.5 ± 0.01$^{a}$ | 0.8 ± 0.00$^{b}$ | 1.2 ± 0.00$^{c}$ | <0.001 | 0.6 ± 0.09$^{b}$ | 0.8 ± 0.01$^{a}$ | 0.6 ± 0.01$^{b}$ | 0.6 ± 0.00$^{b}$ | 0.02 |
| 20:5n-3 | 3.0 ± 0.05$^{a}$ | 3.2 ± 0.03$^{b}$ | 2.9 ± 0.02$^{a}$ | 3.2 ± 0.04$^{c}$ | < .0001 | 2.8 ± 0.00$^{b}$ | 3.5 ± 0.03$^{a}$ | 2.4 ± 0.00$^{c}$ | 2.5 ± 0.03$^{c}$ | < .0001 |
| 22:5n-3 | 1.4 ± 0.00 | 1.5 ± 0.02 | 1.2 ± 0.21 | 1.4 ± 0.23 | 0.55 | 0.9 ± 0.03$^{b}$ | 1.2 ± 0.00$^{a}$ | 0.9 ± 0.03$^{b}$ | 0.9 ± 0.03$^{b}$ | 0.0002 |
| 22:6n-3 | 11.0 ± 0.16$^{a}$ | 13.1 ± 0.15$^{b}$ | 10.7 ± 0.05$^{a}$ | 12.8 ± 0.10$^{b}$ | < .0001 | 9.7 ± 0.19$^{d}$ | 10.8 ± 0.07$^{b}$ | 10.4 ± 0.12$^{c}$ | 12.2 ± 0.03$^{a}$ | < .0001 |
| **Σ n-3** | **20.5 ± 0.26$^{a}$** | **22.6 ± 0.22$^{b}$** | **23.7 ± 0.23$^{b}$** | **29.9 ± 0.09$^{c}$** | **< .0001** | **17.6 ± 0.15$^{d}$** | **19.3 ± 0.20$^{c}$** | **20.6 ± 0.23$^{b}$** | **24.7 ± 0.17$^{a}$** | **< .0001** |
| **n-6/n-3** | **0.38 ± 0.00$^{a}$** | **0.32 ± 0.00$^{b}$** | **0.32 ± 0.00$^{b}$** | **0.24 ± 0.00$^{c}$** | **<0.001** | **0.38 ± 0.00$^{a}$** | **0.31 ± 0.00$^{c}$** | **0.33 ± 0.01$^{b}$** | **0.26 ± 0.00$^{d}$** | **< .0001** |
| **EPA+DHA** | **17.1 ± 0.25$^{a}$** | **20.1 ± 0.20$^{b}$** | **16.6 ± 0.08$^{a}$** | **19.6 ± 0.15$^{b}$** | **< .0001** | **12.5±0.19$^{b}$** | **14.3±0.10$^{a}$** | **12.8±0.12$^{b}$** | **14.7±0.06$^{a}$** | **< .0001** |

nd, not determined.

[1]Includes 15:0, 17:0, 19:0, and 21:0.

[2]Includes 17:1, 20:1n-7, 20:1n-11, 22:1n-7, 22:1n-9, and 24:1n-9.

[3]Includes 18:3n-6, 20:2n-6, 20:3n-6, and 22:4n-6.

Table 8. Lipid content (% of wet weight) and fatty acid composition (% of total) in the muscle of Atlantic salmon fed the experimental diets for 70 days at low (12°C) water temperature. Data are shown as mean values using tank as a statistical unit (n = 3, being each sample represented by a pool of five fish) with their standard errors. Different superscript letters indicate statistically significant differences ($p < 0.05$).

| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value |
|---|---|---|---|---|---|
| Total fat | 5.0 ± 0.14 | 5.3 ± 0.18 | 4.7 ± 0.32 | 5.5 ± 0.20 | 0.12 |
| 14:0 | 3.4 ± 0.02$^{b}$ | 3.8 ± 0.02$^{a}$ | 3.1 ± 0.17$^{c}$ | 2.9 ± 0.01$^{c}$ | 0.0004 |
| 16:0 | 13.7 ± 0.07$^{ab}$ | 14.2 ± 0.22$^{a}$ | 13.2 ± 0.29$^{b}$ | 13.1 ± 0.06$^{b}$ | 0.01 |
| 18:0 | 2.7 ± 0.04 | 2.6 ± 0.39 | 2.7 ± 0.03 | 2.9 ± 0.06 | 0.76 |
| 20:0 | 1.1 ± 0.02$^{c}$ | 1.3 ± 0.01$^{b}$ | 1.3 ± 0.02$^{b}$ | 1.6 ± 0.06$^{a}$ | < .0001 |
| **Σ SFA[1]** | 21.5 ± 0.10$^{ab}$ | 22.5 ± 0.48$^{a}$ | 20.8 ± 0.44$^{b}$ | 20.9 ± 0.16$^{b}$ | 0.03 |
| 16:1n-7 | 2.6 ± 0.03$^{ab}$ | 3.0 ± 0.05$^{a}$ | 2.3 ± 0.31$^{b}$ | 2.1 ± 0.03$^{b}$ | 0.03 |
| 18:1n-9 | 29.5 ± 0.43$^{a}$ | 25.3 ± 0.21$^{b}$ | 28.5 ± 1.11$^{a}$ | 27.2 ± 0.71$^{ab}$ | 0.01 |
| 18:1n-7 | 3.9 ± 0.36 | 3.6 ± 0.45 | 4.1 ± 0.92 | 3.4 ± 0.41 | 0.85 |
| 20:1n-9 | 6.3 ± 0.01$^{b}$ | 7.1 ± 0.08$^{a}$ | 6.0 ± 0.20$^{bc}$ | 5.6 ± 0.20$^{c}$ | 0.0006 |
| 22:1n-11 | 0.8 ± 0.03$^{c}$ | 0.9 ± 0.01$^{bc}$ | 1.0 ± 0.04$^{b}$ | 1.2 ± 0.07$^{a}$ | 0.0007 |
| **Σ MUFA[2]** | 44.1 ± 0.31$^{a}$ | 41.2 ± 0.44$^{bc}$ | 42.7 ± 0.09$^{ab}$ | 40.6 ± 0.83$^{c}$ | 0.005 |
| 18:2n-6 | 5.1 ± 0.03$^{a}$ | 4.3 ± 0.02$^{c}$ | 5.1 ± 0.16$^{a}$ | 4.7 ± 0.12$^{b}$ | 0.001 |
| 20:2n-6 | 0.4 ± 0.00 | 0.4 ± 0.02 | 0.5 ± 0.02 | 0.4 ± 0.02 | 0.19 |
| 20:4n-6 | 0.3 ± 0.01$^{b}$ | 0.2 ± 0.00$^{c}$ | 0.3 ± 0.01$^{ab}$ | 0.3 ± 0.00$^{a}$ | 0.0006 |
| **Σ n-6[3]** | 5.9 ± 0.03$^{a}$ | 5.1 ± 0.06$^{b}$ | 6.0 ± 0.17$^{a}$ | 5.6 ± 0.15$^{a}$ | 0.003 |
| 18:3n-3 | 3.2 ± 0.05$^{c}$ | 2.7 ± 0.03$^{c}$ | 5.1 ± 0.50$^{b}$ | 7.2 ± 0.31$^{a}$ | < .0001 |
| 20:3n-3 | 0.3 ± 0.01$^{c}$ | 0.3 ± 0.00$^{c}$ | 0.5 ± 0.06$^{b}$ | 0.7 ± 0.03$^{a}$ | < .0001 |
| 20:5n-3 | 9.1 ± 0.09$^{b}$ | 10.8 ± 0.04$^{a}$ | 8.4 ± 0.47$^{bc}$ | 7.9 ± 0.15$^{c}$ | 0.0002 |
| 22:5n-3 | 1.1 ± 0.06 | 1.4 ± 0.21 | 1.0 ± 0.04 | 1.0 ± 0.03 | 0.06 |
| 22:6n-3 | 12.2 ± 0.32 | 13.3 ± 13.28 | 13.4 ± 0.28 | 13.9 ± 0.37 | 0.06 |
| **Σ n-3[4]** | 26.0 ± 0.44$^{c}$ | 28.4 ± 0.55$^{b}$ | 28.4 ± 0.34$^{b}$ | 30.6 ± 0.77$^{a}$ | 0.003 |
| **n-6/n-3** | 0.23 ± 0.00$^{a}$ | 0.18 ± 0.01$^{b}$ | 0.21 ± 0.00$^{a}$ | 0.18 ± 0.01$^{b}$ | 0.001 |
| **EPA+DHA** | 21.4 ± 0.37$^{b}$ | 24.0 ± 0.40$^{a}$ | 21.8 ± 0.22$^{b}$ | 21.8 ± 0.44$^{b}$ | 0.003 |

[1]Includes 15:0, 17:0, and 24:0.

[2]Includes 14:1n-5, 16:1n-5, 16:1n-9, 17:1n-7, 20:1n-11, and 22:1n-9.

[3]Includes 18:3n-6.

Table 9. Lipid content (% of wet weight) and fatty acid composition (% of total) in erythrocytes of Atlantic salmon fed the experimental diets for 70 days at low (12°C) water temperature. Data are shown as mean values using tank as a statistical unit (n = 3, being each sample represented by a pool of five fish) with their standard errors. Different superscript letters indicate statistically significant differences ($p < 0.05$).

| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value |
|---|---|---|---|---|---|
| Total fat | 2.6 ± 0.13 | 2.6 ± 0.03 | 2.4 ± 0.05 | 2.6 ± 0.26 | 0.74 |
| 14:0 | 1.3 ± 0.05$^{b}$ | 1.6 ± 0.09$^{a}$ | 1.3 ± 0.03$^{b}$ | 1.3 ± 0.03$^{b}$ | 0.01 |
| 16:0 | 15.1 ± 0.16$^{ab}$ | 15.3 ± 0.10$^{a}$ | 14.5 ± 0.46$^{ab}$ | 14.2 ± 0.22$^{b}$ | 0.08 |
| 18:0 | 2.6 ± 0.09 | 2.4 ± 0.08 | 2.6 ± 0.07 | 2.6 ± 0.09 | 0.25 |
| **Σ SFA[1]** | 20.2 ± 0.12$^{ab}$ | 20.6 ± 0.29$^{a}$ | 19.6 ± 0.29$^{b}$ | 19.5 ± 0.06$^{b}$ | 0.02 |
| 16:1n-7 | 1.5 ± 0.03$^{a}$ | 1.5 ± 0.08$^{a}$ | 1.2 ± 0.04$^{b}$ | 1.2 ± 0.05$^{b}$ | 0.005 |
| 18:1n-11 | 1.3 ± 0.07$^{b}$ | 1.5 ± 0.01$^{a}$ | 1.2 ± 0.02$^{b}$ | 1.3 ± 0.01$^{b}$ | 0.005 |
| 18:1n-9 | 18.1 ± 0.36$^{a}$ | 14.5 ± 0.73$^{c}$ | 17.6 ± 0.33$^{ab}$ | 15.5 ± 1.07$^{bc}$ | 0.02 |
| 18:1n-7 | 1.9 ± 0.01$^{b}$ | 1.9 ± 0.05$^{b}$ | 2.0 ± 0.05$^{ab}$ | 2.1 ± 0.08$^{a}$ | 0.05 |
| 20:1n-9 | 4.2 ± 0.06$^{ab}$ | 4.3 ± 0.12$^{a}$ | 3.8 ± 0.15$^{bc}$ | 3.6 ± 0.15$^{c}$ | 0.02 |
| 22:1n-11 | 3.5 ± 0.17$^{a}$ | 3.7 ± 0.12$^{a}$ | 3.0 ± 0.10$^{b}$ | 3.0 ± 0.12$^{b}$ | 0.008 |
| **Σ MUFA[2]** | 33.9 ± 0.61 | 30.9 ± 1.16 | 32.7 ± 0.10 | 30.9 ± 1.12 | 0.11 |
| 18:2n-6 | 3.0 ± 0.11$^{a}$ | 2.4 ± 0.02$^{c}$ | 3.2 ± 0.05$^{a}$ | 2.7 ± 0.08$^{b}$ | 0.0003 |
| 20:4n-6 | 0.9 ± 0.05 | 0.9 ± 0.10 | 0.9 ± 0.11 | 0.8 ± 0.09 | 0.85 |
| **Σ n-6[3]** | 6.4 ± 0.05 | 5.6 ± 0.25 | 6.7 ± 0.56 | 6.1 ± 0.33 | 0.21 |
| 18:3n-3 | 1.3 ± 0.05$^{c}$ | 1.1 ± 0.01$^{c}$ | 2.4 ± 0.11$^{b}$ | 3.3 ± 0.15$^{a}$ | < .0001 |
| 18:4n-3 | 0.2 ± 0.09 | nd | 0.2 ± 0.12 | nd | |
| 20:3n-3 | 0.2 ± 0.02$^{bc}$ | 0.2 ± 0.09$^{c}$ | 0.5 ± 0.06$^{ab}$ | 0.7 ± 0.06$^{a}$ | 0.0009 |
| 20:4n-3 | 0.7 ± 0.07 | 0.7 ± 0.01 | 0.6 ± 0.06 | 0.4 ± 0.21 | 0.3 |
| 20:5n-3 | 6.1 ± 0.10$^{b}$ | 6.8 ± 0.29$^{a}$ | 5.2 ± 0.08$^{c}$ | 5.2 ± 0.10$^{c}$ | 0.0003 |
| 22:5n-3 | 1.6 ± 0.15 | 1.7 ± 0.06 | 1.4 ± 0.24 | 1.1 ± 0.52 | 0.56 |
| 22:6n-3 | 27.3 ± 0.54 | 30.1 ± 0.66 | 28.3 ± 1.11 | 30.1 ± 0.62 | 0.08 |
| **Σ n-3[4]** | 37.4 ± 0.66 | 40.6 ± 1.04 | 38.6 ± 0.88 | 40.8 ± 0.90 | 0.07 |
| **n-6/n-3** | 0.17 ± 0.00 | 0.14 ± 0.00 | 0.17 ± 0.02 | 0.15 ± 0.01 | 0.1 |
| **Omega-3 index[4]** | 33.4 ± 0.6$^{b}$ | 37.1 ± 0.9$^{a}$ | 33.7 ± 1.1$^{b}$ | 35.6 ± 0.6$^{ab}$ | 0.05 |

nd, not determined

[1]Includes 15:0, 17:0, 20:0, 22:0, and 24:0.

[2]Includes 14:1n-5, 16:1n-5, 16:1n-9, 17:1n-7, 20:1n-11, 20:1n-7, 22:1n-7, and 24:1n-9.

[3]Includes 16:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, and 22:4n-6.

Table 10. Lipid content (% of wet weight), fatty acid composition (% of total), and sterols (mg $kg^{-1}$) in the liver of Atlantic salmon fed the experimental diets for 70 days at low (12°C) water temperature. Data are shown as mean values using tank as a statistical unit (n = 3, being each sample represented by a pool of five fish) with their standard errors. Different superscript letters indicate statistically significant differences ($p<0.05$).

| | LOW FO | HIGH FO | LOW DHA-CA | HIGH DHA-CA | ANOVA p-value |
|---|---|---|---|---|---|
| Total fat | 6.3 ± 0.66 | 6.4 ± 0.25 | 6.3 ± 0.23 | 5.8 ± 0.27 | 0.65 |
| 14:0 | 1.6 ± 0.07$^{ab}$ | 1.8 ± 0.12$^{a}$ | 1.4 ± 0.03$^{b}$ | 1.4 ± 0.04$^{b}$ | 0.02 |
| 16:0 | 18.8 ± 0.54 | 18.5 ± 0.17 | 18.5 ± 0.23 | 18.3 ± 0.21 | 0.76 |
| 18:0 | 4.4 ± 0.20 | 4.4 ± 0.16 | 4.5 ± 0.07 | 4.7 ± 0.13 | 0.39 |
| **Σ SFA[1]** | 25.6 ± 0.87 | 25.6 ± 0.28 | 25.5 ± 0.32 | 25.7 ± 0.27 | 0.99 |
| 16:1n-7 | 1.8 ± 0.10$^{a}$ | 1.5 ± 0.10$^{ab}$ | 1.4 ± 0.07$^{b}$ | 1.3 ± 0.06$^{b}$ | 0.02 |
| 16:1n-9 | 0.4 ± 0.04 | 0.4 ± 0.00 | 0.4 ± 0.02 | 0.4 ± 0.01 | 0.93 |
| 18:1n-9 | 23.3 ± 0.59$^{a}$ | 19.1 ± 0.47$^{c}$ | 21.7 ± 0.29$^{ab}$ | 20.3 ± 0.59$^{bc}$ | 0.002 |
| 20:1n-7 | 3.3 ± 0.07$^{b}$ | 3.7 ± 0.07$^{a}$ | 3.2 ± 0.12$^{bc}$ | 3.0 ± 0.10$^{c}$ | 0.004 |
| 22:1n-11 | 0.5 ± 0.08$^{c}$ | 0.6 ± 0.03$^{bc}$ | 0.8 ± 0.13$^{ab}$ | 1.0 ± 0.03$^{a}$ | 0.01 |
| **Σ MUFA[2]** | 30.3 ± 0.87$^{a}$ | 26.7 ± 0.35$^{b}$ | 28.4 ± 0.29$^{b}$ | 26.9 ± 0.65$^{b}$ | 0.008 |
| 18:2n-6 | 3.0 ± 0.04$^{a}$ | 2.3 ± 0.07$^{b}$ | 3.0 ± 0.07$^{a}$ | 2.6 ± 0.16$^{b}$ | 0.003 |
| 20:2n-6 | 0.7 ± 0.05 | 0.6 ± 0.04 | 0.8 ± 0.06 | 0.7 ± 0.01 | 0.06 |
| 20:3n-6 | 1.1 ± 0.06$^{a}$ | 0.8 ± 0.07$^{b}$ | 1.1 ± 0.11$^{a}$ | 1.0 ± 0.03$^{ab}$ | 0.03 |
| 20:4n-6 | 1.5 ± 0.09 | 1.8 ± 0.09 | 1.7 ± 0.13 | 1.5 ± 0.05 | 0.13 |
| **Σ n-6[3]** | 6.3 ± 0.12$^{a}$ | 5.5 ± 0.14$^{b}$ | 6.6 ± 0.11$^{a}$ | 5.8 ± 0.16$^{b}$ | 0.002 |
| 18:3n-3 | 1.0 ± 0.06$^{c}$ | 0.8 ± 0.04$^{c}$ | 1.7 ± 0.08$^{b}$ | 2.4 ± 0.18$^{a}$ | < .0001 |
| 20:3n-3 | 0.00 ± 0$^{b}$ | 0.2 ± 0.16$^{ab}$ | 0.3 ± 0.07$^{a}$ | 0.5 ± 0.05$^{a}$ | 0.03 |
| 20:5n-3 | 5.1 ± 0.06$^{a}$ | 5.5 ± 0.14$^{a}$ | 4.7 ± 0.14$^{b}$ | 4.6 ± 0.07$^{b}$ | 0.002 |
| 22:5n-3 | 1.8 ± 0.13 | 1.7 ± 0.21 | 1.8 ± 0.16 | 1.7 ± 0.03 | 0.95 |
| 22:6n-3 | 28.1 ± 0.86$^{b}$ | 32.5 ± 0.55$^{a}$ | 30.0 ± 0.84$^{ab}$ | 31.3 ± 1.11$^{a}$ | 0.03 |
| **Σ n-3[4]** | 36.0 ± 0.85$^{b}$ | 40.7 ± 0.68$^{a}$ | 38.5 ± 0.62$^{ab}$ | 40.5 ± 0.96$^{a}$ | 0.01 |
| **n-6/n-3** | 0.17 ± 0.00$^{a}$ | 0.14 ± 0.00$^{b}$ | 0.17 ± 0.00$^{a}$ | 0.14 ± 0.01$^{b}$ | 0.0003 |
| **EPA+DHA** | 33.2 ± 0.90$^{b}$ | 38.0 ± 0.47$^{a}$ | 34.7 ± 0.70$^{b}$ | 35.9 ± 0.15$^{ab}$ | 0.02 |
| | | | | | |
| **Sterols** | | | | | |
| Cholesterol | 3780 ± 425 | 3894 ± 241 | 3840 ± 262 | 3715 ± 437 | 0.69 |
| Σ Phytosterols | 52.1 ± 7.9$^{a}$ | 51.8 ± 15.2$^{a}$ | 67.8 ± 13.4$^{b}$ | 58.2 ± 10.6$^{ab}$ | 0.03 |
| β-Sitosterol | 15.6 ± 5.4 | 16.7 ± 9.2 | 16.6 ± 7.5 | 14.4 ± 5.2 | 0.90 |
| Campesterol | 16.2 ± 5.1$^{a}$ | 12.1 ± 5.5$^{a}$ | 26.1 ± 9.2$^{b}$ | 21.7 ± 5.8$^{b}$ | 0.0005 |
| Brassicasterol | 5.2 ± 2.8 | 5.9 ± 1.9 | 7.7 ± 2.0 | 5.9 ± 1.7 | 0.39 |
| Sitostanol | 2.5 ± 0.8$^{a}$ | 2.6 ± 1.3$^{ab}$ | 3.4 ± 1.0$^{b}$ | 3.3 ± 0.6$^{b}$ | 0.04 |

[1]Includes 15:0, 17:0, 20:0, 22:0, and 24:0.

[2]Includes 14:1n-5, 16:1n-5, 17:1n-7, 18:1n-7, 20:1n-11, 20:1n-9, and 22:1n-9.

[3]Includes 18:3n-6.

[4]Includes 16:2n-3.

Table 11. Selected differentially expressed genes (DEG) in the liver from Atlantic salmon fed diets containing fish oil (FO) or oil from the n-3 rich modified canola (DHA-CA) for 70 days at low (12°C) water temperature. Data are presented as relative to the diet containing high levels of FO, which is regarded as reference level. Significantly up-regulated genes ($log_2$-expression ratio > |0.6| (1.5-fold), $p < 0.05$) are highlighted with red scale.

| | LOW FO | LOW DHA-CA | HIGH DHA-CA |
|---|---|---|---|
| **Total DEG** | | | |
| Up-regulated | 85 | 79 | 13 |
| Down-regulated | 96 | 66 | 4 |
| Diphosphomevalonate decarboxylase | 1.88 | 2.05 | 1.29 |
| Endothelial lipase | 3.14 | 2.44 | 1.45 |
| Farnesyl diphosphate synthase 1 | 2.94 | 3.32 | 1.68 |
| Farnesyl diphosphate synthase 2 | 2.94 | 3.27 | 1.65 |
| Fatty acid-binding protein. brain | 2.50 | 1.88 | 1.15 |
| Hydroxymethylglutaryl-CoA synthase | 2.40 | 2.80 | 1.52 |
| Insulin-induced gene 1 | 1.82 | 2.01 | 1.68 |
| Isopentenyl-diphosphate Delta-isomerase 1 | 2.52 | 2.50 | 1.36 |
| 7-Dehydrocholesterol reductase | 1.65 | 1.44 | 1.41 |
| 7-Dehydrocholesterol reductase | 1.79 | 1.91 | 1.61 |
| Lanosterol 14-alpha demethylase 1 | 2.04 | 1.54 | 1.22 |
| Lanosterol 14-alpha demethylase 2 | 1.53 | 1.81 | 1.26 |
| Lanosterol synthase | 2.14 | 1.90 | 1.47 |
| Lipin 1 | 1.80 | 1.58 | 1.63 |
| Mevalonate kinase | 1.50 | 1.94 | 1.22 |
| Squalene monooxygenase 1 | 1.34 | 1.73 | 1.30 |
| Squalene monooxygenase 2 | 2.26 | 2.42 | 1.61 |
| Squalene synthase | 1.90 | 2.00 | 1.40 |
| Stearoyl-CoA desaturase b | 3.10 | 2.73 | 2.69 |
| Sterol-4-alpha-carboxylate 3-dehydrogenase 1 | 1.77 | 1.58 | 1.26 |
| Sterol-4-alpha-carboxylate 3-dehydrogenase 2 | 1.44 | 1.69 | 1.39 |
| Sterol-C5-desaturase | 1.69 | 1.75 | 1.56 |

Figure 1

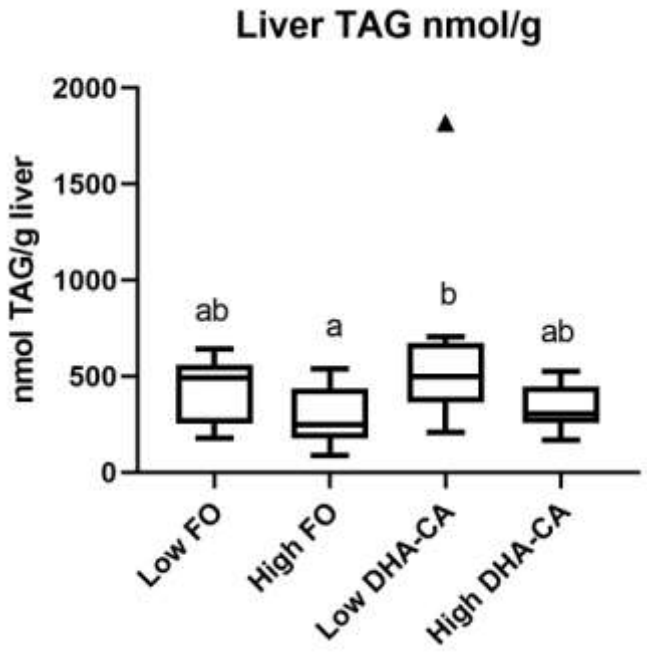

Figure 2

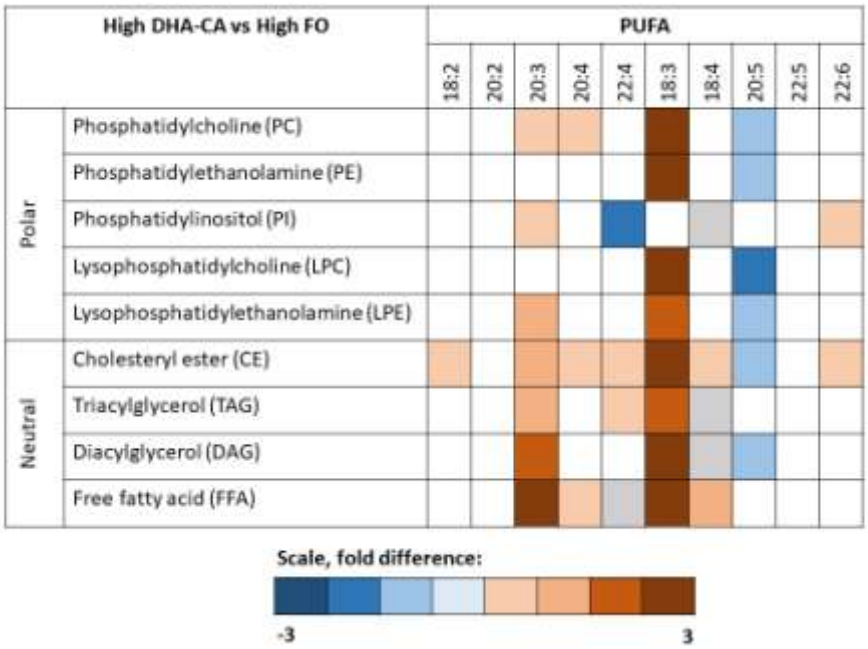

Figure 3