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Oils Derived from GM Crops as Sustainable Solutions to the Supply of Long-Chain Omega-3 for On-Growing Atlantic Bluefin Tuna (*Thunnus thynnus* L.)

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Abstract: Recently *Camelina sativa*, has been genetically modified to produce oils rich in omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), EPA (eicosapentaenoic acid) and EPA + DHA (docosahexaenoic acid). The aim of this study was to test the feasibility of using these novel sources of de novo EPA and EPA + DHA as substitutes for marine oil in feeds for juvenile Atlantic Bluefin tuna (ABT). The results showed the oils were practical sources of n-3 LC-PUFA which could potentially replace fish oil (FO) in feeds for ABT juveniles. Fish fed the test diets (ECO, EPA alone and DCO, EPA + DHA) displayed good growth performance, survival and feed utilisation approaching that of ABT fed the reference diet (MGK) containing marine fish oil with the rank order being MGK > DCO > ECO. The test diets showed positive effects, upregulating the expression of genes of major nuclear receptors and those of lipid metabolism including digestion, LC-PUFA synthesis and antioxidant pathways. The results indicated that the DCO feed containing both DHA and EPA performed better than the ECO feed with much lower DHA. However, feeds formulated with both these oils may still require supplementary DHA to satisfy the high requirement of ABT for this essential nutrient.

Keywords: Atlantic bluefin tuna; GM Camelina; dietary oils; sustainable feeds; hepatic lipid metabolism

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1. Introduction

A decade or more ago, the future of tuna farming in terms of both human and fish nutrition against a scenario of diminishing global supply of marine resources, fishmeal (FM) and fish oil (FO), was envisaged and discussed [1,2]. Part of the solution has come through the partial replacement of FM and FO with plant meals and vegetable oils (VO), respectively [3]. Any formulated feed for farmed tuna would have to be dependent, to some extent, upon global supplies of FM and FO in order to meet both the fish's nutritional requirements and to tailor for market expectations. Fish performance, as well as flesh quality, could be influenced by feeds containing more sustainable alternatives to FM and FO, which in turn could have a greater market impact on tuna than on most other fish species [2].

In comparison to FO, the main issue with terrestrial alternatives such as VO is the lack of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA; ≥20 carbons and ≥3 double bonds), such as the health-beneficial eicosapentanoate (EPA; 20:5n-3) and docosahexaonate (DHA; 22:6n-3). These fatty acids are characteristic of the marine environment, with microalgae and other marine microbes being the primary producers. Consequently, a major undesirable effect of substituting FO with VO is the reduction in levels of LC-PUFA in flesh, which ultimately impacts the human consumer. Therefore, the main issue in replacing dietary FO is maintaining the supply of essential omega-3 LC-PUFA [4]. Very recently, alternative sustainable terrestrial sources of EPA and DHA have become available in the form of oils from microalgae and GM oilseed crops [3,5–10]. Metabolic engineering of oilseed crops, for example, *Camelina sativa*, has allowed the production of "hybrid" VOs rich in omega-3 LC-PUFA, characteristics of the marine environment, as well as shorter chain PUFA such as α-linolenic (LNA; 18:3n-3) and linoleic (LA; 18:2n-6) acids, characteristic of terrestrial environments [11–13]. Oils derived from different iterations of GM Camelina have already been tested in Atlantic salmon (*Salmo salar*) [14–17] as well as in sea bream (*Sparus aurata*) [18] and European sea bass (*Dicentrarchus labrax*) [19]. These new gene technologies can revolutionize the way we produce our food and, in aquaculture, have the potential to release pressure on wild fish stocks and improve the nutritional profiles of farmed fish for human consumption [6,7,9,20].

While uptake, digestion and storage of appropriate lipids improve the survival and growth of all fish species, for highly active migratory predator fish species such as tunas is particularly relevant [1,2]. The presence of n-3 LC-PUFA in fish is derived both from their diet and endogenous biosynthesis, with the latter varying considerably with species [21,22]. Furthermore, the n-3 LC-PUFA, EPA and DHA, are essential for the survival, normal growth and development of most marine fish [23], with DHA in particular shown to be critical for the function of neural and retinal tissues [24–29]. Thus, the replacement of FO with VO lacking EPA and DHA makes farmed fish more dependent on the endogenous production of n-3 LC-PUFA via biosynthetic pathways depending upon the expression of key enzymes such as fatty acyl desaturases (Fads) and elongases of very long chain fatty acids (Elovl) [14,23]. Nonetheless, it has been reported recently that the capability of fish species for de novo synthesis of LC-PUFA is inversely correlated to the trophic level and those carnivorous marine fish species occupying higher levels are unable or exhibit only limited capacity to synthesize LC-PUFA from C_{18} PUFA precursors [22,30]. Thus, top predators such as bluefin tuna species are known to have a poor capacity for the endogenous biosynthesis of EPA and DHA [22,31–33]. Furthermore, limited knowledge exists regarding the metabolic response of these species when fed reduced levels of marine ingredients are largely unknown.

Many enzymes and nuclear receptors (NR) in fish play key roles in lipogenesis and lipolysis including, among others, lipases, Fads, Elovl, transferases, and NR such as peroxisome proliferator-activated receptors (PPAR), sterol regulatory-element binding proteins (SREBP) and liver or retinoid receptors (LXR, RXR). As LC-PUFA are natural ligands that can bind and activate these NR, influencing the circulating levels of lipogenic and lipolytic genes [22,34], dietary n-3 LC-PUFA content affects the expression levels of a range of genes associated with lipid homeostasis and other pathways, such as antioxidant genes [35,36]. Therefore, studying the impacts of dietary lipids on fish metabolism, including lipid and fatty acid compositional changes, as well as the patterns of expression of genes involved in major lipid metabolic pathways in ABT is highly relevant [37–40].

In this study, the efficacy of oils derived from transgenic *Camelina sativa* containing either EPA alone or EPA + DHA was evaluated in juvenile ABT using feeds that reflected current sustainable formulations compared to a commercial-like feed for pacific bluefin tuna (PBT) as a reference diet. The impacts of these new sources of de novo EPA and DHA on ABT growth performance and survival, feed utilisation, tissue fatty acid profiles, liver

and intestine histo-morphology, and nutritional regulation of hepatic and intestinal gene expression were investigated.

2. Materials and Methods

2.1. Experimental Diets Formulation and Manufacturing Process

Two isoproteic and isoenergetic experimental diets were formulated to satisfy the nutritional requirements of ABT. Diet DCO contained oils rich in both EPA + DHA from transgenic Camelina and diet ECO contained the oil that was rich in EPA alone, with no DHA (Table 1). Diets were manufactured by SPAROS Lda., Olhão (Portugal). All powder ingredients were initially mixed and ground in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Augsburg, Germany). The oils were added to the powder mixtures along with approximately 25% water and feed produced by a low-shear and low-temperature extrusion process (ITALPLAST, Bertinoro, Italy). The resulting pellets (1.5 and 2.0 mm) were dried in a convection oven at 45 °C for 4 h (OP 750-UF, LTE Scientifics, Oldham, UK).

Table 1. Formulations of the experimental diets, ECO and DCO, including detailed list of the ingredients used.

Ingredients (%)	ECO	DCO
Fishmeal [1]	12.00	12.00
Fish protein concentrate [2]	30.00	30.00
Squid meal [3]	15.00	15.00
Fish gelatin [4]	9.00	9.00
Gelatinised starch [5]	8.25	8.25
GM *Camelina* oil (DHA + EPA)	0.00	11.90
GM *Camelina* oil (EPA only)	11.90	0.00
Soy lecithin [6]	1.80	1.80
Vitamin and mineral premix [7]	2.00	2.00
Vitamin C [8]	0.10	0.10
Vitamin E [9]	0.04	0.04
Antioxidant [10]	0.30	0.30
Sodium phosphate [11]	4.50	4.50
Selenized yeast [12]	0.25	0.25
L-Histidine [13]	0.25	0.25
L-Taurine [14]	2.00	2.00
L-Glutamic acid [15]	0.20	0.20
Inosine-5-monophosphate [15]	0.01	0.01
Technical additives [16]	2.40	2.40

[1] MicroNorse, Tromsø Fiskeindustri AS, Norway; [2] CPSP90, Sopropêche, France; [3] Sopropêche, France; [4] WEISHARDT International, Slovakia; [5] Pregeflo P100, Roquette, France; [6] P700IPM, LECICO GmbH, Germany; [7] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, ferric sulphate, 6mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middlings; [8] ROVIMIX STAYC35, DSM Nutritional Products, Switzerland; [9] ROVIMIX E50, DSM Nutritional Products, Switzerland; [10] VERDILOX, Kemin Europe NV, Belgium; [11] Sodium phosphate, Vadequímica, Spain; [12] Sel-Plex, Alltech, USA; [13] L-Histidine 98%, Ajinomoto Eurolysine SAS, France; [14] L-Taurine 98%, ORFFA, The Netherlands; [15] Sigma-Aldrich, Burlington, MA, USA; [16] Binder and anti-agglomerating agents.

The formulations and analysed proximate compositions of the test diets and the commercial reference diet (MGK; Magokoro Nishin Marubeni®, Tokyo, Japan) are shown in Tables 1 and 2. Crude protein was approximately 64% on a dry matter basis in the test and reference diets. Crude lipid content was approximately 15% in the test diets and 17% in the reference diet. The caloric contents of the test and reference diets were about 5.5 kcal/g or 23 kJ/g. The reference diet MGK presented the highest level of total saturated fatty acids (due to the highest levels of 16:0 and 18:0, respectively), while the test diets ECO and DCO showed the highest levels of C18 PUFA, 18:1n-9, 18:2n-6 and 18:3n-3. With regard to n-3 LC-PUFA levels, the MGK reference diet presented the highest total level (26.6%) with 9.3% EPA and 14.5% DHA while the test diets showed lower total levels of 12.1% (ECO; 8.4% EPA and 2.2% DHA) and 15.4%, (DCO; 15.3% EPA and 5.8% DHA). It should be noted that while the added oil only contained EPA, the ECO diet contained DHA originating from the protein sources including fish meal, squid meal and fish protein concentrate. The DHA/EPA ratio showed a decreasing trend in the diets in the rank order, MGK (1.6) > DCO (1.1) > ECO (0.3). The overall n-3/n-6 PUFA ratio was around 1.5 in the test diets compared to 3.6 in the reference diet, mainly due to the high levels of 18:2n-6 present in the Camelina oils.

2.2. Experimental Protocol and Fish Husbandry

All experimental procedures followed the Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/EU) and Spanish Legislation (RD 1201/2005 and Law 32/2007) for the use of laboratory animals. Additionally, the experimental protocol was subjected to ethical review by the University of Stirling through the Animal Welfare and Ethical Review Board (reference number AWERB/1819/177).

ABT juveniles were obtained from eggs spawned in the summer of 2019 from captive wild broodstock maintained in a floating net cage located at El Gorguel, (Cartagena coast, SE Spain). The trial was carried out at the Planta Experimental de Cultivos Marinos, Instituto Español de Oceanografía (IEO), Puerto de Mazarrón (Murcia, Spain) following established protocols [41–43]. Briefly, fish were weaned from the live feed stage by being fed gilthead sea bream (*Sparus aurata* L.) yolk sac larvae as prey (piscivorous stage) to formulated feed at 27 days after hatch (dah), using a commercial diet (Magokoro®; MGK; Marubeni Nisshin Feed Co., Tokyo, Japan). This diet has been successfully used as a formulated feed for PBT [44–46]. Next, ABT was weaned using MGK at 0.6 to 0.9 mm pellet sizes and was completely weaned by 32 dah. From day 32 to 40 dah, fish were fed with a mixture (1/1/1, by weight) of MGK and the two experimental diets, ECO and DCO. The experimental feeding trial started at 41 dah and lasted 18 days (up to 59 dah).

The dietary trial was carried out in eight 10 m^3 cylindroconical tanks, with the experimental diets tested in triplicate, whereas the commercial reference diet was run in duplicate tanks. Rearing conditions are summarised in Supplementary Table S1. Tanks in an open flow-through system were used, with incoming seawater first filtered at 10 μm and then UV sterilised. A total of 400 juvenile ABT with an initial average body weight of 3.4 ± 1.9 g were distributed into the experimental tanks (50 fish/tank). Fish were hand-fed

Table 2. Analysed proximate compositions (% dry mass), estimated energy contents and fatty acid profiles (% of total fatty acids) of the reference (MGK) and test (ECO and DCO) diets.

	MGK	ECO	DCO
Analysed composition			
Dry matter (DM,%)	85.6	90.8	91.2
Crude protein (% DM)	63.0	64.1	66.4
Crude lipid (% DM)	17.2	15.2	15.1
Carbohydrate [1] (% DM)	10.8	9.5	9.9
Ash (% DM)	8.9	11.2	8.5

Energy content [2] (kJ/g)	23.5	22.8	23.3
Fatty acids			
14:0	5.2	0.8	0.8
16:0	16.9	10.3	10.1
18:0	4.9	4.8	4.9
Total SFA [3]	**28.2**	**17.7**	**17.8**
16:1n-7	5.6	1.1	1.1
18:1n-9	12.3	18.3	12.8
18:1n-7	3.5	2.0	1.8
20:1n-9	2.3	8.7	7.7
Total MUFA [4]	**31.8**	**33.7**	**27.2**
18:2n-6	5.9	15.2	17.0
20:4n-6	1.3	2.2	1.5
Total n-6 PUFA [5]	**8.3**	**20.2**	**22.0**
18:3n-3	1.3	14.4	15.4
18:4n-3	1.8	1.1	1.1
20:4n-3	0.6	0.9	1.2
20:5n-3	9.3	8.04	5.3
22:5n-3	2.2	0.6	3.1
22:6n-3	14.5	2.2	5.8
Total n-3 PUFA [6]	**30.3**	**28.4**	**32.9**
Total n-3 LC-PUFA	**26.6**	**12.1**	**15.4**
n-3/n-6	3.6	1.4	1.5
DHA/EPA	1.6	0.3	1.1

Data are means of three technical replicates. [1] Carbohydrate% = 100 − (protein% + lipid% + ash); [2] Conversion factors as in [47,48]; [3] Totals include 15:0, 20:0, 22:0 and 24:0; [4] Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; [5] Totals include 18:3n-6, 20:2n-6, 22:4n-6 and 22:5n-6; [6] Totals include 20:3n-3 and 22:3n-3; DCO, diet containing oil with DHA + EPA; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ECO, diet containing oil with EPA alone; MGK, Magokoro reference diet; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. An SD marked 0.0 implies 0.0 < SD < 0.05. Values bearing different superscript letters are significantly different ($p < 0.05$).

To satiation every 2 h during the light hours, eight times per day, taking care not to overfeed. It is important to note that the trial was deliberately limited to 18 days as at this stage the major cause of death in ABT juveniles in tanks is collisions with the tank walls [49] and so the trial duration was determined by considerations of fish welfare. However, the trial produced weight increases of 6.8 to 8.6-fold, which is generally accepted as adequate for fish nutritional trials. Moreover, similarly short-duration trials testing dietary oil sources have been performed successfully with juvenile PBT [50].

2.3. Sampling Protocol and Calculations

At the start of the feeding trial (41 dah), thirty randomly caught ABT juveniles were euthanised by an overdose of anaesthetic (0.02% 2-phenoxyethanol; Sigma, Spain), weights and total lengths were recorded, and individual fish were photographed. At the end of the trial (59 dah), surviving ABT juveniles from all tanks were measured and weighed with final survival (%) calculated by counting juveniles in each tank at the beginning and end of the trial. Several growth performances and feed utilisation parameters such as mean weight (g), mean length (cm), percent weight gain (%), specific growth rate (SGR) as % of daily growth increase, feed conversion ratio (FCR), condition factor (CF) and daily feeding rate (DFR, %) were calculated using the following formulae.

Weight gain (%) = (average weight gain/average initial body weight) × 100

SGR (%.day^{-1}) = [(lnW_2 − lnW_1)/time (days)] × 100, where W_1 and W_2 denote the initial and final weight (g), respectively.

FCR = dry feed intake (g)/wet weight gain (g)

CF = (W/L^3) × 100, where W and L denote wet body weight (g) and fork length (cm), respectively.

DFR (%) = feed intake (dry matter)/100/[(initial fish weight + final fish weight) 18 days fed/2].

Retention of dietary nutrients/energy was estimated as:

% Retention = (final weight × final nutrient/energy content) − (initial weight × initial nutrient/energy content) × 100/nutrient/energy intake [47].

Additionally, at the end of the 18-day feeding period, twelve fish per tank were humanely euthanised by an overdose of anaesthetic (0.02% 2-phenoxyethanol; Sigma, Spain) and killed by a blow to the head. Three fish per tank were rapidly frozen on dry ice for whole body chemical composition. An additional six fish per tank were dissected and the liver and intestine were removed for gene expression analysis. These tissue samples were placed in RNALater, stored at 4 °C overnight and kept at −20 °C until RNA extraction. From the same fish, portions of the liver, anterior and posterior intestine were fixed in 4% buffered formalin for histological analysis. A further three fish per tank were dissected and the liver, intestine, gill, muscle, eye and brain were rapidly frozen on dry ice for fatty acid composition analysis.

2.4. Biochemical Analysis

2.4.1. Proximate Composition of Diets and Whole Fish

Gross proximate compositions of test and control feeds (protein, lipid, ash and moisture) and whole ABT juveniles were determined according to standard procedures [51]. Briefly, moisture contents were obtained after drying the sample in an oven at 110 °C for 24 h and ash contents were determined after incineration at 600 °C for 16 h. Crude protein was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, UK) and crude lipid contents determined gravimetrically after Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus). The caloric contents of the diets were estimated by the indirect method using gross composition values and caloric conversion factors [51,48].

2.4.2. Total Lipid Extraction and Quantification and Fatty Acid Analysis

Total lipids were extracted from feeds and tissues of the experimental fish according to the method of [52]. Approximately 200 mg of ground feed, or ABT tissues were placed in ice-cold chloroform/methanol (2:1, by vol) and homogenised with an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). Next, the non-lipid and lipid layers were separated by addition of 0.88% (*w*/*v*) KCl. The upper aqueous layer was then aspirated and discarded, whereas the lower organic layer was dried under oxygen-free nitrogen. The lipid content was determined gravimetrically after drying overnight in a vacuum desiccator.

Fatty acid methyl esters (FAME) of total lipids were prepared by acid-catalysed transesterification at 50 °C for 16 h according to [53]. Firstly, the FAME were separated and quantified by gas-liquid chromatography (Agilent Technologies 7890B GC System) using a 30 m × 0.32 mm i.d. fused silica capillary column (SUPELCOWAXTM-10, Supelco Inc., Bellefonte, PA, USA) and on-column injection at 50 °C. Hydrogen was used as carrier gas and temperature programming was from 50 °C to 150 °C at 40 °C per min and then to 230 °C at 2.0 °C per min. Then, individual methyl esters were identified by comparison with known standards and by reference to published data [39,40,54]. Agilent Technologies Openlab CDS Chemstation for Windows (version A.02.05.21, Santa Clara, CA, USA) was used to collect and process data.

2.5. Tissue RNA Extraction and cDNA Synthesis

Liver and intestine samples were firstly homogenised in 1 mL of TriReagent® (Sigma-Aldrich, Burlington, MA, USA) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, OK, USA). Total RNA was isolated following the manufacturer's instructions and quantity and quality were determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK). RNA integrity was assessed with electrophoresis using 200 ng of total RNA in a 1% agarose gel. Two micrograms of total RNA and random primers were used to synthesize cDNA in 20 μL reactions and the high-capacity reverse transcription kit without RNase inhibitor (Applied Biosystems, Warrington, UK). Prior to synthesising cDNA, the same amount of RNA (2 μg per fish) from three fish per tank were pooled (two pools per tank) in order to obtain n = 6 per dietary treatment.

2.6. Quantitative RT-Polymerase Chain Reaction Analysis

Quantitative RT-polymerase chain reaction (qPCR) was used to determine the transcript abundance of candidate genes. Genes of interest were involved in several key pathways related to lipid and fatty acid metabolism, nutrient digestion and antioxidant system enzymes. Specifically, qPCR was carried out on NR *pparα*, *pparγ*, *lxr*, *rxr*, *srebp1* and *srebp2*; LC-PUFA biosynthesis genes *fads2d6*, *elovl4* and *elovl5*, lipolysis and lipogenesis genes *fas*, *cpt1*, *aco*, *lpl* and *hmgcl*, antioxidant enzyme genes *sod*, *cat*, *gpx1* and *gpx4* and digestive enzyme genes *anpep*, *tryp*, *alp*, *amy*, *pl*, *pla2*, *bsl1* and *bsl2*. Two reference genes, *Elongation factor-1α* (*elf1α*) and *β-actin* (*bactin*) were used. Serial dilutions (1/5, 1/10, 1/20, 1/50, 1/100, 1/200 and 1/500) of cDNA pooled from the samples were used to evaluate the efficiency of the primers for each gene and was >85% for all primer pairs. qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 10 μL reaction volumes containing 5 μL of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 0.5 μL of the primer corresponding to the analysed gene (10 pmol), 1.5 μL of molecular biology grade water and 2.5 μL of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters included a UDG pre-treatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. Primer sequences for genes are given in Supplementary Table S2.

2.7. Histological Evaluation

Transverse sections of liver and midgut from six fish per tank were fixed in 4% buffered formalin and embedded in paraffin. Four μm sections of the samples were cut on a microtome (Leica RM 2035, Leica Biosystems, Wetzlar, Germany) and subsequently stained with hematoxylin and eosin (H&E) [14]. In addition, the slides containing intestinal samples were stained with Alcian Blue/Periodic acid-Schiff (AB-PAS) to differentiate between neutral/mixed and acidic muco-substances in goblet cells [16]. Stained slides were scanned (Axio Scan Z1, ZEISS, Oberkochen, Germany) and visualised using QuPath 0.2.3 [55]. Digital images were analysed using ImageJ (FIJI) software [56].

Liver micrographs at 20× original magnification were examined for the presence of lipid intracytoplasmic vacuoles in hepatocytes from each dietary group (Supplementary Figure S1). The micrographs were converted into black and white 2-bit images, where white corresponds to the area occupied by lipid droplets and the percentage of the area covered by them was measured. In the intestine, 30 measurements were taken per fish of each evaluated parameter including enterocyte height (hE), enterocyte width (nNE) measured as the number of enterocyte nuclei along a distance of 100 μm, circular muscle thickness (CMT), maximum (Lmax) and minimum (Lmin) lengths of the intestine passing through the center of the lumen in a transverse section, and goblet cells (GB, calculated as

the number of goblet cells per 100,000 μm² of intestinal mucosa) (Supplementary Figure S2). Length measurements are shown in μm.

2.8. Statistical Analysis

Results are presented as means ± SD with n = 3 for biochemical analyses or n = 6 for gene expression. The homogeneity of the variances was checked by the Bartlett test and arc-sin transformation was applied where necessary, before further statistical analysis. The t-test and one-way analysis of variance (ANOVA) was used to assess differences between mean values, followed, when pertinent, by a Tukey multiple comparison test. The null hypothesis was rejected and results were reported as statistically significant when $p < 0.05$ [57]. Molecular biology results were analysed using the relative expression software tool (REST 2009). This software employs a pairwise fixed reallocation randomisation test (10,000 randomisations) with efficiency correction [58] to determine the statistical significance of expression ratios among treatments.

3. Results

3.1. Growth Performance, Survival, Feed Utilisation and Biometry of ABT Juveniles

Table 3 shows the performance, feed utilisation and survival of ABT juveniles fed the reference (MGK) and experimental feeds (ECO and DCO). After 18 days of feeding, ABT juveniles fed all the diets grew more than six-fold in weight. However, total final wet weight also showed significant differences with diet, being the highest in ABT-fed MGK and lowest in fish-fed ECO with that of fish-fed DCO being intermediate, with specific growth rate (SGR) showing the same pattern. Fish total length was also significantly lowest in fish-fed ECO although there were no significant differences detected between fork lengths of fish-fed MGK and DCO. In contrast, no significant differences were observed among the different treatments regarding weight gain percentage. The condition factor was significantly higher in fish fed MGK and ECO than those fed DCO. While daily feeding rate (DFR) was significantly higher in fish fed ECO and DCO compared to fish fed MGK, total feed intake was higher in juveniles fed MGK (29.2 g/fish) than fish fed ECO and DCO, which were not significantly different to each other (23.6 g/fish and 23.1 g/fish for ECO and DCO diets, respectively). Feed conversion ratio (FCR) showed no significant differences among treatments, although the numerical mean values were in reverse rank order DCO < MGK < ECO. Regarding nutrient and energy retention, fish fed MGK and DCO showed higher protein retention values (~28–30%), than fish fed ECO (~26%), and dietary energy retention followed a similar pattern. However, lipid retention was significantly higher in fish fed ECO and DCO (~31%) compared to fish fed MGK, which retained only 20% of dietary lipid. Survival rates varied between 34 and 47% but were not significantly different among treatments due to high variability among the replicates, although mean values were in decreasing order DCO > ECO > MGK. As in other dietary trials with bluefin tunas (both PBT and ABT), many of the deaths observed at this juvenile stage occurred due to collisions with the tank wall and were not related to feed quality [39,49].

Table 3. Fish performance, feed utilisation and analysed proximate composition of juvenile ABT (*T. thynnus*) fed reference (MGK) and test (ECO and DCO) diets for 18 days.

Dietary Treatments	MGK			ECO			DCO		
Initial fork length (cm)	7.0	±	1.0	7.0	±	1.0	7.0	±	1.0
Initial wet weight (g)	3.4	±	1.9	3.4	±	1.9	3.4	±	1.9
Final fork length (cm)	13.3	±	0.4 [a]	12.5	±	0.3 [b]	13.0	±	0.3 [a]
Final wet weight (g)	29.4	±	0.3 [a]	22.9	±	2.0 [c]	24.9	±	1.3 [b]
Weight gain (%)	420.0	±	30.3	327.1	±	197.6	355.7	±	130.2
SGR (%)	18.5	±	0.1 [a]	17.1	±	0.5 [c]	17.6	±	0.3 [b]
CF (g/cm³)	1.16	±	0.01 [a]	1.17	±	0.02 [a]	1.13	±	0.03 [b]

FCR	2.45	±	0.15	2.67	±	0.90	2.10	±	0.57
DFR (%)	2.13	±	0.08 [b]	2.48	±	0.23 [a]	2.37	±	0.07 [a]
Total intake (g/fish)	29.2	±	1.2 [a]	23.6	±	0.9 [b]	23.1	±	1.7 [b]
Protein retention (%)	30.3	±	1.2 [a]	25.8	±	0.6 [b]	28.1	±	0.8 [a]
Lipid retention (%)	20.2	±	0.8 [b]	30.1	±	1.2 [a]	31.8	±	0.9 [a]
Energy retention (%)	23.9	±	0.9 [ab]	23.1	±	0.7 [b]	25.5	±	0.4 [a]
Survival (%)	34.3	±	1.7	30.1	±	10.4	46.3	±	2.4
Chemical composition									
Moisture (%)	76.6	±	0.4	77.2	±	0.8	77.6	±	0.3
Crude protein (% DM)	70.6	±	0.1	67.0	±	0.4	67.1	±	0.2
Crude lipid (% DM)	14.5	±	0.5	14.1	±	0.6	14.3	±	0.2
Carbohydrate [1] (% DM)	0.3	±	0.0	0.6	±	0.0	0.5	±	0.0
Ash (% DM)	14.6	±	0.2b	18.3	±	0.2a	18.1	±	0.4 [a]
Caloric content (kcal/g)	5.4	±	0.1	5.1	±	0.2	5.1	±	0.1

CF, condition factor; DFR, daily feeding rate; FCR, feed conversion ratio; SGR, specific growth rate; DM, dry matter. Results are mean ± SD (n = 12 per replicate and treatment for biometry and n = 3 for treatment). An SD marked 0.0 implies 0.0 < SD < 0.05. Values bearing different superscript letters are significantly different ($p < 0.05$). [1] Carbohydrates = 100 − (lipid-protein-ash).

3.2. Whole Fish Proximate Composition and Total Lipid Fatty Acid Profiles

The analysed proximate compositions, caloric contents and total lipid fatty acid compositions of fish after 18 days of feeding the reference and experimental feeds are displayed in Tables 3 and 4. At the end of the trial, other than higher ash content in fish fed ECO and DCO, no significant differences were observed in gross composition or caloric content among fish from different treatments, with total lipid content similar in all groups (about 14% on a dry matter basis). Total n-3 PUFA was higher in fish fed MGK and DCO (32.5% and 31.3%, respectively), compared to fish fed ECO. However, fish fed MGK showed higher percentages of EPA and DHA, whereas fish fed DCO presented higher values of LNA and n-3 DPA. The proportion of DHA was highest in fish fed MGK (18.6%), lowest in fish fed ECO (4.6%), and intermediate in fish fed DCO (8.8%). In contrast, the percentage of EPA was higher in fish fed MGK and ECO (7.9% and 7.8%, respectively) compared to fish fed DCO (5.1%). The proportion of LNA was higher in fish fed ECO and DCO than fish fed MGK. Total n-6 PUFA, including both LA and arachidonic acid (ARA; 20:4n-6), was higher in fish fed ECO and DCO (about 20%) than in fish fed MGK (9%). Total saturated fatty acids were higher in fish fed MGK (27.3%) compared to fish fed ECO and DCO (about 20%), mainly due to lower levels of 16:0 in the experimental feeds. Total monoenes were highest in fish fed ECO (32.2%), mainly due to 18:1n-9 and 20:1n-9 levels, followed by fish fed MGK (29.6%) and DCO (26.7%).

Table 4. Total lipid contents (% wet weight) and fatty acid compositions (% of total fatty acids) of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles and muscle after 18 days of feeding the reference (MGK) and experimental feeds containing GM *Camelina sativa* oils containing high EPA (ECO) or EPA + DHA (DCO).

	MGK			**ECO**			**DCO**		
Whole fish									
Total lipid (% wet weight)	14.5	±	0.4	14.1	±	0.6	14.3	±	0.2
Fatty acids (% total fatty acids)									
Total saturated [1]	27.3	±	0.3 [a]	20.3	±	1.3 [b]	19.9	±	0.5 [b]
18:1n-9	13.6	±	0.3 [b]	18.3	±	0.4 [a]	14.2	±	0.4 [b]
Total monounsaturated [2]	29.6	±	1.0 [b]	32.2	±	0.6 [a]	26.7	±	0.6 [c]
18:2n-6	5.7	±	0.1 [c]	14.5	±	0.2 [b]	16.2	±	0.0 [a]
20:4n-6	1.8	±	0.2 [b]	2.9	±	0.1 [a]	2.2	±	0.1 [b]
Total n-6 PUFA [3]	9.0	±	0.4 [d]	20.4	±	0.2 [b]	21.4	±	0.1 [a]

18:3n-3	1.2	±	0.1 [b]	10.5	±	0.8 [a]	11.2	±	0.1 [a]
20:5n-3	7.9	±	0.1 [a]	7.8	±	0.7 [a]	5.1	±	0.2 [b]
22:5n-3	2.7	±	0.0 [b]	1.1	±	0.0 [c]	3.6	±	0.1 [a]
22:6n-3	18.6	±	0.9 [a]	4.6	±	0.4 [c]	8.8	±	0.5 [c]
Total n-3 PUFA [4]	32.5	±	0.7 [a]	26.4	±	1.9 [b]	31.3	±	0.8 [a]
n-3/n-6	3.6	±	0.2 [a]	1.3	±	0.0 [b]	1.5	±	0.1 [ab]
EPA/DHA	2.3	±	0.1 [a]	0.6	±	0.0 [c]	1.7	±	0.1 [b]
Muscle									
Total lipid (% wet weight)	1.6	±	0.0	1.7	±	0.2	1.8	±	0.2
Fatty acids (% total fatty acids)									
Total saturated [1]	25.6	±	2.0	23.9	±	2.2	22.5	±	1.1
18:1n-9	11.7	±	1.8 [b]	16.9	±	1.9 [a]	12.3	±	0.2 [b]
Total monounsaturated [2]	23.4	±	3.4 [b]	28.6	±	1.7 [a]	23.2	±	0.7 [b]
18:2n-6	4.8	±	0.2 [c]	12.3	±	0.4 [b]	13.9	±	0.4 [a]
20:4n-6	2.5	±	0.3 [b]	4.5	±	0.3 [a]	3.0	±	0.2 [b]
Total n-6 PUFA [3]	9.2	±	0.0 [b]	19.4	±	0.8 [a]	19.9	±	0.5 [a]
18:3n-3	0.8	±	0.0 [b]	6.3	±	1.1 [a]	7.8	±	0.6 [a]
20:5n-3	7.4	±	0.3 [a]	8.4	±	1.0 [a]	5.6	±	0.1 [b]
22:5n-3	2.6	±	0.1 [b]	1.4	±	0.1 [c]	4.2	±	0.1 [a]
22:6n-3	28.1	±	1.4 [a]	9.0	±	1.6 [c]	14.1	±	0.9 [b]
Total n-3 PUFA [4]	40.2	±	1.1 [a]	26.8	±	2.4 [c]	33.8	±	0.7 [b]
n-3/n-6	4.3	±	0.1 [a]	1.4	±	0.1 [c]	1.7	±	0.1 [b]
EPA/DHA	3.8	±	0.0 [a]	1.1	±	0.2 [c]	2.5	±	0.1 [b]

Results are mean values (n = 3). Different superscript letters denote significant differences among fish fed the different dietary treatments ($p \leq 0.05$). An SD marked 0.0 implies 0.0 < SD < 0.05. [1] Includes 14:0, 15:0, 20:0, 22:0 and 24:0; [2] Includes 16:1n-9, 17:1, 20:1n-11, 20:1n-7 and 22:1n-9; [3] Includes 16:0 DMA, 18:0 DMA and 18:1 DMA; [4] Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6. DMA, dimethyl acetal; PUFA, polyunsaturated fatty acids.

3.3. Tissue Lipid Contents and Fatty Acid Compositions

The total lipid content was highest in the liver of fish fed ECO (17.9%), followed by those fed DCO (13.8%) and finally those fed MGK (7.1%) (Table 5). The proportion of DHA in the liver was highest in fish fed MGK, lowest in fish fed ECO and intermediate in those fed DCO, while there was no significant difference in EPA levels (Table 5). The levels of the C_{18} PUFA, LNA and LA, and total n-6 PUFA were significantly higher in the liver of fish fed ECO and DCO compared to fish fed MGK. Total saturated fatty acids were higher in fish fed MGK than fish fed ECO and DCO, mainly due to higher levels of 16:0 and 18:0. Total monoenes in the liver showed a similar pattern as in whole fish with the rank order being ECO > MGK > DCO although the differences were not statistically significant due to variation.

Table 5. Total lipid contents (% wet weight) and fatty acid compositions (% of total fatty acids) of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles liver and intestine after 18 days of feeding the reference (MGK) and experimental feeds containing GM *Camelina sativa* oils containing high EPA (ECO) or EPA + DHA (DCO) compared to the commercial reference diet (MGK).

	MGK			**ECO**			**DCO**		
Liver									
Total lipid (% wet weight)	7.1	±	3.5 [b]	17.9	±	2.3 [a]	13.8	±	1.6 [b]
Fatty acids (% total fatty acids)									
Total saturated [1]	30.6	±	4.2 [a]	17.6	±	2.8 [b]	16.1	±	0.8 [b]
18:1n-9	10.7	±	4.4 [b]	23.2	±	4.4 [a]	15.9	±	0.7 [ab]
Total monounsaturated [2]	23.1	±	9.1	37.8	±	7.4	28.5	±	1.1

18:2n-6	5.6	±	1.9 [b]	17.1	±	1.4 [a]	19.9	±	0.4 [a]
20:4n-6	3.2	±	1.1	2.0	±	0.7	1.8	±	0.1
Total n-6 PUFA [3]	10.3	±	0.9 [b]	22.6	±	2.8 [a]	25.3	±	0.2 [a]
18:3n-3	0.8	±	0.6 [b]	10.2	±	2.4 [a]	12.7	±	0.9 [a]
20:5n-3	7.8	±	0.5	6.2	±	2.8	5.1	±	0.4
22:5n-3	2.1	±	0.6 [b]	0.8	±	0.3 [c]	3.8	±	0.2 [a]
22:6n-3	22.7	±	7.6 [a]	1.8	±	0.9 [c]	5.3	±	0.3 [b]
Total n-3 PUFA [4]	35.1	±	5.5	21.8	±	7.4	30.1	±	1.9
n-3/n-6	3.5	±	0.8 [a]	0.9	±	0.2 [b]	1.2	±	0.1 [b]
DHA/EPA	3.0	±	1.1 [a]	0.3	±	0.0 [c]	1.1	±	0.1 [b]
Intestine									
Total lipid (% wet weight)	3.2	±	0.2	4.0	±	0.9	3.7	±	0.6
Fatty acids (% total fatty acids)									
Total saturated [1]	32.2	±	0.9 [a]	24.3	±	1.4 [b]	26.2	±	3.9 [ab]
18:1n-9	8.9	±	0.1 [b]	15.2	±	0.8 [a]	11.9	±	2.1 [b]
Total monounsaturated [2]	18.5	±	1.1 [b]	26.0	±	1.7 [a]	21.7	±	4.0 [ab]
18:2n-6	3.6	±	0.2 [b]	13.4	±	0.9 [a]	14.0	±	2.5 [a]
20:4n-6	4.3	±	0.1	5.7	±	0.5	4.3	±	1.4
Total n-6 PUFA [3]	9.8	±	0.3 [b]	21.9	±	0.2 [a]	21.1	±	1.5 [a]
18:3n-3	0.4	±	0.1 [b]	8.9	±	1.3 [a]	8.5	±	3.9 [a]
20:5n-3	6.6	±	0.2	7.1	±	0.7	4.6	±	0.3
22:5n-3	1.7	±	0.1 [b]	1.3	±	0.2 [b]	12.5	±	4.9 [b]
22:6n-3	27.2	±	0.2 [a]	7.5	±	1.2 [b]	12.5	±	4.9 [b]
Total n-3 PUFA [4]	36.7	±	0.1 [a]	26.3	±	0.9 [c]	30.0	±	1.1 [b]
n-3/n-6	3.7	±	0.1 [a]	1.2	±	0.1 [b]	1.4	±	0.1 [b]
DHA/EPA	4.1	±	0.1 [a]	1.1	±	0.1 [b]	2.7	±	1.0 [a]

Results are mean values (n = 3). Different superscript letters denote significant differences among fish fed the different dietary treatments ($p \leq 0.05$). An SD marked 0.0 implies 0.0 < SD < 0.05. [1] Includes 14:0, 15:0, 20:0, 22:0 and 24:0; [2] Includes 16:1n-9, 17:1, 20:1n-11, 20:1n-7 and 22:1n-9; [3] Includes 16:0 DMA, 18:0 DMA and 18:1 DMA; [4] Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6; PUFA, polyunsaturated fatty acids.

The total lipid content of the intestine of ABT juveniles showed no significant differences among diets, with average values ranging between 3.2% and 4.0% (Table 5). The proportions of DHA in intestinal lipids were in the rank order MGK (27.2%) > DCO (12.5%) > ECO (7.5%) although the difference between ECO and DCO was not significant (Table 5). Similarly, the levels of EPA did not show statistically significant differences among treatments. Intestines from fish fed ECO and DCO showed higher values of LNA, LA and total n-6 PUFA compared to fish fed MGK. Total saturated fatty acids were significantly higher in fish fed MGK compared to fish fed ECO, with fish fed DCO intermediate and not different to either. In contrast, total monoenes were higher in fish fed ECO compared to fish fed MGK with fish fed DCO intermediate and not different to either.

The total lipid content of muscle was about 1.7% of wet weight and no significant differences were observed among treatments (Table 4). The proportions of DHA and EPA in the muscle total lipid generally reflected those of their respective diets. Thus, the level of DHA was highest in fish fed MGK, followed by those fed DCO and lastly those fed the ECO diet, with the same trend presented by the n-3/n-6 and DHA/EPA ratios (Table 4). The level of EPA was higher in the muscle of fish fed MGK and ECO compared to fish fed DCO. The percentages of LNA, LA and total n-6 PUFA were all higher in the muscle of fish fed ECO and DCO compared to fish fed MGK. Muscle total lipid showed no significant differences in total saturated fatty acids among the dietary treatments, but

18:1n-9 and total monounsaturated fatty acids were significantly higher in the muscle of fish fed ECO, compared to fish fed MGK and DCO.

The total lipid content in the brain was higher than in muscle at about 7% on a wet weight basis but there was no significant effect of diet (Table 6). The proportions of DHA and the DHA/EPA and n-3/n-6 ratios in the brain were all significantly affected by diet with the rank order being MGK > DCO > ECO (Table 6). However, the level of EPA was significantly higher in the brain of fish fed ECO compared to fish fed MGK or DCO. The proportions of the C_{18} PUFA, LNA and LA, were lower in the brain than in other tissues, but still significantly higher in fish fed ECO and DCO compared to fish fed MGK. Neither total saturated nor total monounsaturated fatty acids, mainly represented by their respective major components (16:0, 18:0 and 18:1n-9), showed any significant differences among dietary treatments.

Table 6. Total lipid contents (% wet weight) and fatty acid compositions (% of total fatty acids) of brain and eyes of Atlantic bluefin tuna (*Thunnus thynnus* L.) after 18 days of feeding the experimental feeds containing GM *Camelina sativa* oils with either high EPA (ECO) or EPA + DHA (DCO) compared to the commercial reference diet (MGK).

	MGK			**ECO**			**DCO**		
Brain									
Total lipid (% WW)	7.6	±	0.5	7.0	±	0.6	6.8	±	0.5
Fatty acids (% total FA)									
Total saturated [1]	36.3	±	0.5	35.6	±	1.5	36.5	±	0.7
18:1n-9									
Total monounsaturated [2]	22.2	±	2.4	25.1	±	1.4	22.3	±	1.2
18:2n-6	1.3	±	0.0 ^{b}	4.2	±	0.3 ^{a}	4.0	±	0.5 ^{a}
20:4n-6	1.7	±	0.1 ^{c}	3.2	±	0.0 ^{a}	2.3	±	0.2 ^{b}
Total n-6 PUFA [3]	4.0	±	0.0 ^{c}	9.3	±	0.5 ^{a}	7.9	±	0.6 ^{b}
18:3n-3	0.2	±	0.0 ^{b}	2.0	±	0.3 ^{a}	1.8	±	0.5 ^{a}
20:5n-3	2.5	±	0.0 ^{b}	4.2	±	0.3 ^{a}	2.4	±	0.2 ^{b}
22:5n-3	2.2	±	0.0 ^{b}	3.8	±	0.1 ^{a}	3.9	±	0.3 ^{a}
22:6n-3	27.6	±	1.6 ^{a}	15.0	±	1.4 ^{c}	20.6	±	0.8 ^{b}
Total n-3 PUFA [4]	33.5	±	1.3 ^{a}	26.3	±	1.1 ^{c}	29.8	±	1.3 ^{b}
n-3/n-6	8.4	±	0.3 ^{a}	2.8	±	0.3 ^{c}	3.8	±	0.2 ^{b}
DHA/EPA	11.2	±	0.4 ^{a}	3.6	±	0.5 ^{c}	8.6	±	0.6 ^{b}
Eye									
Total lipid (% WW)	1.5	±	0.1	1.8	±	0.3	2.0	±	0.2
Fatty acids (% total FA)									
Total saturated [1]	29.0	±	0.2 ^{a}	27.6	±	2.6 ^{ab}	24.2	±	1.1 ^{b}
18:1n-9	13.6	±	0.7 ^{ab}	15.9	±	1.5 ^{a}	13.3	±	0.6 ^{b}
Total monounsaturated [2]	22.7	±	1.6	26.2	±	2.4	23.4	±	0.6
18:2n-6	3.3	±	0.4 ^{c}	98.1	±	0.7 ^{b}	11.9	±	0.6 ^{a}
20:4n-6	1.9	±	0.2 ^{b}	3.4	±	0.3 ^{a}	2.3	±	0.1 ^{b}
Total n-6 PUFA [3]	6.3	±	0.1 ^{c}	14.9	±	0.8 ^{b}	16.7	±	0.7 ^{a}
18:3n-3	0.6	±	0.1 ^{c}	5.6	±	1.3 ^{b}	8.1	±	0.8 ^{a}
20:5n-3	4.7	±	0.2 ^{b}	6.1	±	0.9 ^{a}	4.5	±	0.2 ^{b}
22:5n-3	1.7	±	0.1 ^{b}	1.8	±	0.1 ^{b}	3.0	±	0.1 ^{a}
22:6n-3	31.3	±	2.1 ^{a}	14.7	±	3.2 ^{b}	16.9	±	0.9 ^{b}
Total n-3 PUFA [4]	39.6	±	2.0 ^{a}	29.8	±	4.2 ^{b}	34.7	±	0.4 ^{ab}
n-3/n-6	6.3	±	0.4 ^{a}	2.0	±	0.2 ^{b}	2.1	±	0.1 ^{b}
DHA/EPA	6.6	±	0.2 ^{a}	2.4	±	0.5 ^{c}	3.8	±	0.3 ^{b}

Results are presented as means ± SD (n = 3). An SD marked 0.0 implies 0.0 < SD < 0.05. Different superscript letters denote significant differences among fish fed the different diets ($p \leq 0.05$). [1]

Includes 14:0, 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0; [2] Includes 16:1n-7, 16:1n-9, 17:1, 18:1n-7, 20:1n-9, 20:1n-11, 20:1n-7, 22:1n-9, 22:1n-11 and 24:1n-9; [3] Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6; [4] Includes 18:4n-3, 20:3n-3 and 20:4n-3. PUFA, polyunsaturated fatty acids.

The total lipid content of the eyes was around 1.8% on a wet weight basis and showed no significant differences among dietary treatments (Table 6). The proportion of DHA was higher in lipids in the eyes of fish fed MGK (31.3%) and around double the level of DHA level in the eyes of fish fed both ECO and DCO (14.7% and 16.9% in fish fed ECO and DCO diets, respectively) (Table 6). Similar to the brain, EPA was significantly higher in the eyes of fish fed ECO compared to fish fed MGK and DCO. The proportions of the C_{18} PUFA, LNA and LA, and total n-6 PUFA were significantly higher in the eyes of fish fed ECO and DCO compared to fish fed MGK. Total saturated fatty acids in the eye lipids were significantly higher in fish fed MGK, than fish fed DCO with the level in fish fed ECO intermediate and not significantly different to either. Total monoenes showed no significant differences among the dietary treatments.

3.4. Effect of Diet on Gene Expression

3.4.1. Transcript Abundance of Fatty Acid and LC-PUFA Biosynthesis Genes in Liver and Intestine

The expression of the *fads2d6* gene in the liver was upregulated in fish fed ECO and DCO compared to fish fed MGK and was also higher in fish fed ECO compared to fish fed DCO (Figure 1). While the expression of the *elovl4* elongase gene in the liver of ABT fed DCO was significantly higher than in fish fed the ECO and MGK diets, the expression of *elovl5* was not affected by diet. The expression of the *fas* gene in the liver was significantly upregulated in fish fed ECO and DCO compared to fish fed MGK.

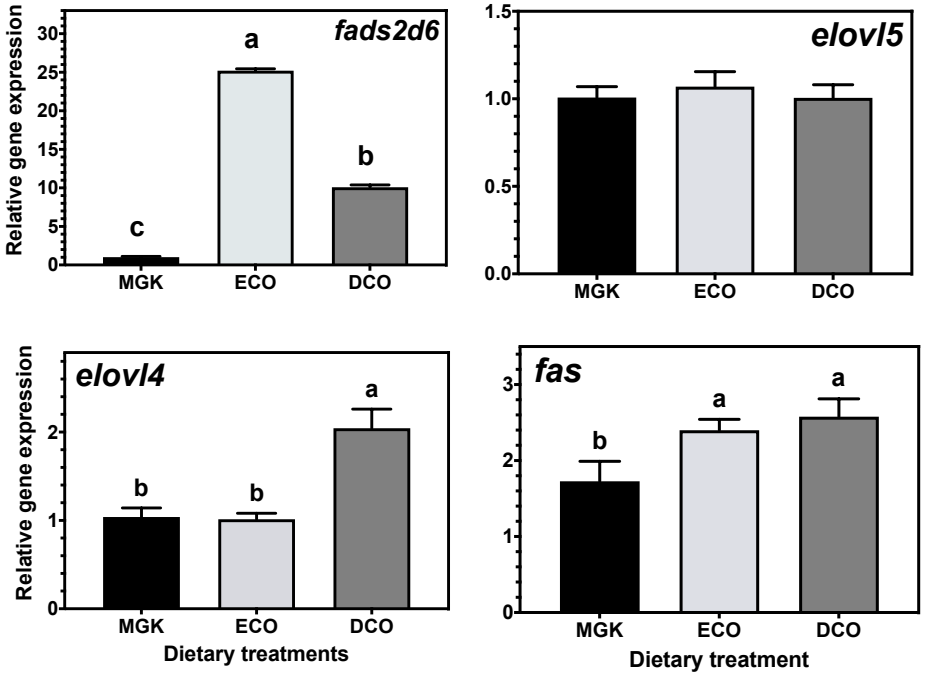

Figure 1. Nutritional regulation of delta-6 fatty acyl desaturase (*fads2d6*), fatty acyl elongases 4 (*elolv4*) and 5 (*elovl5*) and fatty acid synthase (*fas*), gene transcription in liver of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

The expression of the *fads2d6* gene in the intestine was also upregulated in fish fed both ECO and DCO compared to fish fed MGK although the highest expression was in fish fed DCO (Figure 2). The expression of the *elovl5* elongase gene in the intestine was also significantly highest in fish fed DCO, and lowest in fish fed ECO with fish fed MGK showing an intermediate expression level. In contrast, the expression of *elovl4* in the intestine did not show any significant differences among treatments. The expression of the *fas* gene in the intestine showed the same trend as in the liver and as for the *fads2d6* gene in the intestine, expression was highest in the fish fed diet DCO, lowest in the fish fed the reference diet, and intermediate in the fish fed ECO (Figure 2).

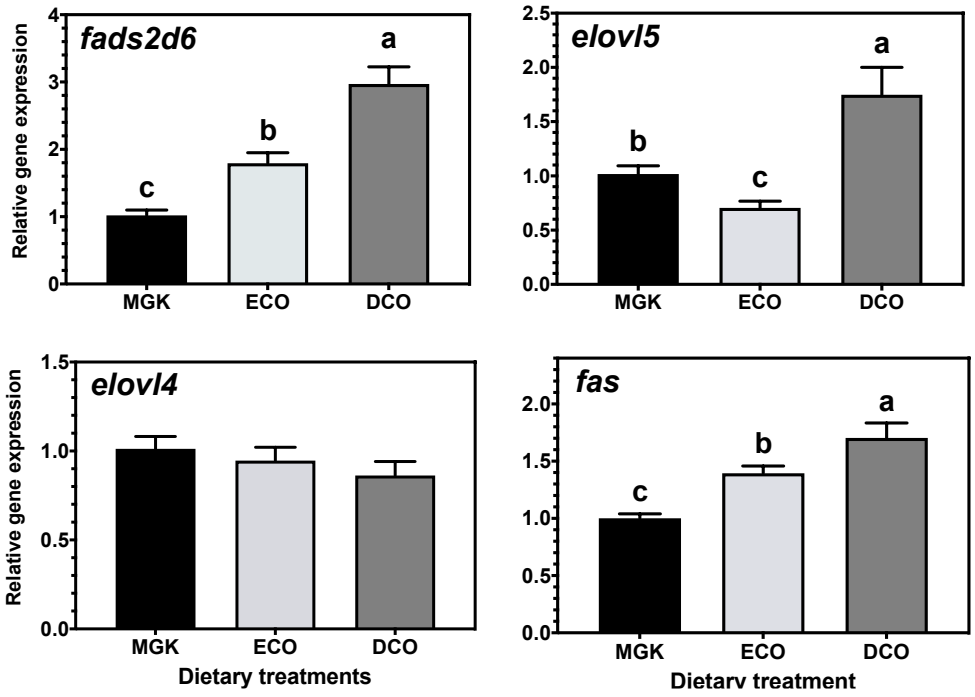

Figure 2. Nutritional regulation of delta-6 fatty acyl desaturase (*fads2d6*), fatty acyl elongases 4 (*elolv4*) and 5 (*elovl5*) and fatty acid synthase (*fas*), gene transcription in intestine of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

3.4.2. Transcript Abundance of Lipid Metabolism Genes in Liver and Intestine

The expression levels of *aco* and *lpl* genes were significantly lower in the liver of fish fed ECO and DCO compared to fish fed MGK (Figure 3). In contrast, the expression levels of the *hmgcl* and *cpt1* genes were significantly upregulated in the liver of fish fed ECO compared to fish fed both MGK and DCO.

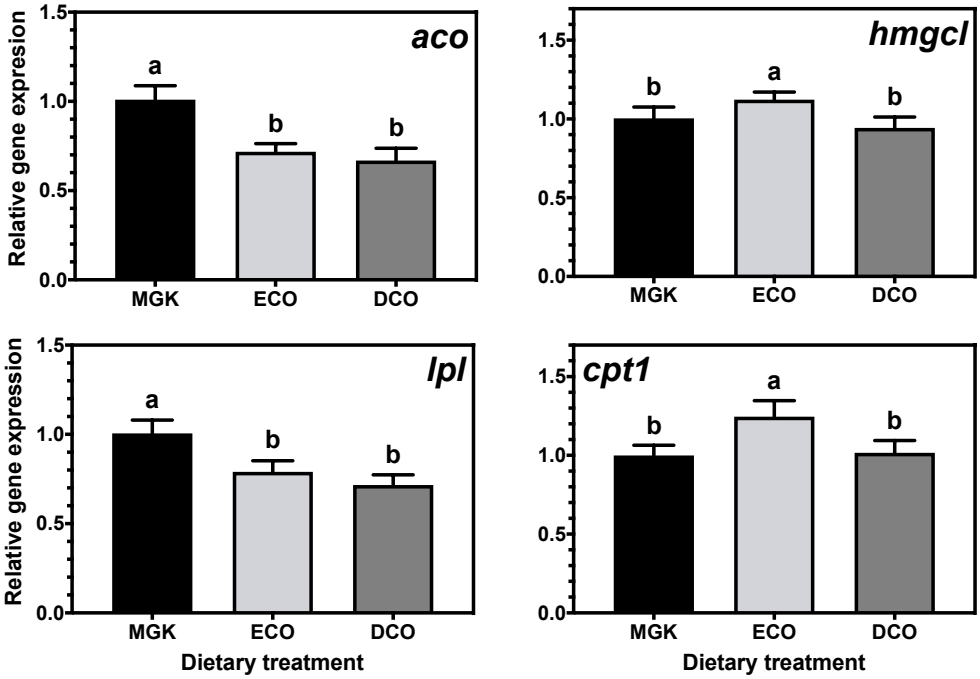

Figure 3. Nutritional regulation acyl coA oxidase (*aco*), lipoprotein lipase (*lpl*), 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*) and carnitine palmitoyl transferase I (*cpt1*) gene transcription in liver of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

Similar to the liver, the expression of the *aco* and *lpl* genes in the intestine presented the same pattern but, in contrast to the liver, both genes were significantly upregulated in the intestine of fish fed diet DCO compared to fish fed the MGK and ECO diets (Figure 4). While the expression of *hmgcl* in the fish intestine was not significantly affected by diet, the expression of *cpt1* in the intestine was higher in the fish fed diet DCO compared to fish fed the ECO diet, with fish fed MGK being intermediate and not different to either.

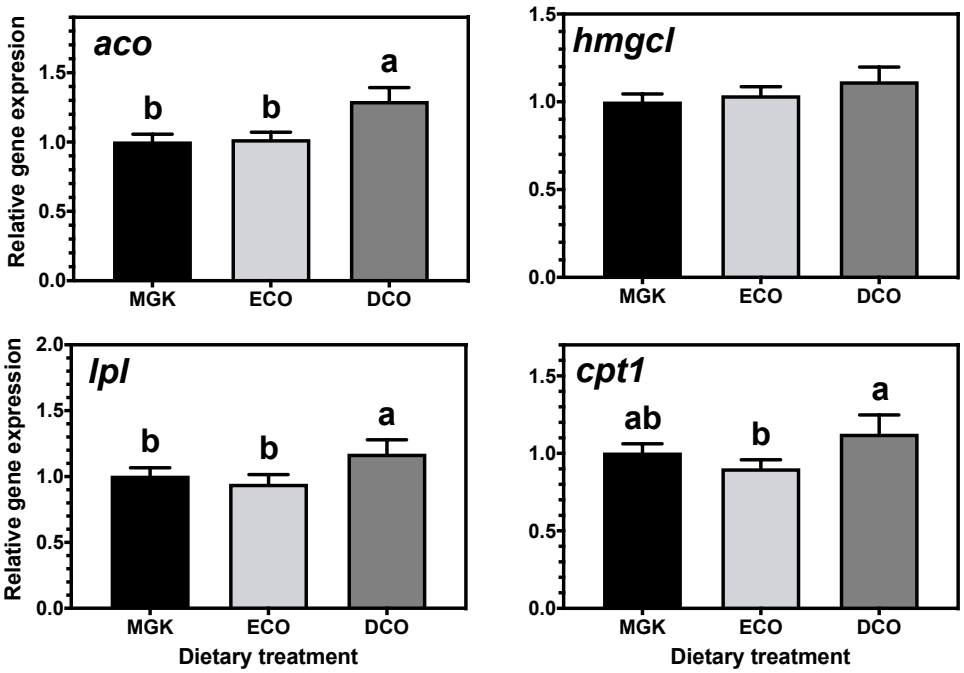

Figure 4. Nutritional regulation acyl coA oxidase (*aco*), lipoprotein lipase (*lpl*), 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*) and carnitine palmitoyl transferase I (*cpt1*) gene transcription in intestine of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

3.4.3. Transcript Abundance of Nuclear Receptor Genes in Liver and Intestine

The expression levels of sterol regulatory-element binding protein genes, *srebp1* and *srebp2*, in the liver fed both ECO and DCO were significantly higher compared to the expression of the genes in the liver of fish fed MGK (Figure 5). On the contrary, the expression levels of the *lxr* and *rxr* transcription factors in the liver were lower in fish fed both the test diets (ECO and DCO) compared to fish fed the MGK reference diet. The expression of *pparα* in the liver was significantly upregulated in fish fed DCO compared to fish fed ECO and MGK whereas, in contrast, the expression of *pparγ* was downregulated in the liver of fish fed the DCO diet compared to fish fed both the ECO and MGK diets.

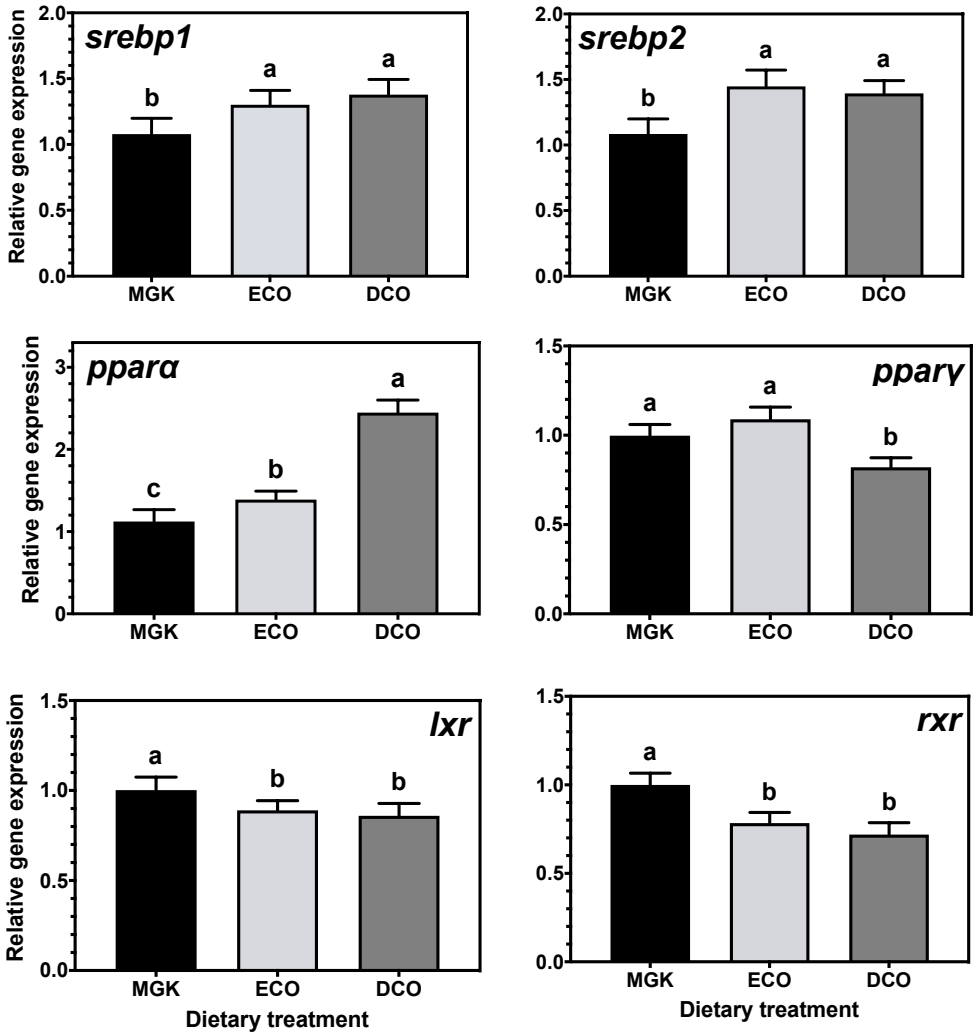

Figure 5. Nutritional regulation of sterol regulatory element-binding protein 1 and 2 (*srebp1*and *srebp2* respectively), peroxisome proliferator-activated receptor alpha (*pparα*), gamma (*pparγ*), liver X receptor (*lxr*) and retinoid X receptor (*rxr*) gene transcription in liver of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

While the expression of *srebp1* in the intestine was significantly upregulated in fish fed both test diets, especially ECO, compared to fish fed MGK, the expression of *srebp2* showed no significant effect of the diet (Figure 6). The expression levels of *pparα* and *pparγ* showed similar patterns in the intestine, being higher in fish fed MGK compared to fish fed ECO, with fish fed DCO showing intermediate levels, not different to fish fed either MGK or ECO.

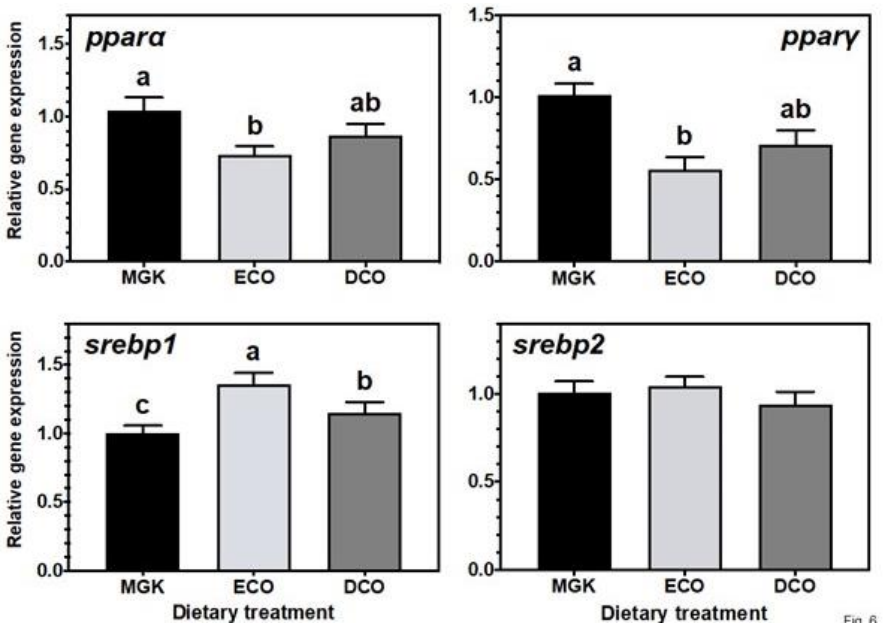

Figure 6. Nutritional regulation of sterol regulatory element-binding protein 1 and 2 (*srebp1*and *srebp2* respectively), peroxisome proliferator-activated receptor alpha (*pparα*) and gamma (*pparγ*) gene transcription in intestine of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

3.4.4. Abundance of Transcripts of Antioxidant Defence Enzyme Genes in Liver

The expression levels of the *cat* and *sod* genes in the liver were significantly upregulated in fish fed DCO compared to fish fed ECO and MGK (Figure 7). While the expression level of the *gpx1* gene was relatively low and showed no significant differences among dietary treatments, the expression of the *gpx4* gene in the liver was significantly upregulated in fish fed both ECO and DCO, especially ECO, compared to fish fed MGK.

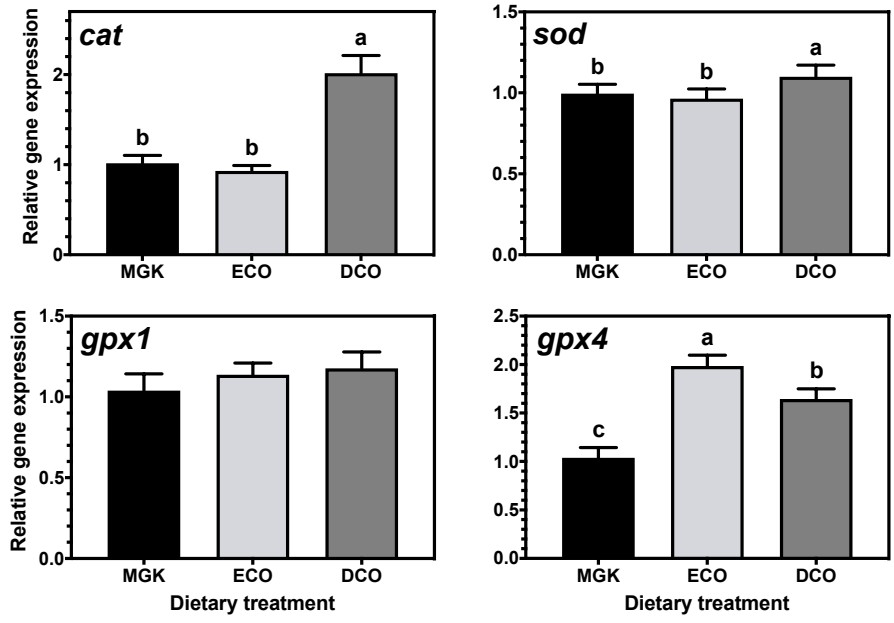

Figure 7. Nutritional regulation of catalase (*cat*), superoxide dismutase (*sod*) and glutathione peroxidase 1 and 4 (*gpx1* and *gpx4*, respectively) gene transcription in liver of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing

either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

3.4.5. Transcript Abundance of Digestive Enzyme Genes in Intestine

The expression of *amino peptidase* gene (*anpep*) in the intestine was downregulated in fish fed ECO and DCO, significantly so with DCO, compared to fish fed MGK (Figure 8). In contrast, *trypsin* (*tryp*), *phospholipase A2* (*pla2*) and *bile salt-activated lipase 2* (*bsl2*) were all upregulated in the intestine of fish fed ECO and DCO compared to fish fed MGK. Furthermore, expression levels of *alkaline phosphatase* (*alp*) and *pancreatic lipase* (*pl*) were upregulated in fish fed diet DCO compared to fish fed the other diets. No significant differences were observed in the expression of *amylase* (*amy*) and *bile salt activated lipase 1* (*bsl1*) in response to diet.

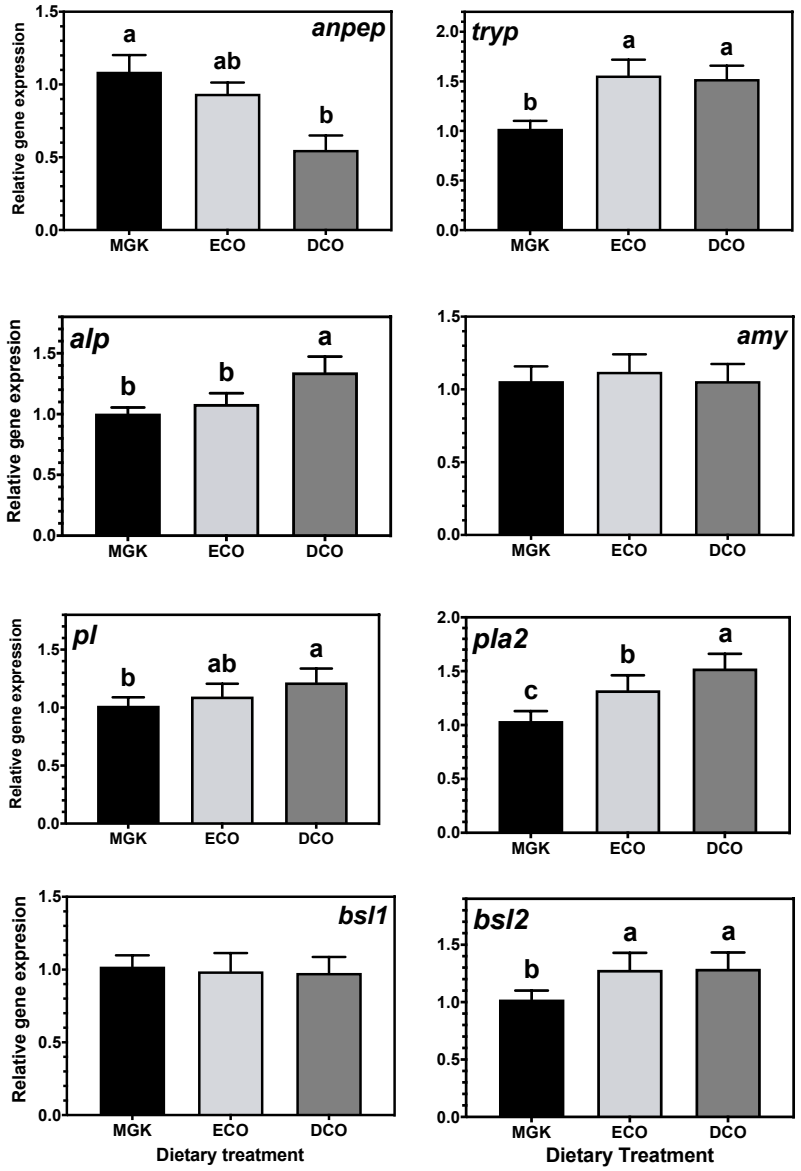

Figure 8. Nutritional regulation of amino peptidase (*anpep*), trypsin (*tryp*), alkaline phosphatase (*alp*), amylase (*amy*), pancreatic lipase (*pl*), phospholipase A2 (*pla2*), bile salt activated lipase 1 (*bsl1*) and bile salt activated lipase 2 (*bsl2*) digestive enzyme gene transcription in intestine of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed a reference diet Magokoro® (MGK) and two test diets containing either EPA (ECO) or EPA + DHA (DCO) oils from transgenic *Camelina sativa* seeds. Values are normalised expression ratios to MGK reference diet and are means ± SD (n = 6). Values

with different superscript letters are significantly different (one-way ANOVA and Tukey test; $p < 0.05$).

3.5. Histological Evaluation

The liver parenchyma did not show any structural abnormalities or melanomacrophage centers among fish fed the different dietary treatments. The storage of lipids was heterogeneous among the dietary groups, with fish fed ECO showing a significantly higher area percentage covered by lipid vacuoles compared to fish fed MGK, with fish fed DCO showing intermediate values (Table 7). Histological examination of the intestine showed no differences in any of the evaluated parameters among fish fed the different diets, with only random specimens showing a moderate degradation of the epithelium and infiltration of immune cells in the mucosa (Table 7).

Table 7. Histological parameters measured in intestine samples.

	MGK	ECO	DCO
LV	5.3 ± 0.5 ^{b}	13.5 ± 2.7 ^{a}	10.6 ± 2.3 ^{ab}
hE	24.6 ± 1.2	25.0 ± 1.3	23.4 ± 3.0
nNE	21.5 ± 1.7	21.3 ± 1.4	20.7 ± 2.1
Lmax	1952.5 ± 29.4	2012.3 ± 30.7	2006.0 ± 42.7
Lmin	1758.1 ± 87.5	1866.5 ± 11.6	1723.4 ± 228.9
CMT	145.6 ± 15.6	142.2 ± 14.5	128.4 ± 8.7
GB	137.6 ± 7.6	133.3 ± 37.9	155.8 ± 36.8

LV, liver vacuolisation; hE, enterocytes height (μm); nNE, enterocyte width; Lmax and Lmin, maximum and minimum intestine lengths on a transverse section (μm); CMT, circular muscle thickness (μm); GB, goblet cells per 100,000 μm^2. ECO, feed containing high EPA Camelina oil; DCO, feed containing EPA + DHA Camelina oil; MGK, commercial reference diet. Different superscript letters denote significant differences among fish fed the different diets ($p \leq 0.05$).

4. Discussion

New, scalable sources of DHA and EPA that can increase the declining n-3 LC-PUFA contents of commercial feeds and, at the same time, do not compromise growth or feed efficiency are required to ensure the sustainable growth of aquaculture. A viable and feasible new source of EPA and DHA in aquafeeds could be the genetic modification of oilseed crops [8]. In the present study, the ability of oils derived from GM-*Camelina sativa*, containing either EPA or EPA + DHA, to promote growth, support tissue n-3 LC-PUFA levels, and impact metabolism were evaluated in ABT for the first time, and benchmarked against commercial feed formulated with marine FO that has been used successfully with PBT.

In the present study, values for growth performance, feed utilisation and survival were better than those obtained previously in ABT juveniles [39] and similar to studies in PBT juveniles of the same size [59]. While some parameters (weight gain %, FCR and survival) showed no difference among treatments, others (final length and weight, SGR, CF, total intake, protein and energy retention) showed significant differences, mostly in the rank order MGK (reference) > DCO ≥ ECO. In contrast, DFR and lipid retention were lower in ABT fed the reference diet than in fish fed the test diets. However, despite the differences, it was clear both test feeds supported good growth and feed efficiency with the DCO diet containing higher DHA performing slightly better than diet ECO. No significant differences among diets were observed in terms of survival, partly due to high variability among treatments. However, studies in PBT juveniles showed that the major mortality at this stage is normally not associated with dietary deficiencies but with stress responses to external stimuli (light, noise, etc.) often resulting in collisions with the tank wall and death of the fish [59–61].

Diet did not affect gross composition (protein, lipid and carbohydrate) or caloric content of whole ABT and there were no differences in lipid contents of the intestine, muscle, brain and eyes among the dietary treatments. Related to the latter, it was noteworthy that *fas* expression (and likely lipogenesis) was upregulated in ABT fed the test feeds, probably in response to the slightly lower lipid content (~15%) of these feeds compared to the reference diet (17%). The lipid content of the liver was higher than in the other tissues reflecting lipid storage in this organ and was increased in fish fed the test diets compared to ABT fed the reference diet. In agreement, hepatocyte lipid vacuolisation was higher in ABT fed the test diets than in fish fed the commercial reference diet. This is a common outcome in many fish, especially marine species when fed VOs [39,62–64]. This may suggest that the fatty acid profiles of VOs could be less efficiently utilised by tuna as a source of metabolic energy, and thus more accumulated in the liver although the precise mechanism is unclear [65,66].

As expected, fatty acid compositions of whole fish and tissues generally reflected those of diets [67]. In whole fish, it was clear that ABT fed the test diets accumulated only around half as much n-3 LC-PUFA as fish fed the high marine ingredient reference diet, and fish fed the DCO diet had a higher DHA level and DHA/EPA ratio than fish fed the ECO diet. However, irrespective of diet, the proportions of total n-3 LC-PUFA and DHA, and DHA/EPA ratio were higher in ABT than in the diet. Therefore, the test diets formulated with GM-*Camelina* oils performed similarly to the reference feed formulated with FO in terms of supply of n-3 LC-PUFA, within the constraints of the lower level of DHA and EPA present in the test feeds. This was consistent with the results obtained previously in similar studies using feeds formulated with these *Camelina* oils in other fish species such as Atlantic salmon, gilthead sea bream and European sea bass [14–18].

Levels of LC-PUFA were similar in the intestine and muscle, and higher compared to levels in the liver, which may reflect the lower lipid levels and thus higher proportions of polar lipids in these tissues. It is well known that EPA and DHA tend to be selectively incorporated into polar lipids that are less responsive to diet in comparison to storage lipids [4,67,68]. In a recent study, marine fish showed net gain and selective deposition of LC-PUFA in their tissues, despite having very limited capacity for endogenous biosynthesis [68]. However, the impact of the different diets was similar in all tissues with the levels of n-3 LC-PUFA being lower in ABT fed the test diets compared to fish fed the reference diet, with tissues of fish fed the DCO diet showing higher DHA and DHA/EPA ratio than fish fed the ECO diet, all in line with the dietary levels of the fatty acids.

While still showing the above pattern, the fatty acid compositions of the brain and eye proved to be the least plastic, with fewer fatty acids showing significant differences among the different diets and the magnitude of the differences being lower than in other tissues. The neural/visual tissues also presented the highest levels of n-3 LC-PUFA, particularly DHA, reflecting the specific fatty acid requirements and metabolism of these tissues, and that their fatty acid profiles are under strict physiological control and, therefore, less representative of diet [28,68]. The specific accumulation of dietary and/or biosynthesised DHA (and ARA) in fish neural tissues, particularly during the development of the brain and retina, is well known [24–29]. This reflects the importance of DHA for neural and visual functions which is supported by the fact that diets deficient in DHA can lead to visual and behavioral impairments that are particularly critical in top visual predators such as ABT [69,70].

The impacts of feeds with low levels of marine ingredients and, thus, low levels of n-3 LC-PUFA on metabolic responses and the nutritional quality of ABT for human consumption are largely unknown. Marine fish evolved in LC-PUFA-rich food webs where there was insufficient selection pressure to develop and/or maintain the enzymatic capacity to biosynthesize LC-PUFA de novo in most species. Indeed, it was demonstrated that the biosynthetic capacity of LC-PUFA from C_{18} PUFA is very restricted in bluefin tuna species [31–33] although the production of DHA from EPA and/or 22:5n-3 is likely possible [22]. The liver and intestine are generally two of the most active tissues in terms

of lipid metabolism and both have been shown to be important sites for LC-PUFA biosynthesis in fish [16,22,67,68,71]. In the present study, the expression of genes of LC-PUFA biosynthesis showed nutritional regulation in response to diet, with increased expression of *fads2d6* in both tissues, and *elovl4* and *elovl5* in the liver and intestine, respectively, in fish fed the test feeds compared to the reference diet. The upregulation of the expression of the *fads2d6* gene in both tissues in fish fed the test diets likely reflects the lower DHA in these diets and thus points to a slight deficiency for ABT juveniles. Thus, the DHA requirement for ABT juveniles is likely above the amount present in the higher of the test diets (DCO, 7.3 mg·g^{-1} dry mass), although it is not possible to speculate if the level in the reference diet (15.0 mg·g^{-1} dry mass) is sufficient.

Generally, when fish are fed low levels of n-3 LC-PUFA, the upregulation of *fads2d6* expression is observed, whereas reduced expression occurs when high dietary levels are ingested [15,72]. In the present study, there were differences between the ECO and DCO feeds in the extent of the upregulation in the different tissues. In the liver, *fads2d6* expression was upregulated by both test diets, but more in the liver of fish fed the ECO diet (lower in DHA) than in those fed DCO. Similar effects on liver *fads2d6* expression in response to transgenic *Camelina sativa* oils were found in Atlantic salmon [16], gilthead sea bream [18] and European sea bass [19]. In contrast, intestinal *fads2d6* expression was higher in ABT fed DCO than in fish fed ECO, with a similar trend reported in gilthead sea bream intestine, while no regulation was observed in European sea bass [18,19]. The response in ABT liver was consistent both with dietary DHA level and previous studies, whereas the response in the intestine was opposite to previous results where fadsd6 was upregulated in marine fish fed lower levels of DHA [18,73–75]. It was also noteworthy that *elovl5* expression was not nutritionally regulated in the liver but was downregulated in the intestine of ABT fed the ECO diet and upregulated in fish fed the DCO diet. Similarly, *elovl5* was not nutritionally regulated in the liver of salmon, gilthead sea bream and European sea bass [16,18,19]. In contrast, *elovl4* was similarly not regulated in the intestine but upregulated in the liver of ABT fed DCO. Irrespective of species or tissue, increased endogenous LC-PUFA biosynthesis capacity seldom compensates completely for the reduction in dietary LC-PUFA [68,76].

Nuclear receptors *pparα* and *srebp1* are implicated in catabolism and fatty acid biosynthesis, respectively [77,78], and their expression can be regulated by dietary fatty acids [79–81]. Specifically, n-3 LC-PUFA, including DHA, can act as ligands for *pparα* and *srebp1*, regulating LC-PUFA biosynthesis [82–85]. In the present study, expression levels of *srebp1* and *pparα* were upregulated in the liver of ABT fed the test diets that contained lower levels of EPA and DHA than the reference diet. In agreement, studies on numerous fish species showed that *srebp1* expression was upregulated when feeds contained low levels of n-3 LC-PUFA [18,32,35,85–87]. The upregulation of *srebp1* in ABT liver and intestine induced LC-PUFA synthesis by upregulating the *srebp1* target genes *fads2d6* and *elovl4* expression in the liver together with the upregulation of *fas*, *fads2d6* and *elovl5* in the intestine, as well as fatty acid synthesis by upregulating the expression of *fas*, with both genes showing a similar pattern of expression as shown previously in ABT larvae [40].

Similarly, the expression of *srebp2*, a regulator of cholesterol synthesis [88,89] was upregulated in the liver but not the intestine by both test diets. In agreement, the hepatic expression of *srebp2* was increased in rainbow trout fed plant-based diets [90] although, contrary to the present study, the expression of *lxr* was unaffected by dietary cholesterol levels. Lxr regulates bile acid biosynthesis from cholesterol in mammals [77] and previous studies in Atlantic salmon [91] and ABT [39] have shown upregulation of *lxr* when fish were fed diets containing VOs. Previously, Zhu et al. [90] explained the lack of dietary regulation of *lxr* and other genes involved in cholesterol excretion and bile acid synthesis by the short duration of the trial (10 weeks). In the present study, this was not the case, as *lxr* was downregulated in ABT fed low EPA + DHA levels after only two weeks, which might indicate species-specific regulation of cholesterol biosynthesis pathways.

Adipocyte function and differentiation are regulated by Pparγ which also has important roles in lipogenesis and lipid storage [34,50,92,93], and its expression was downregulated in the liver of ABT fed DCO and the intestine of fish fed ECO. This might indicate, as reported previously in ABT [39], that given the fast growth rate of ABT juveniles, most of the available dietary resources are used for energy to fuel anabolic processes, given that an increase in biomass prevails over lipid storage. The nuclear receptor RXR forms heterodimers with PPARγ among other NRs, to regulate the transcription of lipid metabolism genes, and its transcript levels are known to be decreased in vitro in response to dietary LA and LNA [66], which agrees with the results obtained in vivo in ABT in the present study.

In fish, an effective antioxidant system needs to operate in order to maintain health and prevent adverse effects of oxidation. In this study, the mRNA levels of antioxidant enzyme genes in the liver were influenced by diet. In this respect, *cat* and *sod* were upregulated in the liver of ABT fed DCO, which contained the highest of n-6 and n-3 PUFA and the lowest MUFA levels. It is known that high substitution of FO by VO can lead to reduced expression of both *cat* and *sod* [94]. This result is quite surprising given that both ECO and DCO oils could be considered as a "blend" of FO and VO, but still the expression profile between fish fed both treatments differed greatly. In contrast, the expression profile of *glutathione peroxidases* was generally similar between fish fed ECO and DCO. It has been demonstrated recently that the nuclear factor erythoid 2-related factor 2 (Nrf2) can modify mRNA levels of antioxidant response element (ARE)-containing genes such as *cat* and *sod* [95], which could explain why the direction of regulation diverged between these two enzymes and *gpx* in the liver of fish fed the GM oil derived feeds.

The expression of *gpx4*, a mitochondrial enzyme that acts mainly on peroxidised fatty acids of membrane phospholipids, was significantly downregulated in fish fed the commercial control diet with the highest levels of n-3 LC-PUFA. High dietary levels of EPA + DHA are known to suppress the hepatic expression of *gpx4* in sea bream [96], in agreement with the present results. This pattern of expression was not observed in the cytosolic enzyme *gpx1*, which could indicate that fish prime the in situ repair of oxidised phospholipids instead of the turnover of membrane phospholipids from the cytosolic pool, as had been speculated previously [96].

The intestinal expression of *amy* did not present any nutritional regulation in response to dietary treatment and had previously shown a lack of rhythmicity in ABT juveniles [97]. This is no surprise, as in carnivorous fish, such as ABT, lipid and protein pathways dominate intermediary metabolism given that carbohydrate is only a minor component of their natural diet [1,2,67]. In contrast, the expression of proteolytic digestive enzymes was impacted by dietary treatment. Given that the commercial control diet contained a different blend of raw materials, it is not surprising to find differences in the expression levels of *tryp* between ABT fed this treatment and fish fed the experimental diets, as has been found in other fish species [98,99]. The fish fed the DCO diet displayed the highest intestinal mRNA levels of *alp*. Previously, European sea bass fed VO diets displayed higher alkaline phosphatase activity than fish fed a FO-rich diet [62], which could be linked to the slower release of proteases into the intestinal lumen due to a decrease in transit rate in fish fed VO [100]. The opposite pattern of expression was observed for *anpep*, where DCO-fed fish displayed the lowest mRNA level. Another potential explanation for the differences observed in protease activities could be due to different levels of antinutritional factors present in the diets as it is known that the inhibition of proteases can be due to the presence of protease inhibitors.

Regarding the expression levels of lipases, the lowest expression levels of *pl*, *pla2* and *bsl2* were observed in the intestines of fish fed the reference MGK diet. In general terms, high expression of these lipases might be a direct response to the lower lipid levels of the two experimental feeds (15 vs. 17%) and/or high C_{18} PUFA and low LC-PUFA levels. Curiously, low expression levels of *pla2* have been associated with high dietary LNA

levels in Senegalese sole (*Solea senegalensis*) intestine, particularly after stress [101]. However, in the aforementioned trial, the levels of dietary DHA were similar among the treatments whereas in the present study they were 6.5- and 2.5-times higher in diet MGK compared to diets ECO and DCO, respectively. It is known that low dietary DHA levels can increase adiposity and plasma lipid levels [102] that, in turn, could explain the high expression levels of lipases in fish fed the diets with the lowest DHA (ECO and DCO). Apart from participating in phospholipid digestion and metabolism, PLA2 plays crucial roles in several other cellular responses such as providing precursors for eicosanoid production. Indeed, the expression of *pla2* has been used as an indicator of intestinal integrity in fish [103]. Nevertheless, despite the relatively high intestinal *pla2* mRNA levels found in the present study, no signs of inflammation were observed histologically in the intestine, which suggests that the upregulation of *pla2* in fish fed the experimental feeds is probably related to phospholipid metabolism rather than inflammatory processes.

5. Conclusions

The present study represents the first time that oils from transgenic oilseed crops have been tested as dietary ingredients in extruded pelleted feeds for ABT juveniles. The oils containing either EPA alone (diet ECO) and or both EPA and DHA (diet DCO) were shown to be viable new sustainable sources of n-3 LC-PUFA and potential candidates to replace dietary FO in feeds for ABT juveniles. Fish fed the experimental ECO and DCO diets showed acceptable growth performance, feed utilisation and survival broadly comparable to ABT fed a commercial reference diet (MGK) with the rank order being generally MGK > DCO > ECO. The study not only tested the efficacy of the new oils as sources of n-3 LC-PUFA to support ABT production but also provided some insight into lipid and LC-PUFA metabolism in different tissues at biochemical and molecular levels. The test diets showed positive effects in upregulating the expression of genes involved in LC-PUFA synthesis and modulating lipid metabolism via impacts on transcription factors. Moreover, the test diets also showed positive effects on the expression of digestive enzyme genes and influenced antioxidant enzyme genes. Nonetheless, mild steatosis was observed in ECO-fed fish, probably related to the low dietary levels of DHA. While the DCO diet performed slightly better than the ECO diet in terms of weight and length, likely due to the higher level of DHA, even diets formulated with the DCO oil should include a supplement of DHA to fully satisfy the essential requirement of ABT juveniles.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7060366/s1, Figure S1. Representative liver photomicrographs (20×; H&E) of Atlantic Bluefin tuna fed ECO, DCO and MGK diets; Figure S2. Representative intestine photomicrographs of Atlantic Bluefin tuna. (a) Measurement of enterocyte height (hE) and width (nNE; 40×; H&E); (b) Measurement of circular muscle thickness (CMT; 20×; H&E); (c) Minimum (Lmin) and maximum (Lmax) length of the transverse section (10×; H&E); (d) Detail of intestinal vili. Arrows point to goblet cells which are blue-stained (40×; PAS); Table S1. Rearing conditions for the feeding trial of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles; Table S2. Sequence, annealing temperature (Tm) and size of the fragment produced by the primer pairs used for quantitative PCR (qPCR).

Author Contributions: Conceptualisation, M.B.B., D.R.T. and G.M.; methodology, A.O., F.d.l.G. and J.A.N.; formal analysis, M.B.B., M.S., D.G.-S. and X.G.; resources, J.A.N.; writing—original draft preparation, M.B.B.; writing—review and editing, D.R.T., M.S., J.A.N. and G.M.; project administration, A.O. and G.M.; funding acquisition, M.B.B., A.O., F.d.l.G., J.A.N. and G.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experimental procedures followed the Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/EU) and Spanish Legislation (RD 1201/2005 and Law 32/2007) for the use of laboratory animals. Additionally, the experimental protocol was subjected to ethical review by the University of Stirling through the Animal Welfare and Ethical Review Board (reference number AWERB/1819/177).

Data Availability Statement: The majority of data used to support the findings of this study are included within the article. Scanned histological slides are available upon request to the authors.

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