Oil from transgenic *Camelina sativa* containing over 25 % n-3 long-chain polyunsaturated fatty acids as the major lipid source in feed for Atlantic salmon (*Salmo salar*)

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Abstract

Facing a bottleneck in the growth of aquaculture, and a gap in the supply and demand of the highly beneficial omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), sustainable alternatives to traditional marine–based feeds are required. Therefore, in the present trial, a novel oil obtained from a genetically engineered oilseed crop, *Camelina sativa*, that supplied over 25 % n-3 LC-PUFA was tested as a sole dietary added lipid source in Atlantic salmon (*Salmo salar*) feed. Three groups of fish were fed for 12 weeks three experimental diets with the same basal composition and containing 20 % added oil supplied by either a blend of fish oil and rapeseed oil (1:3) (COM) reflecting current commercial formulations, wild-type Camelina oil (WCO) or the novel transgenic Camelina oil (TCO). There were no negative effects on the growth, survival rate or health of the fish. The whole fish and flesh n-3 LC-PUFA levels were highest in fish fed TCO with levels almost and more than 2-fold higher compared to those of fish fed the COM and WCO diets, respectively. Diet TCO had no negative impacts on the evaluated immune and physiological parameters of head kidney monocytes. The transcriptomic responses of liver and mid-intestine showed only mild effects on metabolism genes. Overall, the results clearly indicated that the oil from transgenic Camelina was highly efficient in supplying n-3 LC-PUFA providing levels double that obtained with a current commercial standard, and similar to those a decade ago prior to substantial dietary fishmeal and oil replacement.

1. Introduction

Fish is recognized as a nutritious and healthy food in part due to the fact that they are naturally rich in the health promoting omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA)[1,2]. Much of the fish consumed today is farmed[3], many reared on feeds containing high levels of more sustainable raw materials such as plant meals and oils of agricultural origin. Such terrestrial ingredients are devoid of n-3 LC-PUFA and, therefore, the use of these plant ingredients translates into reduced levels of beneficial n-3 LC-PUFA in the fillet of the fish, as has been documented in farmed Atlantic salmon (*Salmo salar*) from 2006 onwards[4]. This means that larger or more portions of farmed fish must be consumed in order to obtain the recommended EPA and DHA intake suggested by different agencies (e.g. 5 and 6).

Oilseed crops are major agricultural products with a highly organised and well-established infrastructure for their cultivation, harvest, and processing, as well as distribution, marketing and utilisation of vegetable oils (VO)[7]. Thus, VO have been the main alternatives to dietary fish oil (FO) in aquafeeds and, while these oils are devoid of n-3 LC-PUFA, they can be rich in shorter chain PUFA such as 18:3n-3. This opens up the possibility for genetic modification to enable the desaturation and elongation of 18:3n-3 to EPA and DHA. Recently, transgenic *Camelina sativa* crops, capable of producing either EPA or EPA and DHA in their seeds, have been developed[8,9]. Two such oils have been evaluated as replacements for dietary FO in feeds for Atlantic salmon (*Salmo salar*)[10-13] and gilthead sea bream (*Sparus aurata*)[14] showing promising results, enabling good fish growth and deposition of n-3 LC-PUFA in tissues including flesh. In contrast, feeds formulated with high levels of conventional plant ingredients, including VO, can adversely affect fish health through impacts on immune and stress responses[15]. A well-balanced dietary fatty acid profile, particularly in terms of LC-PUFA, is a determinant of animal health and welfare and, thus, the replacement of FO by VO can alter this balance. In previous trials, no major impacts on fish health were observed after feeding fish with oil from transgenic Camelina supplying 12% EPA+DHA with, specifically, no detrimental effects on immune function[12] or response after an environmental stress[13]. However, the outcome of stress responses in fish fed VO will depend on the intensity and type of the stressor as well as its duration[16] and, therefore, different stressors may have different effects on particular immune or inflammatory responses.

In previous studies, oils from transgenic Camelina supplying either 20% EPA or 6% each of EPA and DHA were tested in Atlantic salmon against "gold standard" feeds formulated with high fishmeal and FO[10-12]. The oil supplying 6% each of EPA and DHA was subsequently tested in Atlantic salmon against feeds formulated with lower fishmeal and FO[13]. In the present study, a third generation oil supplying levels of EPA, DHA and total n-3 LC-PUFA greater than most FO was tested in feeds for Atlantic salmon with even lower levels of fishmeal and FO reflecting current commercial salmon feeds. Thus, triplicate groups of Atlantic salmon were fed one of three experimental diets formulated with 30 % fishmeal and 43 % plant meals that contained either a mix of FO and rapeseed oil (1:3) (diet COM), wild-type Camelina oil (diet WCO) or transgenic Camelina oil (diet TCO) in order to evaluate the capability of the new transgenic oil to restore EPA and DHA levels in farmed salmon to those of a decade ago before the development of sustainable feeds[4]. Therefore, feeds were formulated with lower levels of fishmeal (30 % vs. 49 or 35 % previously) and, in the reference (COM) diet, FO (5 % vs. 17.5 or 10 % previously) to reflect current feed formulations and n-3 LC-PUFA levels. The impacts of diet TCO on fish growth performance and tissue fatty acid composition were assessed, and influences on fish metabolism were determined by investigating gene expression via liver and pyloric caeca (PC) transcriptomic responses. Additionally, at the end of the experimental trial an *ex vivo* challenge was performed on head kidney monocytes in order to assess potential impacts on the immune response.

2. Materials and Methods

2.1 Diets and feeding trial

The feeding trial was carried out at the facilities of the Institute of Marine Research (Matre, Norway) from December 2016 to February 2017. A total of 297 post-smolt Atlantic salmon (initial weight 133.0 ± 1.6) were distributed into nine 400 L squared flow-through seawater tanks (33 fish per tank) and fed twice daily with one of the three feeds in triplicate for 12 weeks. Fish were fed to satiety+ to ensure that feed availability did not restrict growth. Water temperature was maintained at 13 °C throughout the experimental period. The three feeds were formulated to be essentially isonitrogenous and isolipidic and produced by vacuum coating identical basal extruded pellets with either FO/rapeseed oil (COM), wild-type Camelina oil (WCO) or high EPA+DHA-Camelina oil (TCO) (Table 1). Fatty acid profiles of the constituent oils used in the present trial (Supplementary Table 1) resulted in percentages of both EPA and DHA in diet TCO that were almost 3-fold higher than in the other two dietary treatments (Table 1). The proportions of n-6 and total PUFA were also higher in the TCO diet whereas this feed showed the lowest levels of monounsaturated fatty acids. Yttrium oxide (Y_2O_3, >99.9 % purity, Strem chemicals, Bischheim, France) was added to the feeds as an inert marker in order to determine lipid and fatty acid digestibility. All procedures were approved by the Norwegian Animal Experiment Committee (Forsøksdyrutvalget), experiment ID.8089.

2.2 Sample collection

At the end of the feeding trial 13 fish per tank were killed by an overdose of metacaine sulphonate (> 150 mg l^{-1}, FINQUEL vet., ScanAqua AS, Årnes, Norway). Fish were measured (weight and length) and blood from 4 fish per tank collected via the caudal vein using 1 ml heparinised syringes fitted with 20G needles, and whole blood used for haematocrit determination. Two whole fish per tank were frozen (-20 °C) for biochemical proximate composition analysis, and liver and pyloric caeca were collected from 4 fish per tank were stabilized in RNA Later (Sigma, Poole, UK) and frozen at -20 °C until further RNA extraction. After sampling the fish remaining in the tanks continued to be fed the same feeds as prior to sampling for a further three days at which point faeces were collected and pooled by tank (n = 3). Briefly, fish were killed by an overdose of metacaine sulphonate as

above and faecal samples collected from the hind gut region by gently squeezing the ventral abdominal area[17]. Faeces were initially stored at -20 °C and freeze dried prior to analyses.

2.3 Proximate composition

Feeds were ground and whole fish were pooled per tank (n = 3 per dietary treatment) and homogenised before determination of proximate composition according to standard procedures[18]. Moisture contents were obtained after drying in an oven for 24 h at 110 °C. Ash content was determined after incinerating the samples at a temperature of 600 °C for 20 h. Crude protein content was measured by determining nitrogen content (N x 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK) and crude lipid determined gravimetically after Soxhlet lipid extraction with petroleum ether (Fisher Scientific UK, Loughborough) using a Tecator Soxtec system 2050 Auto Extraction apparatus (Foss, Warrington, UK).

2.4 Calculations

Biometric parameters were estimated as follows: Fulton's condition factor (k) = 100 * (W/L^3), where W is the final weight (g) and L is the total length (cm). Specific growth rate (SGR) = 100 * (lnWo – ln Wf) * D^{-1}, where Wo and Wf are the initial and final weights (tanks means, n = 3), respectively, and D represents the number of feeding days.

2.5 Lipid content and fatty acid composition

Total lipid was extracted from feeds, faeces and whole fish, and flesh, liver, head kidney, midgut, and hindgut homogenates prepared from three pooled fish per tank (n = 3 per treatment) by homogenising approximately 1 g samples in chloroform/methanol (2:1, v/v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK), with content determined gravimetrically[19]. Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 ˚C for 16 h[20], and FAME extracted and quantified by a gas chromatography (AutoSystem XL, Perkin Elmer, Waltham, MA) with Total Chrom Version 6.3.1 software. The GC was equipped with a fused silica capillary column coated with chemically-bonded polyethylene glycol (CP-Wax 52CB, 25 m × 0.25 mm i.d; Varian, Palo Alto, CA), auto-injector (1 µl, inlet temperature 250 ºC) and a flame ionisation detector (FID, 280 ºC). Helium was the carrier gas and the oven temperature program was 90 ºC for 1 min, increased to 150 ºC at 30 min^{-1} and finally raised to 225 ºC at 3

ºC min^{-1} and held for 7 min.. Individual methyl esters were identified by comparison with known standards and a well-characterised fish oil, and also by reference to published data[21].

2.6 Digestibility calculation:

The apparent digestibility coefficients (ADC) of lipid and selected fatty acids were calculated as: 100 – [100 x (Y_2O_3 concentration in feed/Y_2O_3 concentration in faeces) x (lipid or fatty acid concentration in faeces/lipid or fatty acid concentration in feed)]. The concentration of individual fatty acids in diets and faeces were calculated based on the relative proportion of each fatty acid compared with a known amount of internal standard (17:0) added and the total lipid content determined in the samples.

2.7 Isolation and primary culture of head kidney monocytes/macrophages

Four fish per tank were killed by an overdose of metacaine sulphonate (as above) and head kidneys removed, pooled (n = 2 per tank) and transferred to Leibovitz L-15 medium (Sigma, Oslo, Norway) supplemented with 10 U ml^{-1} heparin, 1.8 mM glucose, 10 mM HEPES, 20 U Penicillin ml^{-1}, 20 μg Streptomycin ml^{-1} and 0.05 μg Amphotericin B ml^{-1}. Head kidneys were carefully sieved through a 100 μm nylon cell strainer and the cell suspension loaded onto discontinuous 36 % / 54 % Percoll (GE Healthcare, Oslo, Norway) gradients followed by centrifugation at 400 g for 30 min at 4 °C. The interface was collected and washed twice with L-15 medium (without heparin) followed by centrifugation at 400 g for 5 min at 4 °C. Finally, cells were diluted in L-15 medium and seeded at a density of 1×10^7 cells per well in 96-well or 6-well culture plates (Nunc, Roskile, Denmark) in aliquots of 50 μl $well^{-1}$ or 1 ml $well^{-1}$, respectively. The culture plates were incubated at 12 °C overnight and the cells washed twice in 50 ml L-15 and incubated in L-15 for further studies.

2.8 LPS challenge

Isolated macrophage cultures (6-well plates; n = 6) were stimulated with 20 μg ml^{-1} crude *E. coli* LPS (0111:B4; Sigma Aldrich, Oslo, Norway). Samples were collected prior to the addition of LPS (0 h) and after incubation at 12 °C for sequential sampling points (6 and 24 h) cells were harvested by adding 1 ml of TRI Reagent® (Sigma, Oslo, Norway) lysis buffer and stored at -70 °C until RNA extraction.

2.9 Respiratory burst activity

Respiratory burst activity was determined by the reduction of nitroblue tetrazolium (NBT) to formazan[22,23] Cells were incubated in L-15 medium supplemented with 1 mg ml^{-1} NBT and 1 µg ml^{-1} phorbol myristate acetate (PMA) for 30 min at 12 °C. Cells were then washed twice with pre-warmed phosphate-buffered saline (PBS) and fixed in absolute methanol for 10 min, washed once with 70 % methanol to remove extracellular formazan, air-dried and then intracellular formazan was dissolved in 120 µl 2 M KOH and 140 µl DMSO. Absorbance was measured in a microplate reader at 630 nm using a KOH/DMSO blank. The mean number of cells, obtained from at least three wells was determined by counting nuclei after removal of medium and addition of 100 µl lysis buffer containing 0.1 M citric acid, 1 % Tween 20 and 0.05 % crystal violet. Results were presented as absorbance per 10^5 cells.

2.10 Phagocytosis

Phagocytic activity was determined by a modification of the method of Pulsford et al.[24] using opsonised yeast as engulfed material. For opsonisation, yeast was sterilized in boiling water and, after several washes with PBS, yeast were incubated overnight with foetal bovine serum (FBS) at room temperature[25]. Head kidney cells were mixed with opsonised yeast to obtain an approximate ratio of 10:1 yeast to kidney cells. Phagocytosis proceeded for 1 h at 26 °C, and was then terminated by washing with ice-cold PBS. Phagocytosis was evaluated by observation by inverted light microscopy[26]. At least one hundred cells were counted and phagocytosis was expressed as Phagocytosis percentage (PP), which was calculated:

$$PP = \left(\frac{no\ of\ cells\ ingesting\ yeasts}{no\ of\ adherent\ cells\ observed}\right) \times \left(\frac{no\ of\ yeast\ ingested}{no\ of\ adherent\ cells\ observed}\right)$$

2.11 RNA extraction and cDNA synthesis

Individual liver and pyloric caeca samples from eighteen fish per dietary treatment were homogenised in 1 ml of TriReagent® (Sigma-Aldrich, Dorset, UK) to extract total RNA following the producer's protocol. Quantity and quality of RNA was determined by spectrophometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK). The same protocol was used to extract RNA from macrophage samples. cDNA was synthesised using a high capacity reverse transcription kit utilising 2 µg of total RNA and random primers in a total reaction volume of 20 µl following the manufacturer's protocol (Applied Biosystems,

Warrington, UK). The samples were pooled to obtain n = 6 per dietary treatment. A dilution of 1:20 was applied to the resulting cDNA using milliQ water.

2.12 Liver and pyloric caeca transcriptome - microarray hybridisations and image analysis

Transcriptomic analysis of liver and pyloric caeca was performed using a custom-designed 4 x 44 k Atlantic salmon oligo microarray (Agilent Technologies, Wokingham, UK; ArrayExpress accession no. A-MEXP-2065). The salmon microarray and laboratory procedures utilised have been widely used and validated in many previous studies[10-11; 27-30]. Replicate RNA samples were amplified using TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit, (Epicentre Technologies Corporation, Madison, Wisconsin, USA) following recommended procedures. Aminoallyl-amplified RNA (aRNA) samples were labelled with Cy3 dye (GE HealthCare Life Sciences, Buckinghamshire, UK) while a pool of all aRNA samples was labelled with Cy5 dye (GE HealthCare Life Sciences) and used as a common reference in a dual-label common reference design, and finally hybridised. Scanning was performed using a GenePix 4200 AL Scanner (Molecular Devices (UK) Ltd., Wokingham, UK), and the resulting images analysed with Agilent Feature Extraction Software v.9.5 (Agilent Technologies) to extract intensity values and identify features. Features considered outliers (i.e., defined as those probes whose background intensity was between the 0.05^{th} and 99.95^{th} percentile of the distribution) in two or more replicates within at least one treatment were excluded from further analyses. Additionally, features consistently expressed just above background noise (defined as those features whose intensity was lower than the 5^{th} percentile of the distribution in 75 % or more of the analysed samples) were also removed. The full protocol for microarray and data analysis has been reported previously[10]. The full data set supporting the results is available in MIAME-compliant format in the ArrayExpress repository under accession number E-MTAB-6228.

2.13 Quantitative PCR gene expression

Gene expression levels were determined by real-time quantitative RT-PCR in liver, pyloric caeca and macrophages as described previously[31]. Genes involved in LC-PUFA biosynthesis (*fads2d6, delta-6 fatty acyl desaturase*; *fads2d5, delta-5 fatty acyl desaturase*; *elovl2, fatty acyl elongase 2*; *elovl5a, fatty acyl elongase 5 isoform a*; *elovl5b, fatty acyl elongase isoform b*) were measured in liver and pyloric caeca whereas genes involved in inflammation (*il1b, interleukin 1 beta*; *cox2, cyclooxygenase 2, inos, inducible nitric oxide synthase* and *tnfα, tumor necrosis factor alpha;*) and antibacterial activity (*cath, cathelicidin* and *hepc, hepcidin*)

were measured in macrophages (Supplementary Table 2). Results were normalised using reference genes, *cofilin 2* (*cfl2*), *elongation factor 1 alpha* (*ef1α*) and *ribosomal protein L2* (*rpl2*). Primers were designed using Primer 3 in regions that included the microarray probes[32]. Quantitative PCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific), 1 µl of primer corresponding to the analysed gene (10 pmol), 3 µl of molecular biology grade water and 5 µl of cDNA, with the exception of the reference genes, which were determined using 2 µl of cDNA. In addition, amplifications were carried out with a systematic negative control (NTC-no template control) containing no cDNA. Standard amplification parameters contained an UDG pre-treatment at 50 °C for 2 min, an initial activation 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.

2.12 Statistical Analysis:

All data are means ± S.D. (n = 3) unless otherwise specified. Percentage data were subjected to arcsin square-root transformation prior to statistical analyses. Data were tested for normality and homogeneity of variances with Levene's test prior to one-way analysis of variance (ANOVA) followed by a Tukey and post-hoc test. All statistical analyses including the gene expression results were performed using SPSS software (IBM SPSS Statistics 23; SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Fish growth performance and biochemical composition

No significant differences were observed between the three dietary groups at the end of the feeding period in any of the evaluated growth or biometric parameters (Table 2). There was a 100 % survival rate and no presence of disease was observed. Fish fed diet TCO had a lower lipid content than fish fed the COM and WCO diets, but there were no differences in protein, ash or dry matter contents of whole fish (Table 2).

3.2 Lipid and fatty acid digestibility

Differences in apparent digestibility coefficient (ADC) of dietary lipid were observed among the dietary treatments with TCO showing the lowest value (Table 3). The ADCs of saturated

and monounsaturated fatty acids varied between about 87 - 94 % and 91 – 98 %, respectively, with the rank order generally being COM > WCO > TCO (Table 3). The ADC for n-6, n-3 and total PUFA were all 97 - 98 % with few significant differences between feeds although the diet WCO showed the lowest ADC for EPA, DHA, 22:5n-3 (docosapentaenoic acid, DPA) and 20:4n-6 (arachidonic acid, ARA). The COM diet showed the lowest ADC for 20:3n-3, probably reflecting the very low dietary content of this fatty acid (Table 1).

3.3 Lipid contents and fatty acid compositions of whole fish and tissues

The lower lipid content of whole fish fed diet TCO compared to fish fed COM (Table 2) was reflected in the lipid content of flesh (whole muscle) although there was no significant difference to fish fed WCO (Table 4). In contrast, diet had no effect on the lipid contents of liver and head kidney (Table 5).

3.4 Fatty acid compositions of whole fish and tissues

The proportion of total n-3 LC-PUFA in whole body of Atlantic salmon fed diet TCO was around double that of fish fed diets WCO or COM (Table 4). Specifically, the relative contents of EPA, DPA and DHA were highest in fish fed TCO with fish fed COM and WCO showing similar lower values. The proportions of 18:2n-6, ARA and n-6 PUFA were also highest in TCO-fed fish with COM-fed fish showing the lowest proportions of 18:2n-6 and total n-6 PUFA. Differences were also observed in total saturated and monoenes with COM-fed fish showing the lowest and highest contents respectively.

Flesh of fish fed TCO displayed the highest contents of the n-3 LC-PUFA, EPA, DPA and DHA, and ARA (Table 4). In COM-fed fish, flesh showed the highest proportions of total saturates and 18:1n-9, and the lowest contents of 18:3n-3, whereas WCO-fed fish contained the highest percentage of this fatty acid. Relative levels of total n-3 PUFA, EPA and DPA in liver and head kidney were highest in fish fed TCO, although total n-3 LC-PUFA levels in TCO-fed fish was not different to WCO-fed fish. Although DHA level was highest in fish fed TCO in both liver and head kidney, variation in the data rendered the dietary differences not significant (Table 5).

A PCA analysis was applied to the whole fish and tissue fatty acid profiles (percentage) of fish fed the three experimental feeds. The first two principal components explained 82.8 % of variance (Fig. 1). The score plot showed some separation between tissues of fish fed COM

and those fed both WCO and TCO. There was an overlap between tissues of fish fed WCO and TCO, although liver from fish fed TCO showed a distinct profile, clustering at the left side of the plot (red diamonds). The fatty acid vectors representing the MUFA 16:1n-7, 18:1n-7, 18:1n-9 and 22:1n-11 appeared to be correlated with fish fed the COM diet. The n-6 and n-3 PUFA (18:2n-6, 18-3n-3 and 18:4n-3) vectors lie towards fish fed WCO whereas the n-6 and n-3 LC-PUFA (20:4n-6, 20:5n-3, and 22:6n-3) point towards TCO-fed fish, as well as livers from WCO-fed fish.

3.5 Liver transcriptome

In liver, a total of 312 unique genes were differentially expressed ($p < 0.05$; fold-change, FC > 1.3) in salmon fed the TCO diet versus the COM diet whereas 249 genes were affected when comparing WCO-fed fish with COM-fed fish (Table 6). The highest number (506) of differentially expressed genes (DEG) was observed when comparing the hepatic transcriptomes of TCO-fed and WCO-fed fish. Among these unique probes, 86 DEG were commonly regulated when comparing fish fed TCO with fish fed either COM or WCO, whereas 45 were found when TCO- and WCO-fed fish were compared to COM-fed fish and only 37 when fish fed TCO and COM were compared to those fed WCO (Fig. 2A).

In order to elucidate the genes exclusively affected by TCO, KEGG Orthology (KO) numbers were assigned to the 86 genes commonly affected by TCO (Supplementary Table 3), and genes mapped to a known compendium of metabolic pathways (KEGG). Enrichr, an integrative web-based software application[33], was used to build a network with the enriched gene sets (Fig. 3A). Many of the categories represented belonged to metabolism with high representation of lipid metabolism including “fatty acid degradation”, “sphingolipid metabolism” and “terpenoid backbone biosynthesis”, as well as amino acid metabolism including “alanine, aspartate and glutamate metabolism” and “other glycan degradation”. Genes belonging to other categories such as “protein processing in the endoplasmic reticulum”, and “DNA replication” were also highly represented. At a gene level, all the genes presented the same direction of regulation (up or down) between both contrasts and a similar FC. Expression of a set of candidate LC-PUFA biosynthesis genes was determined by qPCR with *fads2d5* and *fads2d6* showing up-regulation in fish fed WCO, with no difference between fish fed TCO and COM (Fig. 4). No differences were observed among the expression of the other LC-PUFA biosynthesis genes evaluated by qPCR.

3.6 Pyloric caeca transcriptome

A greater number of DEG was found in pyloric caeca compared to liver when comparing fish fed either diets TCO or WCO to fish fed COM (804 and 971 DEG, respectively) (Table 6). However when comparing fish fed TCO with fish fed WCO, the number of DEG was much lower in pyloric caeca compared to liver (279 *versus* 506). There were differences in the distribution of genes depending on FC, with a high number of DEG showing FC >2.5, particularly among down-regulated genes.

Only 61 genes were commonly regulated in the pyloric caeca of fish fed TCO compared with fish fed COM and WCO (Fig. 2B). Network analysis of the enriched categories showed that most of the genes were related to metabolism, although many also belonged to "mTOR signalling pathway" and "regulation of autophagy" (Fig. 3B). After removing non-annotated genes, 24 unique genes could be identified as being commonly affected by TCO in pyloric caeca (Supplementary Table 4). All the genes presented the same direction of regulation and similar FC in both contrasts (TCO v. COM and TCO v. WCO). High FCs were observed particularly in genes related to carbohydrate metabolism (*N-acetylgalactosaminide alpha-2,6-sialyltransferase* and *beta-mannosidase*), which were up-regulated in TCO-fed fish, particularly when compared with WCO-fed fish (FC of 12.7 and 6.8, respectively). In pyloric caeca, while there appeared to be a trend for lower expression of most of the LC-PUFA biosynthesis genes in fish fed TCO compared to fish fed COM, but this was only significant with *elovl5b* (Fig. 5).

3.7 Head kidney cell LPS challenge and gene expression

Diet did not significantly affect leucocyte respiratory burst activity, although there was a trend toward lower values in TCO-fed fish ($p = 0.057$; Table 7). Similarly, no differences were observed in phagocytic activity of monocytes/macrophages from fish fed the three dietary treatments ($p = 0.854$; Table 7).

Atlantic salmon monocytes were exposed to LPS to study the effect on gene expression at 0, 6 and 24 h after addition. The exposure to LPS triggered a response in all of the genes evaluated with highest expression levels observed 24 h after application of the challenge (Fig. 6). The FC varied among the studied genes, with the strongest regulation in *cox2* (approx. 2500 FC) and the lowest in *inos* (approx. 4 FC). Two-way ANOVA showed gene expression was highly affected by sampling time ($p < 0.001$), but diet did not affect expression of any of the studied genes and there was no interaction between diet and sample time.

Discussion

The benefits of n-3 LC-PUFA on several aspects of human health are widely known and appreciated (Calder, 2017). However, recent studies have highlighted the gradual decrease of these essential fatty acids in the flesh of farmed salmon, reflecting the necessary development of sustainable feeds increasingly formulated with lower levels of marine ingredients that supply the n-3 LC-PUFA[4]. Previous studies demonstrated the feasibility of oils obtained *de novo* from transgenic *Camelina sativa* to supply n-3 LC-PUFA in feeds for Atlantic salmon and sea bream, maintaining growth and enhancing n-3 LC-PUFA contents in fillet compared to fish reared on feeds formulated with conventional VO[10-14]. The earlier oils from transgenic Camelina supplied either 20 % EPA or 6 % each of EPA and DHA and were compared to feeds formulated with high fishmeal and FO as "gold standards"[10-12,14] and also a feed with lower fishmeal and FO[13]. In the present study, the latest generation of transgenic oil supplied levels of EPA, DHA and total n-3 LC-PUFA as high as many FO, or higher than those from the northern hemisphere, and was specifically tested in order to evaluate its capability to maintain the sustainability of feeds while simultaneously restoring levels of n-3 LC-PUFA in farmed salmon to those of a decade ago, before the development of low marine feeds[4].

In the present study, the COM diet was formulated to reflect current commercial feed formulations for salmon and thus included levels of fishmeal and FO that resulted in a dietary n-3 LC-PUFA level of around 7 % of total dietary fatty acids. Replacing the added oil with the oil from transgenic Camelina resulted in the n-3 LC-PUFA content of the TCO diet being almost 24 % of total fatty acids that, in turn, almost doubled the EPA+DHA and n-3 LC-PUFA contents of flesh of the TCO-fed fish compared to fish fed the COM diet. While the fish in the present trial were not market size and so the data obtained are not directly comparable to the data presented in (4), they nonetheless indicate the potential of this 3rd generation transgenically-derived oil to supply sufficient n-3 LC-PUFA to restore EPA+DHA levels in farmed salmon to levels last seen a decade ago, thus retaining all the positive health benefits associated with the consumption of fish.

As in previous trials, no differences in term of growth were observed between fish fed the different feeds, which was not unexpected given that the levels of n-3 LC-PUFA contained in all the feeds were sufficient to cover the requirements for this species. For instance, total n-3 LC-PUFA in fish fed diet WCO was still ~5.5 % of total fatty acids representing ~ 1.1 % of diet, already above the reported EFA requirement level without even considering the high dietary level of 18:3n-3[34]. In agreement with previous trials, Atlantic salmon fed TCO

showed a reduced deposition of lipid in whole body and flesh, and, although not always significant, other tissues[12], which could be attributed to the higher contents of EPA and DHA that are known to have anti-adipogenic effects in mammals[35]. Additionally, the microarray revealed that *acsl*, a gene involved in lipid biosynthesis, was commonly down-regulated in TCO-fed fish when compared to fish fed COM or WCO, which could indicate inhibition of lipogenesis. Interestingly, the expression of *lpl* was also down-regulated in TCO-fed fish, which may or may not be consistent with lower lipid levels in tissues. However, at least three different transcripts exist for *lpl* in Atlantic salmon and different patterns of nutritional regulation for each isoform have been demonstrated[36].

Although there were no effects on growth performance, some differences were observed in lipid and fatty acid digestibility. The ADC for lipid was generally high and affected by dietary lipid source, being slightly higher in the COM and WCO feeds than in TCO feed. Previous studies in several teleost species generally reported highest lipid ADC in feeds containing FO[37,38]. Indeed, in our previous studies, feeds for Atlantic salmon formulated with either wild-type or transgenic Camelina oil displayed higher lipid ADC than feeds containing FO[11,12]. However the FO-based feeds were formulated with high levels of fishmeal (49 %) and FO (18 %) and thus the content of n-3 LC-PUFA was much higher than in the VO-based feeds, whereas in the present trial diet TCO contained the highest contents of n-3 LC-PUFA. These data suggest that lipid ADC is dependent on the fatty acid profile of the feeds and high contents of n-3 LC-PUFA enhance lipid digestibility as these fatty acids generally have high ADC[39,40]. Consistent with this, the ADC for the n-3 LC-PUFA were high in the present trial and highest in fish fed the TCO diet. Individual fatty acid ADC were also consistent with previous studies where digestibility decreased with increasing chain length, but increased with increasing degree of unsaturation[39,41-42]. While water temperature can also impact the ADC of fatty acids, particularly when dietary FO is substituted by VO[42], the trial was performed at a constant controlled temperature throughout and so differences in digestibility of individual fatty acids between diets cannot be attributed to water temperature. Overall, diet TCO had no major negative effects on fatty acid digestibilities and differences between diets could be attributed to the different fatty acid profile of the feeds.

Although diet WCO contained the lowest levels of DHA and n-3 LC-PUFA, fish fed this feed did not show major differences in the contents of DHA in whole fish or tissues compared to fish fed the COM diet. Anadromous species, such as the salmonids, have the capability to biosynthesize LC-PUFA, with liver and intestine being the most active metabolic sites[43,44]. In this respect, fish fed WCO showed up-regulation of hepatic *fads2d6*

and *fad62d5* as well as intestinal *elovl5b*, which could have contributed in the levels of n-3 LC-PUFA being similar in COM- and WCO-fed fish. Although the COM diet had only a slightly higher level of DHA compared to WCO (3.7 and 2.7 %, respectively) and much lower than in TCO (9.0 %) expression levels of desaturases and elongases were similar to that in fish fed TCO. However, the level of 18:3n-3 was almost 4 times higher in diet WCO than in the COM diet and it has been suggested that up-regulation of the LC-PUFA biosynthetic pathway in fish fed VO was induced by the level of dietary precursor (18:3n-3) as well as low levels of pathway products (e.g. DHA)[45]. Therefore, the lower expression of these enzymes in liver and pyloric caeca of fish fed COM compared to fish fed WCO could be related to the low dietary level of the substrate 18:3n-3.

The PCA analysis partly reflected the physiological functions of each tissue. In general terms, the fatty acid profiles of whole fish and tissues largely reflected that of the diet, consistent with previous studies in Atlantic salmon[31,46,47]. The four distinct quarters of the plot represent significantly different fatty acid profiles, distinguishing three groups, one per dietary treatment. The fatty acid vectors representing n-6 and n-3 LC-PUFA have significant loadings on the "TCO" side of the plot, which is directly related to the diet fatty acid profiles. This also explains why 18:1n-9 vector is placed towards "COM" as this fatty acid is in high concentration in rapeseed oil, which constitutes 75 % of the added oil in that diet. Liver fatty acid profiles from fish fed WCO are located towards the left side of the plot, where fish fed TCO are placed, which reflects the capacity of liver for the synthesis of LC-PUFA. Head kidney of fish fed diet TCO tended to cluster on the left panel reflecting differences in head kidney DHA contents among fish fed the different diets. Although these differences were not significant in the present trial they were consistent with previous studies in Atlantic salmon, which showed DHA levels in head kidney reflected the reduced DHA content of VO-based diets[48].

The head kidney in fish is an integral tissue of the immune system[49,50], and dietary lipid and PUFA content are known to influence immune function and thus health status of fish[51-53]. In the present study, no differences were observed in the cellular immune functions evaluated, such as monocyte phagocytic activity or respiratory burst. Previous studies with sustainable feeds have found contradictory results regarding these immune parameters. While Atlantic salmon fed diets with different VO (100 % and 50 % substitution of FO, respectively) did not show altered phagocytic activity nor respiratory burst[54,55] and rainbow trout (*Oncorhynchus mykiss*) fed increasing concentrations of ARA showed no differences in phagocytosis[56], sea bream fed diets containing VO (100 % substitution of FO) showed

reduced phagocytic activity[57]. Differences in the dietary ARA:EPA ratio can alter the production of immunologically active eicosanoids derived from these fatty acids[58], affecting signal transduction and regulation of gene expression[59]. Despite this, the functional assays show no negative effects of the oil from transgenic Camelina on the fish immune system. Furthermore, the expression of several genes related to inflammation and antibacterial activity evaluated in monocytes isolated from fish after LPS challenge showed no differences in any of the genes among the dietary treatments at either sampling point. Previously, the expression of the pro-inflammatory cytokines *il1b* and *tnfa* was not changed 12 h after LPS stimulation in Atlantic salmon after modulation of membrane lipids[55]. Therefore, our results were consistent with replacement of dietary FO by VO in Atlantic salmon feeds having relatively little influence on inflammatory functions of leukocytes. However, in other teleost species, dietary VO can have adverse effects on health/immune system through increasing time to moderate the response up to 7 days after challenge when 70 % of FO was substituted by VO[60]. In this respect, the expression of several inflammatory cytokines as well as the anti-bactericidal gene *cath* started to return basal levels only 72 h after challenging Atlantic salmon monocytes with LPS[61].

Transcriptomic analyses showed that diet regulated expression of a lower number of genes in liver than in pyloric caeca, with most of the pathways enriched in liver belonging to metabolism, which was consistent given the functions of this tissue. One of the genes commonly regulated by TCO was *acsl*, which was down-regulated in fish fed TCO compared to fish fed the other two diets. This gene was also down-regulated in salmon fed a similar EPA+DHA-containing Camelina oil in a previous trial[12]. After LC-PUFA enters a cell, *acsl* converts them to fatty acyl-CoAs that can have numerous metabolic fates, including incorporation into triacylglycerol or phospholipids, or substrates for β-oxidation and protein acylation[62]. Interestingly, a recent study reported up-regulation of acsl3 in hepatopancreas of grass carp (*Ctenopharyngodon idellus*) with increasing levels of dietary ARA[63], which is the opposite trend to that described here, as the TCO feed contained a higher level of ARA than the other diets. However, previous studies in Atlantic salmon showed no differences in the expression of *acsl* in liver between fish fed FO and VO, which had different dietary ARA contents[64]. These authors suggested that this gene may function as a general fatty acid activator for several lipid metabolism pathways not directly related to β-oxidation. This was consistent with the present results, given that TCO-fed fish had the lowest lipid content in body and flesh that, in turn, could be related to enhanced β-oxidation in fish fed this diet. Among the DEG commonly regulated in fish fed TCO, a transcription factor, *pparα*, was

down-regulated in TCO compared to fish fed the other diets. Up-regulation in the expression of this enzyme, concomitant with the expression of *fads2*, was observed previously in rainbow trout after substitution of dietary FO with VO[65].

The intestine is not only the site of nutrient uptake, but also plays an active role in metabolism. Indeed, two genes of carbohydrate metabolism were up-regulated in fish fed TCO compared to fish fed the other diets despite the feeds not differing in carbohydrate content. In an earlier trial with salmon fed a previous version of transgenic Camelina oil, intestinal expression of genes of carbohydrate metabolism such as *glucose-6-phosphate isomerase* (*g6pd*) and *glycerol kinase 5* (*gk5*) was up-regulated[13]. Previous studies also reported similar effects, probably reflecting that pathways of lipogenesis, lipolysis, glycolysis, gluconeogenesis and pentose phosphate shunt are all interrelated in the regulation of energy homeostasis[66]. No genes were found to be commonly regulated in liver and pyloric caeca in fish fed TCO, probably reflecting the differing functional roles of each tissue.

The present study showed that salmon fed the new oil from transgenic *Camelina sativa* supplying ~ 24 % n-3 LC-PUFA could accumulate almost double the level of EPA and DHA in their body and fillet than fish fed a feed reflecting current commercial formulations containing low levels of marine ingredients. This oil also supported good growth without apparently compromising fish health and immune response as shown by the *ex vivo* macrophage challenge and tissue transcriptomic responses. Although fish were not of harvest size (final weight, ~400 g), these data suggest that the new oil from a transgenic oilseed crop has the potential to provide a new generation of salmon feeds that, while maintaining current levels of sustainability, are also able to restore the EPA+DHA levels in harvest-size (~ 4-5 Kg) farmed salmon to those last seen a decade ago before the development of low marine feeds.

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Conflict of Interest

None

Authorship

The authors contributed as follows to the manuscript: study concept and design: J.A.N., O.T., D.R.T., R.E.O.; formal analysis: M.B.B., K.L., M.S., L.H.; funding acquisition: J.A.N., D.R.T., R.E.O.; writing – original draft: M.B.B., D.R.T.; writing – review and editing: K.L., M.S., O.S., S.U., F.N., O.T., J.A.N., R.E.O.

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745

Figure Legends

Fig. 1. Principal component analysis (PCA) of fatty acid profiles (percentage of total fatty acids) of tissues from Atlantic salmon fed the experimental feeds for 12 weeks. Fish fed fish /rapeseed oil (COM), black; Fish fed wild-type Camelina oil, light grey (WCO); Fish fed transgenic Camelina oil, dark grey (TCO); filled circle, head kidney; filled square, flesh; diamond, liver.

Fig. 2. Venn diagram representing genes differentially expressed in liver (A) and pyloric caeca (B) of Atlantic salmon fed the experimental diets (Welch t-test; $p < 0.05$, $FC > 1.3$). Non-annotated genes and features corresponding to the same gene are not represented. COM, fish/rapeseed oil feed; TCO, transgenic Camelina oil feed; WCO, wild-type camelina oil feed.

Fig. 3. Metabolic categories enriched with genes commonly regulated in fish fed transgenic Camelina oil. Gene networks in liver (A) and pyloric caeca (B) were produced using Enrichr web-application.

Fig. 4. Expression of genes of the LC-PUFA biosynthesis pathway in liver of Atlantic salmon as determined by qPCR. Results are normalised expression ratios (means ± SEM; n = 6). Different superscript letters denote differences among treatments as identified by one-way ANOVA. COM, fish/rapeseed oil feed; TCO, transgenic camelina oil feed; WCO, wild type camelina oil feed. *elovl2*, fatty acyl elongase 2; *elovl5a*, fatty acyl elongase 5 isoform a; *elovl5b*, fatty acyl elongase 5 isoform b; *fads2d6*, delta-6 fatty acyl desaturase; *fads2d5*, delta-5 fatty acyl desaturase.

Fig. 5. Expression of genes of the LC-PUFA biosynthesis pathway in pyloric caeca of Atlantic salmon as determined by qPCR. Results are normalised expression ratios (means ± SEM; n = 6). Different superscript letters denote differences among treatments identified by one-way ANOVA. COM, fish/rapeseed oil feed; TCO, transgenic camelina oil feed; WCO, wild-type camelina oil feed. Gene abbreviations as described in legend to Fig. 4.

Fig. 6. Expression of genes of inflammation and antibacterial activity in head kidney monocytes/macrophages of Atlantic salmon at the end of the experimental trial before (0 h), or 6 h and 24 h after challenging the cells with LPS. Results are expressed as normalised expression ratios (means ± SEM; n = 6). COM, fish/rapeseed oil feed; TCO, transgenic camelina oil feed; WCO, wild-type camelina oil feed; *il1b*, interleukin 1 beta; *tnfα*, tumor

777 necrosis factor alpha; *cox2*, cyclooxygenase 2; *inos*, inducible nitric oxide synthase; *cath*,
778 cathelicidin; *hepc*, hepcidin.

779

780 **Table 1**. Formulations, analysed proximate compositions and selected fatty acid profiles of
781 the experimental diets.

	COM	WCO	TCO
Feed Ingredients (%)			
Fish Meal	30.00	30.00	30.00
Soy Protein Concentrate (> 62 %)	10.00	10.00	10.00
Pea Protein (> 72 %)	10.00	10.00	10.00
Wheat Gluten	5.00	5.00	5.00
Maize Gluten	5.00	5.00	5.00
Wheat	12.95	12.95	12.95
Fish oil	5.00	0.00	0.00
Rapeseed oil	15.00	0.00	0.00
Camelina oil (wild-type)	0.00	20.00	0.00
Camelina oil (transgenic)	0.00	0.00	20.00
Mineral and vitamin premixes	2.05	2.05	2.05
Yttrium oxide	0.05	0.05	0.05
Analysed composition			
Dry matter (%)	92.6	93.9	92.2
Protein (%)	45.1	46.4	48.6
Lipid (%)	23.4	19.7	18.6
Ash (%)	7.1	7.4	7.6
Energy (kJ g^{-1})	23.0	22.1	22.4
Analysed fatty acid profile (%)			
16:0	9.3	8.6	9.5
Total saturated[*]	14.1	14.2	19.9
18:1n-9	42.4	15.8	7.8
Total monounsaturated[†]	54.2	35.7	18.6
18:2n-6	15.7	18.2	19.8
20:4n-6	0.2	0.2	2.2
Total n-6 PUFA[‡]	16.2	19.9	25.9
18:3n-3	6.8	22.8	8.7
20:3n-3	0.1	0.8	0.9
20:5n-3	3.2	2.4	9.4
22:5n-3	0.4	0.3	3.9
22:6n-3	3.7	2.7	9.0
Total n-3 PUFA[ǁ]	15.0	29.7	35.2
Total PUFA	31.7	50.1	61.5
Total n-3 LC-PUFA	7.5	5.5	23.9

782 COM, fish oil/rapeseed oil feed; WCO, wild-type camelina oil feed; TCO, transgenic
783 camelina oil feed; LC-PUFA, long-chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-
784 3, 22:5n-3, 22:6n-3). [*]Contains 14:0, 18:0 and 20:0; [†]Contains 16:1n-7, 18:1n-7, 20:1n-11,
785 20:1n-9, 20:1n-6, 22:1n-11 and 22:1n-9; [‡]Contains 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-
786 6. [ǁ]Contains 18:4n-3, 20:3n-3 and 20:4n-3.

Table 2. Growth performance, biometric parameters, and biochemical composition of whole fish after feeding the experimental diets for 12 weeks. Data are presented as means ± SD (n = 3).

	COM		**WCO**		**TCO**		**p value**
	Mean	SD	Mean	SD	Mean	SD	
Final weight (g)	412.2	5.6	397.7	9.2	406.2	18.2	0.447
Length (cm)	31.4	0.3	31.2	0.2	31.6	0.5	0.400
Gutted (g)	367.1	14.4	349.5	15.2	365.2	17.6	0.388
HSI	1.0	0.0	1.0	0.0	1.0	0.1	0.856
VSI	6.2	0.2	6.1	0.2	5.9	0.2	0.177
Haematocrit	33.7	0.6	32.4	0.8	33.9	0.8	0.231
				Whole body composition (% wet weight)			
Crude protein	17.9	0.3	18.2	0.2	18.0	0.6	0.640
Crude lipid	11.7[a]	0.5	10.9[a]	0.3	9.8[b]	0.2	0.013
Ash	1.6	0.0	1.6	0.0	1.7	0.0	0.178
Dry matter	31.5	0.2	32.3	0.6	31.0	0.5	0.270

COM, fish oil/rapeseed oil feed; WCO, diet containing wild-type camelina; TCO, transgenic camelina oil feed; HSI, hepato-somatic index; VSI, viscero-somatic index.

Table 3. Apparent digestibility coefficients (ADC) of total lipid and individual fatty acids. Data are presented as means ± SD (n = 3).

	COM		WCO		TCO	
	Mean	SD	Mean	SD	Mean	SD
Total lipid	94.6^{a}	0.3	93.7^{a}	0.1	90.7^{b}	0.7
14:0	95.9^{a}	0.4	95.0^{a}	1.6	92.5^{b}	0.1
15:0	94.6^{a}	0.2	92.7^{ab}	2.1	89.6^{b}	0.8
16:0	93.9^{a}	0.4	92.4^{ab}	2.3	89.7^{b}	0.8
18:0	91.8^{a}	0.8	89.6^{ab}	3.6	85.1^{b}	1.5
Total saturated	93.7^{a}	0.5	91.4^{ab}	2.8	86.7^{b}	1.3
16:1n-7	97.9^{a}	0.5	97.1^{ab}	0.9	96.0^{b}	0.7
18:1n-9	98.1^{a}	0.4	96.7^{a}	0.2	94.4^{b}	1.1
18:1n-7	97.7^{a}	0.4	95.8^{b}	0.2	95.6^{b}	0.6
20:1n-9	96.5	0.7	97.0	0.2	96.0	0.5
20:1n-7	95.4	0.7	96.5	0.2	95.0	0.6
22:1n-11	96.2^{a}	0.7	95.2^{a}	1.7	91.1^{b}	1.9
22:1n-9	96.2^{a}	0.7	94.9^{ab}	2.4	91.5^{b}	1.2
Total monoenes	97.9^{a}	0.4	96.4^{ab}	1.2	94.6^{b}	0.6
18:2n-6	97.8	0.4	97.3	0.5	97.2	0.5
20:2n-6	90.6^{b}	3.4	97.4^{a}	0.9	92.5^{ab}	1.4
20:4n-6	97.2^{ab}	0.2	95.0^{b}	0.0	99.2^{a}	1.1
Total n-6 PUFA	97.7	0.4	97.2	0.5	97.5	0.5
18:3n-3	98.7	0.3	98.8	0.3	98.3	0.3
18:4n-3	98.8	0.4	97.5	0.9	99.1	0.3
20:3n-3	85.7^{b}	5.1	97.5^{a}	1.0	97.0^{a}	0.3
20:4n-3	96.1^{ab}	1.6	91.7^{b}	2.5	98.4^{a}	0.5
20:5n-3	98.3^{a}	0.3	97.0^{b}	0.4	98.5^{a}	0.3
22:5n-3	95.4^{b}	1.0	92.7^{c}	1.1	98.4^{a}	0.4
22:6n-3	95.6^{a}	0.5	92.2^{b}	0.7	96.2^{a}	0.8
Total n-3 PUFA	97.7	0.4	97.9	0.4	97.9	0.4
Total PUFA	98.3	1.3	97.6	0.4	97.7	0.5

COM, fish oil/rapeseed oil feed; WCO, wild-type camelina oil feed; TCO, transgenic camelina oil feed; LC-PUFA, long-chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3). Different superscript letters denote statistically significant differences between the treatments as determined by one-way ANOVA with Tukey's and Duncan comparison test ($p < 0.05$).

Table 4. Lipid contents (percentage of wet weight) and fatty acid compositions (percentage of total fatty acids) of total lipid of whole body and flesh (muscle) of Atlantic salmon after feeding the experimental diets for 12 weeks. Data are presented as means ± SD (n = 3).

	COM		WCO		TCO	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Whole body						
Lipid content	11.7[a]	0.5	10.9[a]	0.3	9. 8[b]	0.2
16:0	10.6	0.3	10.9	0.1	11.0	0.1
Total saturated[*]	16.1[c]	0.3	17.1[b]	0.2	19.1[a]	0.3
18:1n-9	38.6[a]	0.7	22.9[b]	0.3	18.5[c]	0.9
Total monoenes†	52.3[a]	0.6	40.3[b]	0.2	31.2[c]	0.8
18:2n-6	13.1[c]	0.2	14.4[b]	0.1	16.1[a]	0.5
20:4n-6	0.3[b]	0.0	0.3[b]	0.0	1.2[a]	0.2
Total n-6 PUFA‡	15.1[c]	0.2	17.0[b]	0.0	21.1[a]	0.2
18:3n-3	4.9[c]	0.1	13.2[a]	0.2	6.5[b]	0.1
20:5n-3	2.2[b]	0.2	2.1[b]	0.1	5.2[a]	0.3
22:5n-3	0.9[b]	0.1	0.8[b]	0.0	3.1[a]	0.1
22:6n-3	6.4[b]	0.6	5.7[b]	0.0	10.1[a]	0.3
Total n-3 PUFAǁ	16.2[c]	0.9	25.3[b]	0.0	28.3[a]	0.7
EPA + DHA	8.5[b]	0.8	7.8[b]	0.1	15.3[a]	0.6
Total n-3 LC-PUFA	8.7	1.2	8.7	1.6	11.2	1.5
Flesh						
Lipid content	3.9[a]	1.5	2.6[ab]	0.5	2.5[b]	0.6
16:0	11.7	0.8	11.7	0.3	12.1	0.5
Total saturated*	17.2[b]	1.0	17.6[b]	0.2	19.7[a]	0.4
18:1n-9	34.6[a]	1.9	19.1[b]	1.3	14.7[c]	2.0
Total monoenes†	46.0[a]	2.5	33.4[b]	1.8	24.6[c]	3.3
18:2n-6	12.2[b]	0.6	12.8[b]	0.3	13.7[a]	0.4
20:4n-6	0.5[b]	0.1	0.5[b]	0.1	1.7[a]	0.2
Total n-6 PUFA‡	14.2[b]	0.6	15.3[b]	0.2	18.2[a]	0.5
18:3n-3	5.0[c]	0.2	12.7[a]	0.4	6.3[b]	0.4
20:5n-3	2.9[b]	0.5	3.3[b]	0.4	6.5[a]	0.8
22:5n-3	1.1[b]	0.1	1.1[b]	0.1	3.5[a]	0.3
22:6n-3	10.2[b]	0.6	12.4[b]	0.8	17.7[a]	2.0
Total n-3 PUFAǁ	22.6[b]	2.2	33.8[a]	1.9	37.6[a]	3.0
EPA + DHA	13.1[b]	2.2	15.6[b]	1.6	24.2[a]	2.6
Total n-3 LC-PUFA	16.5[b]	0.8	18.7[b]	0.6	29.3[a]	0.8

COM, fish oil/rapeseed oil feed; WCO, wild-type camelina oil feed; TCO, transgenic camelina oil feed; LC- PUFA, long-chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3). Different superscript letters within a row denote significant differences among diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$). [*]Contains 14:0, 15:0, 18:0, 20:0 and 22:0; [†]Contains 16:1n-7, 18:1n-7, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1; [‡]Contains 20:2n-6 and 20:3n-6; [ǁ]Contains 18:4n-3, 20:3n-3 and 20:4n-3.

Table 5. Lipid contents (percentage of wet weight) and fatty acid compositions (percentage of total fatty acids) of total lipid of liver and head kidney of Atlantic salmon after feeding the experimental diets for 12 weeks. Data presented as means ± SD (n = 3).

	COM		WCO		TCO	
	Mean	SD	Mean	SD	Mean	SD
Liver						
Lipid content	4.7	1.0	4.4	0.9	4.1	0.5
16:0	13.2	1.7	14.0	1.4	15.0	0.9
Total saturated[*]	19.3[b]	1.9	20.4[ab]	1.3	23.5[a]	1.0
18:1n-9	24.9[a]	5.5	15.7[b]	2.5	10.4[b]	0.5
Total monoenes[†]	32.8[a]	6.4	24.3[ab]	3.8	16.1[b]	1.0
18:2n-6	8.5	1.0	9.1	0.9	7.6	0.5
20:4n-6	2.5[b]	0.5	2.3[b]	0.3	6.3[a]	0.0
Total n-6 PUFA[‡]	14.0[b]	0.3	15.0[b]	0.7	17.3[a]	0.4
18:3n-3	2.6[b]	0.2	6.7[a]	1.1	3.1[b]	0.5
20:5n-3	5.5[b]	0.7	6.4[b]	0.9	8.2[a]	0.6
22:5n-3	1.6[b]	0.2	1.6[b]	0.3	3.5[a]	0.4
22:6n-3	22.8	4.5	22.2	3.3	26.2	1.2
Total n-3 PUFA[ǁ]	33.9[b]	5.0	40.3[ab]	2.9	43.1[a]	0.6
EPA + DHA	28.4	5.1	28.5	3.7	34.4	1.0
Total n-3 LC-PUFA	30.8[b]	2.1	31.8[ab]	1.4	39.1[a]	0.5
Head kidney						
Lipid content	5.6	1.1	4.2	0.4	4.1	1.0
16:0	12.6	0.4	14.0	1.0	14.4	1.6
Total saturated[*]	18.1[b]	0.6	20.0[ab]	1.1	22.3[a]	2.1
18:1n-9	32.2[a]	2.2	18.8[b]	1.3	14.2[b]	2.1
Total monoenes[†]	43.7[a]	2.5	32.0[b]	1.8	23.4[c]	2.8
18:2n-6	11.3	0.4	11.8	1.0	11.9	1.6
20:4n-6	1.0[b]	0.2	1.3[b]	0.3	4.2[a]	1.1
Total n-6 PUFA[‡]	13.7[b]	0.4	14.9[b]	1.1	18.4[a]	0.6
18:3n-3	4.2[b]	0.1	10.3[a]	1.5	4.9[b]	0.7
20:5n-3	4.0[b]	0.5	4.8[b]	1.3	7.1[a]	0.5
22:5n-3	1.1[b]	0.2	1.0[b]	0.1	2.8[a]	0.2
22:6n-3	11.6	1.5	13.2	2.0	16.5	2.7
Total n-3 PUFA[ǁ]	22.8[b]	2.1	32.6[a]	1.8	34.1[a]	2.2
EPA + DHA	15.7[b]	2.0	18.1[ab]	3.3	23.6[a]	3.2
Total n-3 LC-PUFA	17.5[b]	0.6	20.2[b]	1.0	27.7[a]	1.2

COM, fish oil/rapeseed oil feed; WCO, wild-type camelina oil feed; TCO, transgenic camelina oil feed; LC- PUFA, long-chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3). Different superscript letters within a row denote significant differences among diets as determined by one-way ANOVA with Tukey's comparison test ($p < 0.05$). [*]Contains 14:0, 15:0, 18:0, 20:0 and 22:0; [†]Contains 16:1n-7, 18:1n-7, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1; [‡]Contains 20:2n-6 and 20:3n-6; [ǁ]Contains 18:4n-3, 20:3n-3 and 20:4n-3.

Table 6. Summary of liver and pyloric caeca microarray analysis after removing duplicated probes

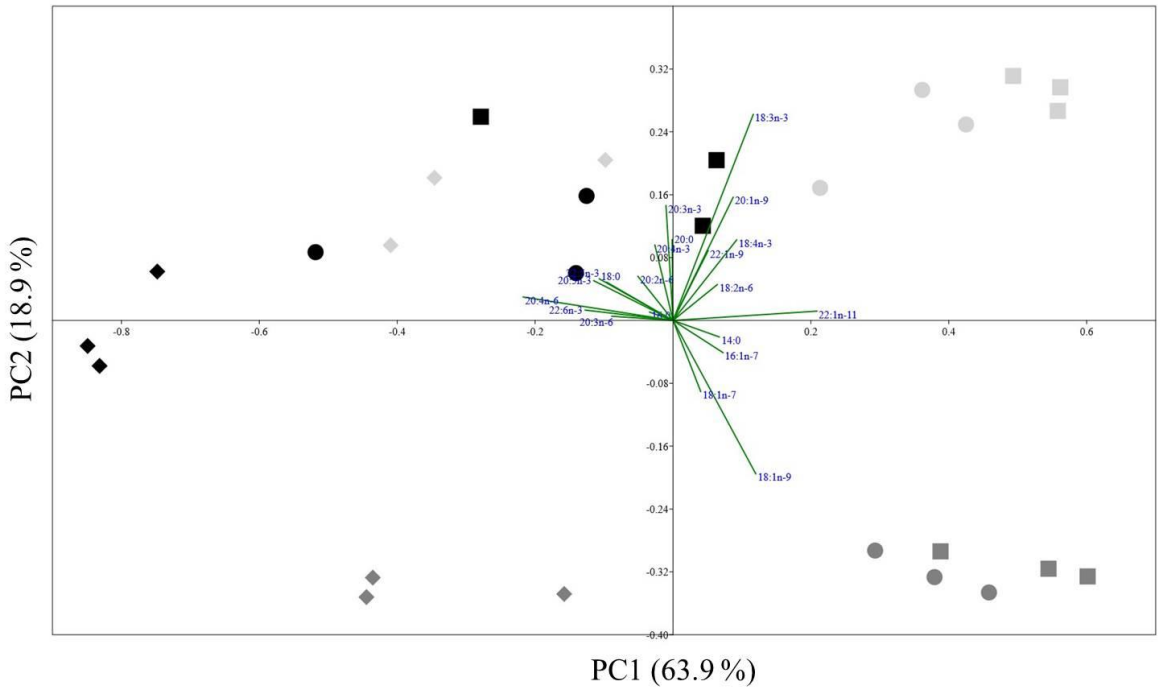
	COM/WCO	COM/TCO	TCO/WCO
Liver			
Total no. of probes		44000	
Total no. of DEG	249	312	506
Up-regulated genes	**169**	**262**	**89**
FC 1.3 – 1.5	58 (34.5 %)	75 (28.6 %)	25 (28.1 %)
FC 1.5 – 2.5	88 (52.4 %)	139 (53.1 %)	50 (56.2 %)
FC > 2.5	22 (13.1 %)	48 (18.3 %)	14 (15.7 %)
Down-regulated genes	**80**	**50**	**417**
FC 1.3 – 1.5	23 (28.7 %)	18 (36 %)	157 (37.6 %)
FC 1.5 – 2.5	48 (60 %)	28 (56 %)	211 (50.6 %)
FC > 2.5	9 (11.3 %)	4 (8 %)	49 (11.8 %)
Pyloric caeca			
Total no. of probes		44000	
Total no. of DEG	971	804	279
Up-regulated genes	**918**	**729**	**182**
FC 1.3 – 1.5	518 (56.4 %)	421 (57.8 %)	55 (30.1 %)
FC 1.5 – 2.5	263 (28.6 %)	207 (28.4 %)	62 (33.9 %)
FC > 2.5	137 (14.9 %)	101 (13.9 %)	66 (36.1 %)
Down-regulated genes	**53**	**75**	**97**
FC 1.3 – 1.5	5 (9.4 %)	14 (19.8 %)	20 (20.6 %)
FC 1.5 – 2.5	24 (45.3 %)	29 (39.2 %)	37 (38.1 %)
FC > 2.5	24 (45.3 %)	31 (41.9 %)	40 (41.2 %)

COM, fish oil/rapeseed oil feed; WCO, wild-type camelina oil feed; TCO, transgenic camelina oil feed; DEG, differently expressed gene; FC, fold change.

884 **Table 7**. Respiratory burst (absorbance per 1 x 10^5 cells) and phagocytic activity of
885 macrophages isolated from experimental fish after 12 weeks of feeding the experimental
886 diets.
887

	COM		WCO		TCO		P value
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Respiratory burst	0.16	0.04	0.18	0.04	0.15	0.03	0.057
Phagocytic activity	50.0	5.9	53.3	8.1	47.8	7.6	0.854

888
889 COM, fish oil/rapeseed oil feed; WCO, wild-type camelina oil feed; TCO, transgenic
890 camelina oil feed.
891
892



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895 Figure 1.

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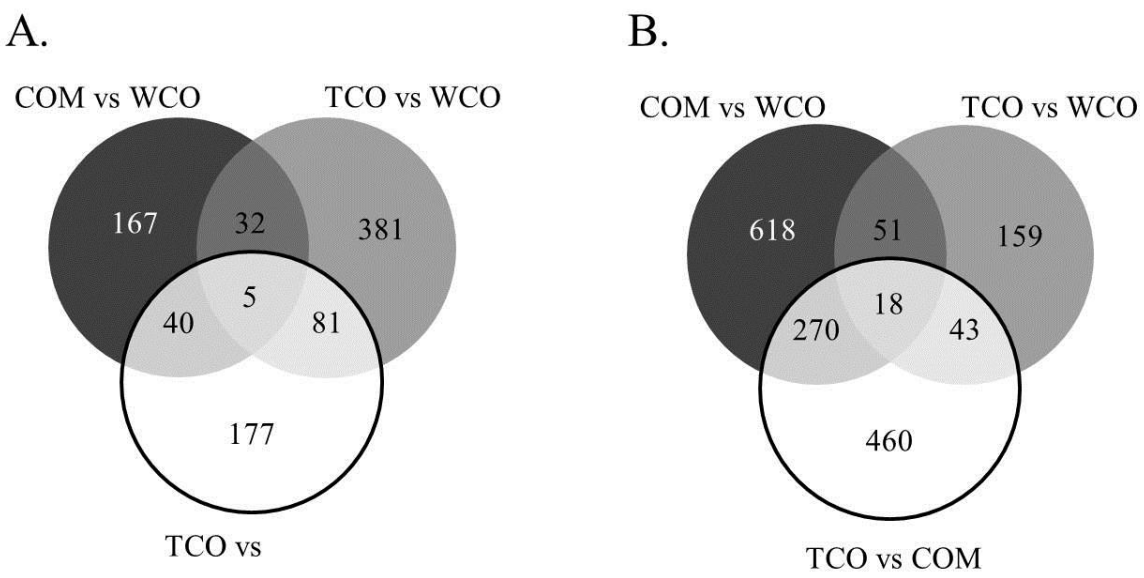

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899 Figure 2

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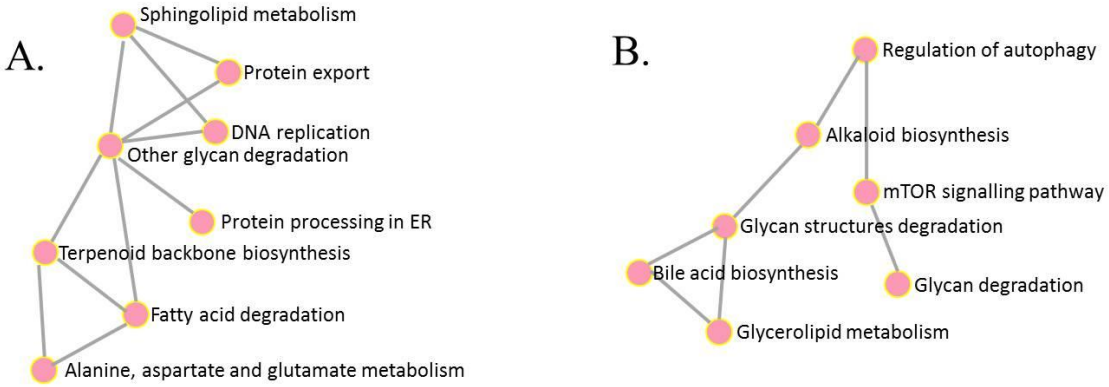

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903 Figure 3

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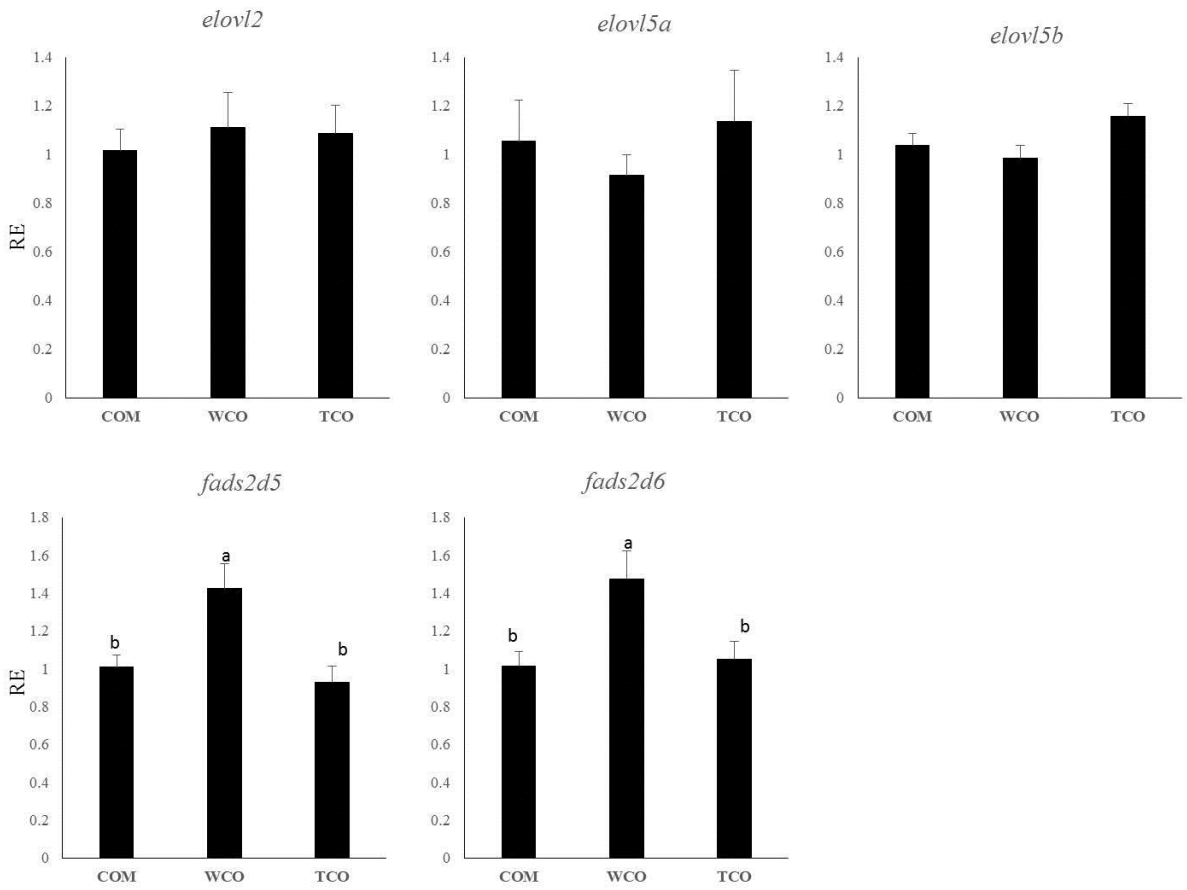

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907 Figure 4

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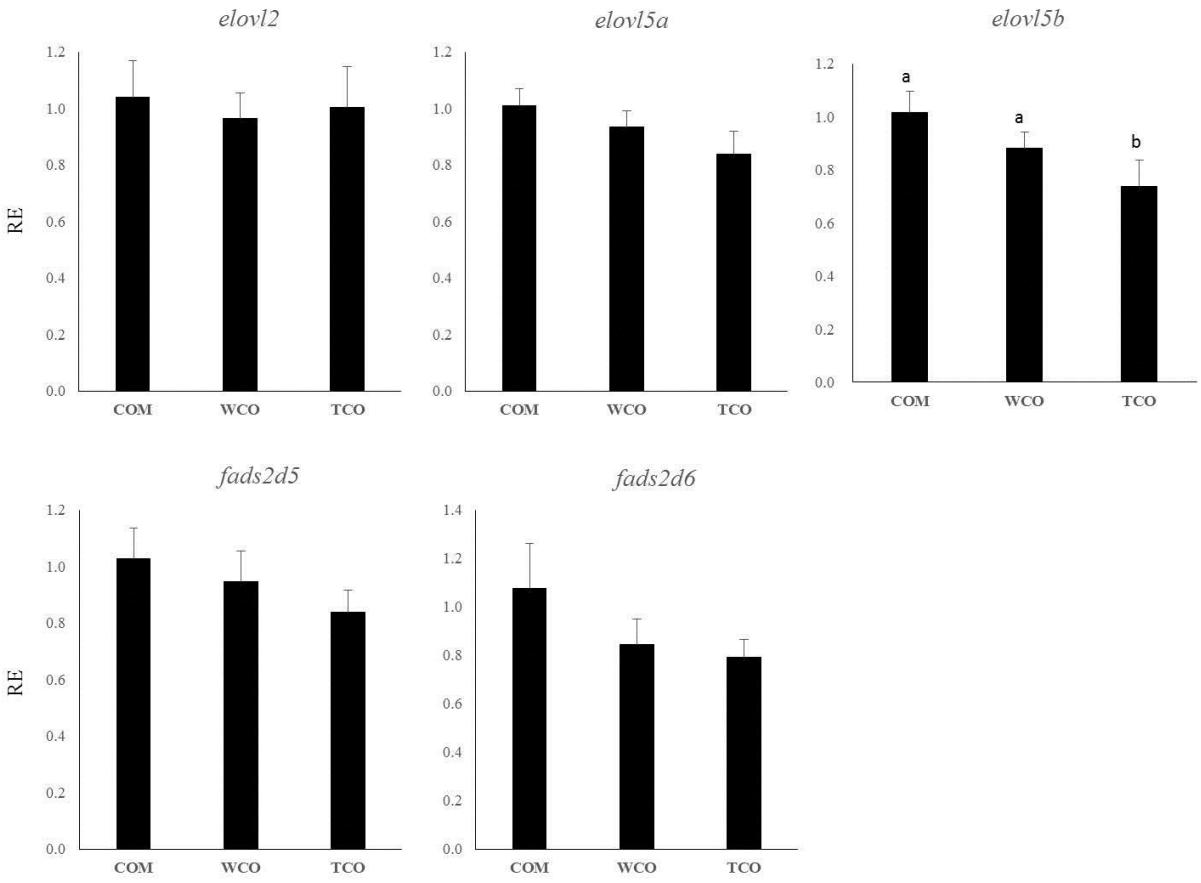
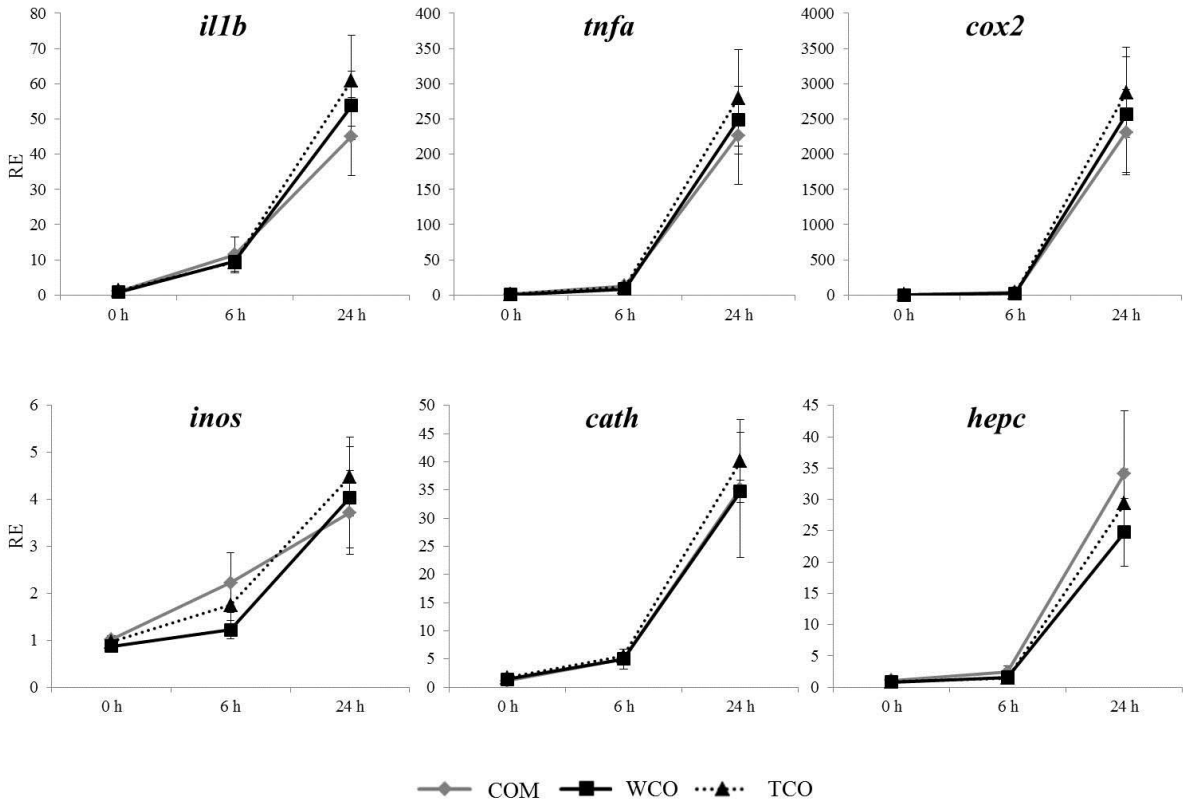


Figure 5



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917 Figure 6