A nutritionally-enhanced oil from transgenic Camelina sativa effectively replaces fish oil as a source of eicosapentaenoic acid for fish

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For humans a daily intake of up to 500 mg omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) is recommended, amounting to an annual requirement of 1.25 million metric tonnes (mt) for a population of 7 billion people. The annual global supply of n-3 LC-PUFA cannot meet this level of requirement and so there is a large gap between supply and demand. The dietary source of n-3 LC-PUFA, fish and seafood, is increasingly provided by aquaculture but using fish oil in feeds to supply n-3 LC-PUFA is unsustainable. Therefore, new sources of n-3 LC-PUFA are required to supply the demand from aquaculture and direct human consumption. One approach is metabolically engineering oilseed crops to synthesize n-3 LC-PUFA in seeds. Transgenic Camelina sativa expressing algal genes was used to produce an oil containing n-3 LC-PUFA to replace fish oil in salmon feeds. The oil had no detrimental effects on fish performance, metabolic responses or the nutritional quality of the fillets of the farmed fish.
developing a novel, renewable supply of n-3 LC-PUFA is the metabolic engineering of oilseed crops with the capacity to synthesize these bioactive fatty acids in seeds. Production of EPA and DHA in seeds was initially demonstrated in the model plant Arabidopsis, and was recently reported in an oilseed crop, Camelina sativa or false flax, a member of the Brassicaceae family and an ancient oilcrop that in the wild type produces an oil with up to 45% of fatty acids as α-linolenic acid (LNA; 18:3n-3). In the present study, C. sativa was transformed with a suite of five microalgal genes to produce a higher plant source of n-3 LC-PUFA. The extracted seed oil, containing 20% of total fatty acids as EPA, was investigated as a replacement for marine fish oil in feeds for Atlantic salmon (Salmo salar). Triplicate groups of salmon were fed one of three experimental diets containing fish oil (FO), wild-type Camelina oil (WCO) or EPA-Camelina oil (ECO) as the sole added lipid source for 7-weeks. The results showed that growth performance, feed efficiency, fish health and nutritional quality in terms of EPA, DHA for the human consumer were all unaffected by replacing FO with ECO. Metabolic analysis confirmed the EPA to DHA pathway was active in liver and transcriptomic analysis indicated that the EPA:DHA ratio had greater influence on gene expression than absolute level of EPA. This translational research has demonstrated that n-3 LC-PUFA enriched oils from transgenic oilseed crops can be effective substitutes for fish oil in feeds for Atlantic salmon, capable of maintaining n-3 LC-PUFA levels in farmed fish. Thus, oils extracted from modified oilseed crops represent a potential solution to supplying the growing demand for these critically important dietary nutrients.

Results

Seeds from transgenic Camelina sativa effectively accumulated over 20% of EPA. The analysis of fatty acid composition of the oil (abbreviated to Tr-CO, for transgenic Camelina oil) extracted from the seeds of transgenic Camelina demonstrated accumulation of significant levels of EPA (>20%; Table 1), as high as those found in many fish oils. This increased the total n-3 PUFA content of Tr-CO to a level similar to fish oil and was accompanied by a reduction in 18:3n-3 compared to the wild-type Camelina oil (Wt-CO). As expected, no DHA was detected in the terrestrial-origin oils. Monoenoic fatty acids, particularly 18:1n-9 and 20:1n-9 were increased due to the increased percentages of 18:3n-6, 20:3n-6 and 20:4n-6, 20:2n-6 and total n-6 PUFA compared to the FO feed (Table 1). Furthermore, the ECO feed had increased percentages of n-3 PUFA (especially 14:0 and 16:0), 16:1n-7, 18:1n-7 and n-3 PUFA (18:4n-3, 20:5n-3 and 22:6n-3) and decreased levels of 18:3n-3, 20:1n-9, 18:2n-6 and n-6 PUFA compared to the FO feed (Table 1). The analysis of fatty acid composition of the oil.

Fatty acid compositions of feeds reflected the lipid source utilised in the formulations. In general terms, the vegetable oil-based feeds, WCO and ECO, had decreased proportions of saturated fatty acids (especially 14:0 and 16:0), 16:1n-7, 18:1n-7 and n-3 PUFA (18:4n-3, 20:5n-3 and 22:6n-3) and increased levels of 18:3n-3, 20:1n-9, 18:2n-6, 20:2n-6 and total n-6 PUFA compared to the FO feed (Table 1). Furthermore, the ECO feed had increased percentages of n-3 PUFA (20:4n-3, 20:5n-3), n-6 PUFA (18:2n-6, 18:3n-6, 20:3n-6, and 20:4n-6) and saturated fatty acids, and decreased monounsaturated fatty acids (18:1n-9, 20:1n-9) in the ECO feed compared to the WCO feed (Table 1). In particular, the ECO feed displayed an EPA level of almost 19% of total fatty acids, similar to that of FO (~16%), whereas EPA content in the WCO feed was just over 2%, being derived from fishmeal employed in the formulation of the

| Table 1 | Fatty acid composition (percentage of fatty acids) of the oils and feeds |
| Oils | Fish | Wt-CO | Tr-CO | FO | WCO | ECO |
|------|------|------|------|----|-----|-----|
| 14:0 | 7.50 | 0.06 | n.d. | 7.20 | 1.16 | 1.30 |
| 16:0 | 17.98 | 5.14 | 6.65 | 18.96 | 7.79 | 9.03 |
| 18:0 | 3.56 | 2.63 | 5.57 | 3.72 | 2.70 | 4.64 |
| 20:0 | 0.22 | 1.58 | 2.37 | 0.21 | 1.22 | 2.06 |
| Σ saturated | 29.91 | 9.86 | 15.54 | 30.69 | 13.20 | 17.38 |
| 16:1n-7 | 8.90 | 0.12 | 1.78 | 7.96 | 1.30 | 1.25 |
| 18:1n-9 | 7.70 | 17.35 | 6.36 | 9.84 | 16.10 | 6.10 |
| 20:1n-9 | 3.07 | 1.09 | 0.00 | 3.30 | 2.24 | 1.85 |
| 22:1n-11 | 1.02 | 2.55 | 0.96 | 2.47 | 12.72 | 6.68 |
| Σ monounsaturated | 22.77 | 36.39 | 16.88 | 26.58 | 36.42 | 18.22 |
| 18:2n-6 | 1.21 | 19.26 | 19.80 | 3.60 | 17.23 | 19.12 |
| 18:3n-6 | 0.31 | 0.00 | 1.77 | 0.25 | 0.05 | 1.61 |
| 20:2n-6 | 0.14 | 1.53 | 1.84 | 0.16 | 1.23 | 1.21 |
| 20:3n-6 | 0.20 | 0.00 | 1.19 | 0.07 | 0.00 | 1.19 |
| 20:4n-6 | 0.98 | 0.06 | 2.86 | 0.88 | 0.13 | 2.54 |
| Σ n-6 PUFA | 3.30 | 20.85 | 27.46 | 5.28 | 18.65 | 25.72 |
| 18:3n-3 | 0.85 | 31.88 | 11.17 | 1.01 | 24.96 | 10.45 |
| 18:4n-3 | 3.18 | 0.00 | 1.00 | 2.45 | 0.44 | 1.40 |
| 20:3n-3 | 0.08 | 1.01 | 1.35 | 0.07 | 0.76 | 0.97 |
| 20:4n-3 | 0.81 | 0.00 | 3.30 | 0.66 | 0.10 | 3.00 |
| 20:5n-3 | 18.76 | 0.00 | 20.36 | 15.95 | 2.14 | 18.75 |
| 22:5n-3 | 2.15 | 0.00 | 1.42 | 1.81 | 0.27 | 1.02 |
| 22:6n-3 | 12.35 | 0.00 | 0.00 | 11.23 | 2.50 | 2.42 |
| Σ n-3 PUFA | 38.20 | 32.89 | 38.60 | 33.19 | 31.17 | 38.03 |
| Σ PUFA | 47.32 | 53.74 | 66.06 | 42.73 | 50.38 | 64.40 |
| Total n-3 LC-PUFA | 34.05 | 0.00 | 25.08 | 29.55 | 5.01 | 25.19 |

Data expressed as means of three technical replicates per batch of diet.
1 contains 15:0, 20:0 and 24:0; 2 contains 16:1n-9, 20:1n-11, 20:1n-7, 22:1n-9 and 24:1n-9; 3 contains C16 PUFA; Fish and FO, fish oil and respective feed; LC-PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 and 22:6n-3); n.d. not detected; Tr-CO and ECO, oil from transgenic Camelina and respective feed; Wt-CO and WCO, oil from wild-type Camelina and respective feed. 4 contains C16 PUFA. Fish and FO, fish oil and respective feed; LC-PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 and 22:6n-3); n.d. not detected; Tr-CO and ECO, oil from transgenic Camelina and respective feed; Wt-CO and WCO, oil from wild-type Camelina and respective feed.
experimental feeds. Furthermore, around 25% DHA was present in both WCO and ECO feeds (as a result of the addition of fishmeal).

There was no difference in growth performance and feed efficiency between fish fed ECO and FO. At the end of the trial, fish from all dietary treatments more than doubled their weight and there were no mortalities (Table 2). There were no significant differences in final weight, total length or specific growth rate between fish fed the different diets. Other fish performance and basic health indicators such as hepatosomatic and viscerosomatic index (HSI and VSI respectively) and Fulton’s condition factor (k) were unaffected. Feed intake and feed efficiency, as determined by feed conversion ratio, were also not affected by dietary treatment (Table 2). Proximate analysis of whole fish showed that fish fed the ECO diet had similar protein, dry matter and ash compositions to those fed the FO diet, but lipid contents were higher in fish fed the ECO diet. The fatty acid profile of muscle tissue from ECO-fed fish reflected dietary fatty acids. The fatty acid profile of muscle tissue (flesh) of fish fed the ECO diet reflected the dietary fatty acid composition (Table 4). Therefore, flesh of fish fed the ECO diet had a higher EPA level than fish fed both FO and WCO diets. Similarly, the level of docosapentaenoic acid (DPA; 22:5n-3) in flesh of ECO-fed fish was similar to that found in FO-fed fish. The levels of DHA in flesh were not influenced by dietary EPA, and reflected dietary DHA contents with similar levels found between fish fed ECO and WCO diets. Total n-3 LC-PUFA (20:4n-3 + EPA + DPA + DHA) was higher in fish fed the ECO diet (23.8%) than in fish fed WCO (12.6%), but lower than in FO-fed fish (29.3%). Total saturated fatty acids in flesh of fish fed the ECO diet were intermediate between the levels in fish fed the FO and WCO diets, which were higher and lower, respectively. However, monounsaturated fatty acid content was lowest in fish fed the ECO diet, and total n-6 PUFA levels of flesh were higher in fish fed both vegetable oil diets (WCO and ECO) compared to fish fed the FO diet, reflecting dietary levels.

High EPA levels in liver of fish fed the ECO diet indicated active DHA biosynthesis. Similar to muscle tissue, the EPA level in liver of fish fed the ECO diet was higher than in fish fed either of the other diets (Table 5). However, in contrast to muscle, DPA level in liver was also highest in fish fed the ECO diet compared to fish fed either the FO or WCO diets. Furthermore, liver DHA contents differed significantly, being higher in liver of ECO-fed fish than in liver of fish fed WCO, but lower than in fish fed FO. The higher DPA and DHA levels in liver of ECO-fed fish compared to those in livers of WCO-fed fish suggested active biosynthesis and accumulation of these fatty acids in fish fed the high EPA feed. In contrast, the highest percentages of metabolites derived from 18:3n-3 (18:4n-3, 20:3n-3, 20:4n-3, 22:3n-3, 22:4n-3, 22:5n-3 and 22:6n-3) in liver was also highest in fish fed the ECO diet compared to fish fed either the FO or WCO diets.

### Table 2 | Growth performance, survival, feed utilisation and basic biometry over the 7-week experimental period

| Parameter       | FO           | WCO          | ECO           |
|-----------------|--------------|--------------|---------------|
| Final weight (g) | 196.5 ± 26.3 | 205.0 ± 28.5 | 207.9 ± 26.5  |
| Total length (cm)| 25.0 ± 1.0  | 24.8 ± 1.4  | 25.0 ± 1.0    |
| Survival (%)     | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0   |
| HSI (%)          | 0.9 ± 0.1   | 0.9 ± 0.1   | 0.9 ± 0.1     |
| VSI (%)          | 9.5 ± 0.9   | 9.6 ± 1.2   | 9.9 ± 0.7     |
| FL (g/tank)      | 4200.0 ± 155.0 | 4203.0 ± 97.0 | 4257.0 ± 216.0 |
| FCR              | 0.9 ± 0.0   | 0.9 ± 0.0   | 0.9 ± 0.0     |
| SGR              | 1.9 ± 0.0   | 1.9 ± 0.0   | 2.0 ± 0.1     |
| k                | 1.3 ± 0.0   | 1.3 ± 0.0   | 1.3 ± 0.0     |

Data are means ± SD (n = 3). There were no significant differences between treatments in any parameter. FCR, feed conversion ratio; FL, feed intake; HSI, hepatosomatic index; k, condition factor; SGR, specific growth rate; VSI, viscerosomatic index.

### Table 3 | Whole body proximate composition (%) at initiation of the dietary trial and after 7 weeks of feeding the experimental diets

| Parameter     | Initial | FO     | WCO    | ECO     |
|---------------|---------|--------|--------|---------|
| Dry matter    | 28.4 ± 1.2 | 29.8 ± 0.3 | 30.7 ± 0.4 | 30.3 ± 0.1 |
| Protein       | 17.8 ± 0.5 | 17.5 ± 0.0 | 17.5 ± 0.2 | 17.3 ± 0.1 |
| Lipid         | 8.0 ± 0.2  | 9.7 ± 0.2  | 10.6 ± 0.3 | 10.4 ± 0.1  |
| Ash           | 2.2 ± 0.6  | 2.0 ± 0.0  | 1.9 ± 0.0  | 1.9 ± 0.1  |

Data are means ± SD (n = 3). Different superscript letters within a row denote significant differences among diets. Statistical differences were determined by one-way ANOVA with Tukey’s comparison test (p < 0.05).

### Table 4 | Lipid content (percentage of wet weight) and fatty acid compositions (percentage of total fatty acids) of total lipid from muscle tissue (flesh) of Atlantic salmon after 7 weeks of feeding the experimental diets

| Fatty acid     | FO       | WCO     | ECO     |
|----------------|----------|---------|---------|
| Total saturated1 | 26.9 ± 0.3 | 17.3 ± 0.2 | 20.0 ± 0.2 |
| EPA            | 14.0     | 5.0 ± 0.1 | 1.6 ± 0.1 |
| DHA            | 16.0     | 17.3 ± 0.1 | 11.0 ± 0.1 |
| DPA            | 18.0     | 3.8 ± 0.0  | 3.4 ± 0.1  |
| Total n-6 PUFA3 | 7.5 ± 0.2 | 16.1 ± 0.1 | 20.2 ± 0.2 |
| 18:3n-3        | 18.3-n3  | 1.8 ± 0.0  | 14.6 ± 0.2 |
| 18:4n-3        | 18.4-n3  | 1.4 ± 0.0  | 2.0 ± 0.1 |
| 20:3n-3        | 20.3-n3  | 0.2 ± 0.0  | 0.6 ± 0.0  |
| 20:5n-3        | 20.5-n3  | 1.1 ± 0.1  | 1.1 ± 0.0  |
| 22:5n-3        | 22.5-n3  | 3.1 ± 0.1  | 0.9 ± 0.0  |
| 22:6n-3        | 22.6-n3  | 15.8 ± 0.9 | 7.7 ± 0.5  | 8.2 ± 0.1 |
| Total n-3 PUFA4 | 32.7 ± 0.9 | 30.2 ± 0.8 | 33.0 ± 0.6 |
| 18:3n-3        | 18.3-n3  | 3.3 ± 0.4  | 24.6 ± 0.7  |
| 20:4n-3        | 20.4-n3  | 5.2 ± 0.3  | 5.6 ± 0.4  |
| 20:5n-3        | 20.5-n3  | 3.3 ± 0.2  | 2.9 ± 0.1  |
| 20:6n-3        | 20.6-n3  | 0.3 ± 0.0  | 0.8 ± 0.0  |

Data expressed as means ± SD (n = 3). Different superscript letters within a row denote significant differences among diets. Statistical differences were determined by one-way ANOVA with Tukey’s comparison test (p < 0.05).

1contains C16 PUFA. ECO, feed containing oil from transgenic Camelina; FO, fish oil feed; LC-PUFA, long-chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3); WCO, feed containing oil from wild-type Camelina.

**References**

1. **High DHA levels in liver of fish fed the ECO diet indicated active DHA biosynthesis.** Similar to muscle tissue, the EPA level in liver of fish fed the ECO diet was higher than in fish fed either of the other diets (Table 5). However, in contrast to muscle, DPA level in liver was also highest in fish fed the ECO diet compared to fish fed either the FO or WCO diets. Furthermore, liver DHA contents differed significantly, being higher in liver of ECO-fed fish than in liver of fish fed WCO, but lower than in fish fed FO. The higher DPA and DHA levels in liver of ECO-fed fish compared to those in livers of WCO-fed fish suggested active biosynthesis and accumulation of these fatty acids in fish fed the high EPA feed. In contrast, the highest percentages of metabolites derived from 18:3n-3 (18:4n-3, 20:3n-3 or 20:4n-3) were observed in liver of fish fed WCO. There was higher n-6 PUFA in livers of fish fed both the vegetable oil diets.
The metabolic response in liver of fish fed ECO was more similar to that in fish fed WCO compared to fish fed FO. Statistical analysis of the microarray data returned a list of 2817 and 1361 DEG in liver of salmon fed ECO were compared to fish fed FO or WCO respectively, (p < 0.05; Fig. 2A). From these transcripts, 2389 were exclusive to the ECO v. FO comparison (p < 0.05), of which 747 were regulated at a fold change (|FC| > 1.5 (31.3% of the total number of transcripts) (Fig. 2B). When comparing liver from salmon fed ECO with WCO, 933 probes exclusive to this contrast were identified, of which 319 had a FC > 1.5 (34.2%) (Figs. 2A and 2B). A total of 428 transcripts were ECO specific (common to both contrasts, Fig. 2A) and, of these, only 9 showed expression changes in opposite directions (ECO/FO and ECO/WCO), indicating that the transcriptomic response was consistent. After removing non-annotated genes, KEGG analysis of the common 428 transcripts returned 148 KO terms at p < 0.05 and revealed that the most affected biological categories were metabolism (32%), translation (17%) and transcription (13%) (Fig. 2C). More comprehensive analysis of the top 100 most significant hits and fold differences showed increased representation of metabolism, and particularly lipid metabolism (Supplementary Table 3). The differing distribution was mainly due to the repetition of multiple features of isopentenyl-diphosphate delta isomerase (idi) gene and to the lower significances found in the other categories. The high fold-changes (FC) found in the lipid metabolism category were noteworthy, especially when comparing ECO-fed fish to FO-fed fish, with an average FC of -4.14 for idi, -4.69 for farnesyl pyrophosphate synthetase (fpps) or -4.95 for squalene epoxidase (sae), all genes involved in cholesterol/isoprenoid biosynthesis. Carbohydrate metabolism category was also augmented and it is remarkable the high FC (-6.18) in the ECO/FO contrast of the MGC80816 protein, involved in butanoate metabolism. Other categories affected were translation (16.7%), transport (6.3%), protein folding (4.2%), signalling (4.2%), transcription (2.1%) and digestive system (2.1%).

The microarray analyses data were fully validated by qPCR of selected genes. Validation of the microarray data was performed by comparing the expression of 10 genes, selected according to their significant up- or down-regulation including some belonging to the LC-PUFA biosynthesis pathway that were identified by KEGG analysis as being significantly affected by diet (Table 6). Good correspondence in terms of intensity (FC) and direction of change (up- or down-regulation) was observed among the studied genes (90%, 9 out of 10). However, the match was less consistent in terms of significance (p value) when comparing microarray and qPCR results which could be due to the relatively small changes in expression observed in all the genes for both analyses.

No transgenic EPA-Camelina DNA fragments could be detected in liver, pyloric caeca or muscle. All salmon tissues tested negative for the presence of the Camelina T-DNA gene construct, as monitored by the use of nptII primers, directed towards the transgene construct sequence, whereas all tissues tested positive to salmon growth hormone gene, gh (Fig. 3).

Discussion
Inclusion of the EPA-rich Camelina oil in diets for juvenile Atlantic salmon had no detrimental effects on fish growth or performance after seven weeks of feeding the experimental diets. Furthermore, performance did not differ between fish fed any of the three diets, confirming that the oil from transgenic Camelina could be employed as a replacement for fish oil in feeds for Atlantic salmon. The fish

### Table 5: Lipid content (percentage of wet weight) and fatty acid compositions (percentage of total fatty acids) of total lipid from liver of Atlantic salmon after 7 weeks of feeding the experimental diets

|        | FO          | WCO         | ECO          |
|--------|-------------|-------------|--------------|
| Lipid  | 3.6 ± 0.1   | 5.9 ± 0.8   | 4.2 ± 0.2    |
| 14:0   | 2.1 ± 0.1   | 1.3 ± 0.1   | 1.1 ± 0.1    |
| 16:0   | 18.7 ± 0.9  | 11.0 ± 1.7  | 14.1 ± 0.8   |
| 18:0   | 4.9 ± 0.4   | 4.8 ± 0.1   | 5.7 ± 0.1    |
| 20:0   | 0.1 ± 0.0   | 0.4 ± 0.0   | 0.5 ± 0.0    |
| Total saturated¹ | 26.3 ± 0.5  | 17.8 ± 1.6  | 21.6 ± 0.9   |
| 16:1n-7 | 2.7 ± 0.2   | 1.8 ± 0.1   | 1.5 ± 0.0    |
| 18:1n-9 | 10.5 ± 1.1  | 22.0 ± 2.8  | 11.8 ± 0.5   |
| 18:1n-7 | 2.7 ± 0.2   | 2.2 ± 0.0   | 1.9 ± 0.1    |
| 20:1n-11 | 0.1 ± 0.0   | 0.1 ± 0.0   | 0.1 ± 0.0    |
| 20:1n-9 | 1.2 ± 0.1   | 5.9 ± 0.9   | 2.0 ± 0.3    |
| 20:1n-7 | 0.2 ± 0.0   | 0.2 ± 0.0   | 0.2 ± 0.0    |
| 22:1n-11 | 0.4 ± 0.1   | 0.5 ± 0.1   | 0.4 ± 0.0    |
| 22:1n-9 | 0.1 ± 0.0   | 0.6 ± 0.1   | 0.2 ± 0.0    |
| Total monoenes² | 19.3 ± 1.7  | 34.4 ± 4.0  | 19.2 ± 0.8   |
| 18:2n-6 | 2.9 ± 0.4   | 10.8 ± 0.8  | 8.2 ± 0.7    |
| 18:3n-6 | 0.0 ± 0.0   | 0.4 ± 0.0   | 0.3 ± 0.0    |
| 18:4n-3 | 0.5 ± 0.1   | 1.8 ± 0.1   | 1.6 ± 0.2    |
| 20:3n-6 | 0.2 ± 0.1   | 1.3 ± 0.2   | 1.3 ± 0.2    |
| 20:4n-3 | 3.1 ± 0.2   | 1.3 ± 0.3   | 4.1 ± 0.2    |
| Total n-6 PUFA³ | 7.3 ± 0.4   | 15.5 ± 0.5  | 15.8 ± 0.7   |
| 18:3n-3 | 1.0 ± 0.2   | 9.1 ± 0.8   | 3.9 ± 0.5    |
| 18:4n-3 | 0.3 ± 0.1   | 1.7 ± 0.1   | 0.6 ± 0.0    |
| 20:3n-3 | 0.2 ± 0.0   | 1.2 ± 0.1   | 0.8 ± 0.1    |
| 20:4n-3 | 0.9 ± 0.1   | 2.1 ± 0.2   | 2.9 ± 0.1    |
| 20:5n-3 | 9.8 ± 0.2   | 4.1 ± 0.7   | 10.9 ± 0.2   |
| 22:5n-3 | 3.3 ± 0.1   | 1.1 ± 0.2   | 4.3 ± 0.2    |
| 22:6n-3 | 31.5 ± 1.9  | 12.8 ± 2.8  | 20.9 ± 1.6   |
| Total n-3 PUFA⁴ | 46.9 ± 1.6  | 32.2 ± 2.9  | 43.2 ± 0.6   |
| 20:5n-3 | 54.4 ± 1.2  | 47.0 ± 2.5  | 59.3 ± 1.1   |
| Total n-3LC-PUFA | 45.5 ± 2.0  | 20.1 ± 3.6  | 38.0 ± 1.2   |

Data expressed as means ± SD (n = 3). Different superscript letters within a row denote significant differences among diets. Statistical differences were determined by one-way ANOVA with Tukey’s comparison test (p < 0.05).

¹contains 15:0, 22:0 and 24:0; ²contains 16:1n-9 and 24:1n-9; ³contains 22:4n-6 and 22:5n-6; ⁴contains C16 PUFA. ECO, feed containing oil from transgenic Camelina; FO, fish oil feed; LC-PUFA, long chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3); WCO, feed containing oil from wild-type Camelina.
performance data obtained were consistent with those reported prev-
iously in other feeding trials where fish oil was substituted by equi-
valent wild-type Camelina oil in juvenile Atlantic cod (Gadus
morhua)\(^3\)2 and Atlantic salmon\(^3\)3. In contrast, cod fed a diet formu-
lated with defatted fishmeal and regular (wild-type) Camelina oil
displayed lower weight gain than fish fed non-defatted fishmeal
together with regular CO due to the requirements for essential fatty
acids (n-3 LC-PUFA) not being met by the former diet\(^3\)4. It should be
noted, therefore, that in this present trial, growth performance was
not impacted in WCO-fed fish because the feeds were formulated
with non-defatted fishmeal that contained sufficient n-3 LC-PUFA
to satisfy salmon essential fatty acids (EFA) requirements\(^7\)5. This was
planned, as the present trial was specifically designed to investi-
gate the effectiveness of the oil from transgenic Camelina (Tr-CO) in
supplying high levels of n-3 LC-PUFA (in this case EPA), well in
excess of EFA requirement levels, so that the dietary n-3 LC-PUFA
would be largely deposited and stored by the fish.

Therefore, it is important to stress that n-3 LC-PUFA in fish
feeds can be defined at three levels\(^1\)7. Firstly, the minimum level
required to satisfy EFA requirements and thus prevent deficiency
signs\(^5\). This level is low (<1% of diet for salmonids species) and
relatively easy to supply even with today’s current high demand
for fish oil\(^7\)6. The second level is that required to sustain max-
imum growth and optimum health in fish being fed modern high-
energy diets\(^7\)7. It is largely unknown in most fish species and,
although higher than the minimum level, it is still relatively low.
The third level, and the subject of the present study, is the level we
must provide to ensure that the nutritional quality of farmed fish
is maintained such that it contains n-3 LC-PUFA at similar or
higher levels than can be found in wild fish. This dietary level far
exceeds the biological requirements of the fish itself and cannot
currently be met without the use of high levels of marine fish oil
and fishmeal in the feeds, but ultimately reflects the expectations
of consumer and their perception of fish such as salmon as health-
beneficial.

In addition to its effectiveness as a substitute for dietary fish oil, the
Tr-CO represented a high-EPA/zero DHA oil that has been hitherto
unavailable and so presents unique oil with which to study n-3 LC-
PUFA metabolism, including EPA to DHA pathways, in vertebrates.
In this respect, several interesting results were obtained using this
EPA-only oil. The utilisation of dietary vegetable oils has been associ-
ated with increased lipid deposition in fish tissues\(^3\)2–3,33–37. Results
from the present study supported this, as whole fish and liver lipid content was higher in fish fed both ECO and WCO than in fish fed FO. The reduced dietary n-3 LC-PUFA, that are known to suppress TAG accumulation in mammalian pre-adipocytes or lipid accumulation in Atlantic salmon adipocytes, may be the mechanism for the increased lipid deposition in fish fed vegetable oils. Indeed, fish fed WCO showed increased levels of TAG, which is in agreement with previous studies. However, fish fed the ECO diet had similar liver n-3 PUFA levels (other than DHA) as FO-fed fish, and showed increased hepatic lipid levels, which could indicate that the factor determining the increased adiposity could be reduced DHA levels, rather than overall n-3 PUFA levels.

The ECO diet had differing effects on fatty acid deposition depending on tissue. As expected of a tissue that serves as a lipid/energy store in salmon, muscle largely reflected dietary fatty acid composition. Higher levels of desaturation products of 18:3n-3 and 18:2n-6 observed in muscle of ECO-fed fish compared to WCO-fed fish probably reflected the higher dietary content of these fatty acids rather than active biosynthesis. Nonetheless, limited activation of the LC-PUFA biosynthesis pathway was observed in fish fed WCO as shown by increased 18:4n-3 compared to fish fed FO. Increased elongation products of 18:3n-3 and 18:2n-6, 20:3n-3 and 20:2n-6, observed in fish fed both ECO and WCO compared to fish fed FO were consistent with data from many studies that report an increase in these elongation products after feeding vegetable oils in fish species. Inhibition of this elongation may be another example of a DHA effect that dietary EPA alone cannot replicate.

Importantly though, muscle DHA did not vary between fish fed the ECO or WCO diets and, although its level was higher than that found in those diets, this was likely due to selective retention in Atlantic salmon adipocytes, may be the mechanism for the increased lipid deposition in fish fed vegetable oils. Indeed, fish fed WCO showed increased levels of TAG, which is in agreement with previous studies. However, fish fed the ECO diet had similar liver n-3 PUFA levels (other than DHA) as FO-fed fish, and showed increased hepatic lipid levels, which could indicate that the factor determining the increased adiposity could be reduced DHA levels, rather than overall n-3 PUFA levels.

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of DHA reported previously in many studies where salmon were fed dietary vegetable oil\textsuperscript{33,43–44}. Most importantly, from a human health viewpoint, the ECO-fed salmon would have more beneficial effects compared to WCO-fed fish due to total n-3 LC-PUFA content in muscle being nearly twice that in fish fed WCO, albeit not as high as in fish fed FO.

In contrast to muscle, liver exhibited active LC-PUFA biosynthesis in fish fed the ECO diet, with higher DHA levels than those found in fish fed WCO. These results were supported by up-regulation of \textit{fads2d5} and \textit{elovl2} gene expression in the liver of fish fed ECO, as expected for the operation of the LC-PUFA biosynthesis pathway in salmonids including the Sprecher shunt\textsuperscript{45}. These genes were up-regulated in Atlantic salmon juveniles fed a vegetable oil enriched with EPA, whereas, the inclusion of EPA plus DHA inhibited their expression\textsuperscript{46}. The results in the present study indicate that the relative lack of dietary DHA in diets ECO and WCO induced expression of the enzymes involved in its biosynthesis, and that high dietary EPA (as in ECO) did not reduce their expression. Interestingly, production of shorter chain products derived from dietary EPA (as in ECO) did not reduce their expression. Further, the level of 22.5 MJ kg\textsuperscript{-1} of energy (long day) at 250 J cm\textsuperscript{-2} sec\textsuperscript{-1}. Oil (7 g) was produced from seeds (approximately 20 kg) by cold-pressing and solvent extraction to maximise yield (~35%) (PPM, Magdeburg, Germany). The anti-oxidant ethoxyquin was added to stabilise the final product.

Diet and feeding trial. Three isoenergetic diets were formulated to satisfy the nutritional requirements of salmonid fish (Supplementary Table 4)\textsuperscript{3}. The diets supplied 46 kg kg\textsuperscript{-1} crude protein and 21 kg kg\textsuperscript{-1} crude lipid at a crude energy level of 22.5 MJ kg\textsuperscript{-1} and were manufactured at BioMar Tech-Centre (Brande, Denmark). The three feeds were produced by vacuum coating identical dry basal extruded pellets with either fish oil (BO), wild-type Camelina oil (WCO) or EPA-Camelina oil (ECO) (Supplementary Table 4). Non-defatted fishmeal was employed as the major protein source to ensure EFA requirements were met\textsuperscript{17}. A total of 405 juveniles (post-smolt) Atlantic salmon with an average body weight of 82.5 ± 8.1 g (mean ± S.D.) were distributed between 9 seawater tanks (45 per tank) and fed one of the three experimental diets in triplicate for 7 weeks. Prior to the start of the experimental period fish were fed a standard commercial feed containing 30% fish oil and 70% rapeseed oil. The experimental system comprised 1 m\textsuperscript{3}, 500 L tanks supplied by flow-through seawater (15 L min\textsuperscript{-1}) at ambient temperature that averaged 10.2 ± 0.6 °C. Experimental feeds were delivered in excess by automatic disc feeders with an automated uneven feed collection system in order to determine accurate feed efficiency. At the end of the trial fish were weighed and growth rate, feed efficiency and biometric parameters calculated as follows: Specific growth rate (SGR) = 100 * (lnWf - lnW0)/t, where W0 = initial weight (g) and Wf = final weight (g) at time t (days), Feed conversion ratio (FCR) = F/ (BW - Bo), where F = feed intake (g), Bo = initial biomass (g) and BF = final biomass (g). Fulton’s condition factor (k) = 100 * (W/L\textsuperscript{3}), where W is the final weight (g) and L is the total length (cm). Liver and empty gastrointestinal tract were used to calculate hepatosomatic index (HSI) = liver weight (g) * 100/Wf (g) and visceral somatic index (VSI) = liver + gastrointestinal tract (g) * 100/Wf (g).

Methods

Metabolic engineering of Camelina. A construct containing a cassette of five genes was used for transformation\textsuperscript{30}. Briefly, the five-gene construct contained a set of genes optimised for EPA synthesis: a \(\Delta^5\)-desaturase gene from \textit{Ostreococcus tauri} (Ots\(\Delta^5\)), a \(\Delta^6\) fatty acid elongase gene from \textit{Phymcomitrella patons} (PSE1), a \(\Delta^5\)-desaturase gene from \textit{Travastochoytrium} sp. (Tc\(\Delta^5\)), a \(\Delta^12\)-desaturase gene from \textit{Phytophthora soaje} (Ps\(\Delta^12\)) and an \(\Delta^3\)-desaturase from \textit{Phytophthora infestans} (Ps-\(\Delta^3\)) as described in detail previously\textsuperscript{30}. All genes were individually cloned under the control of seed-specific promoters, and then combined into a single T-DNA transformation vector as described previously\textsuperscript{30}. The destination vector contained an NPTII gene with the nos promoter as a selection marker. All open reading frames for desaturases and elongases were re-synthesised and codon-optimised for expression in \textit{C. sativa}.

Production of Camelina oil. \textit{C. sativa} was grown in a controlled-environment chamber at 23 °C day/18 °C night, 50–60% humidity, and kept under a 16 h photoperiod (long day) at 250 J cm\textsuperscript{-2} sec\textsuperscript{-1}. Oil (7 kg) was produced from seeds (approximately 20 kg) by cold-pressing and solvent extraction to maximise yield (~35%) (PPM, Magdeburg, Germany). The anti-oxidant ethoxyquin was added to stabilise the final product.

Diet and feeding trial. Three isonitrogenous and isoenergetic diets were formulated to satisfy the nutritional requirements of salmonid fish (Supplementary Table 4)\textsuperscript{3}. The diets supplied 46 kg kg\textsuperscript{-1} crude protein and 21 kg kg\textsuperscript{-1} crude lipid at a crude energy level of 22.5 MJ kg\textsuperscript{-1} and were manufactured at BioMar Tech-Centre (Brande, Denmark). The three feeds were produced by vacuum coating identical dry basal extruded pellets with either fish oil (BO), wild-type Camelina oil (WCO) or EPA-Camelina oil (ECO) (Supplementary Table 4). Non-defatted fishmeal was employed as the major protein source to ensure EFA requirements were met\textsuperscript{17}. A total of 405 juveniles (post-smolt) Atlantic salmon with an average body weight of 82.5 ± 8.1 g (mean ± S.D.) were distributed between 9 seawater tanks (45 per tank) and fed one of the three experimental diets in triplicate for 7 weeks. Prior to the start of the experimental period fish were fed a standard commercial feed containing 30% fish oil and 70% rapeseed oil. The experimental system comprised 1 m\textsuperscript{3}, 500 L tanks supplied by flow-through seawater (15 L min\textsuperscript{-1}) at ambient temperature that averaged 10.2 ± 0.6 °C. Experimental feeds were delivered in excess by automatic disc feeders with an automated uneven feed collection system in order to determine accurate feed efficiency. At the end of the trial fish were weighed and growth rate, feed efficiency and biometric parameters calculated as follows: Specific growth rate (SGR) = 100 * (lnWf - lnW0)/t, where W0 = initial weight (g) and Wf = final weight (g) at time t (days), Feed conversion ratio (FCR) = F/ (BW - Bo), where F = feed intake (g), Bo = initial biomass (g) and BF = final biomass (g). Fulton’s condition factor (k) = 100 * (W/L\textsuperscript{3}), where W is the final weight (g) and L is the total length (cm). Liver and empty gastrointestinal tract were used to calculate hepatosomatic index (HSI) = liver weight (g) * 100/Wf (g) and visceral somatic index (VSI) = liver + gastrointestinal tract (g) * 100/Wf (g).
Sample collection. At the end of the trial, the fish were not fed for 48 h prior to being anaesthetised and killed by overdose with metacaine sulphonate (MS222). Three whole fish per tank (9 per treatment) were frozen for analyses of proximate composition. A further 9 fish per tank were used for biometric measurements (hepato-somatic and viscera-somatic indices) and tissue analyses. Samples of flesh (Norwegian quality cut; NQC) and liver from 3 fish per tank were immediately frozen in liquid nitrogen and stored at −70 °C prior to analysis. Further samples of liver were collected from six fish per treatment (two per tank) and stabilised in RNAlater® (Sigma, Poole, UK) prior to RNA extraction.

Proximate composition. Diets and whole fish were ground before determination of proximate composition according to standard procedures45. Fish were pooled per tank and three technical replicates for single batch diet were analysed. Moisture contents were obtained after drying in an oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h. Crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK) and crude lipid content determined gravimetrically after Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus).

Lipid class and fatty acid compositions. Samples of muscle (flesh) and liver from three fish per tank were prepared as pooled homogenates and total lipid extracted from 1 g by homogenising in chloroform/methanol (2:1, v/v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK), and content determined gravimetrically46. Lipid classes were separated by double-development, high-performance thin-layer chromatography (HPTLC)47. Total lipid samples (1–2 μg) were applied and the plates developed in methyl acetate/isopropanol/chloroform/methanol/25% aqueous KCl (25:45:25:10:9, vol/vol). Excess solvent was evaporated via air drying and vacuum desiccation and plates developed to 9.5 cm using a solvent mixture containing isooxazene/diethyl ether/aetic acid (80: 20: 1, by vol.). Lipids were visualised by spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and charting plates at 160 °C for 20 min. Lipid classes were quantified by densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16; CAMAG, Muttenz, Switzerland). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 °C for 16 h, with heptadecanoic acid (17:0) as an internal standard. FAME were extracted and purified as described previously48. FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionization detector. Data were collected and processed using Chromcard for Windows (version 2.01; Theromoquest Italia S.p.A., Goettingen, Germany) on a Welch (unpaired unequal variance) t-test, at 0.05 significance. No multiple test correction was employed as previous analyses indicated that they were over-conservative for these nutritional data49. Data were submitted to the Kyoto Encyclopedia of Genes and Genomes (KEGG) for biological function analysis. Gene expression results were assembled using the relative expression software tool (REST 2009), which employs a pairwise fixed reallocation randomisation test (10,000 randomisations) with efficiency correction50 to determine the statistical significance of expression ratios (gene expression fold changes) between two treatments.

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Acknowledgments

M.B.B. and this project were partly funded by a UK Biotechnology and Biological Sciences Research Council (BBSRC) Industrial Partnership Award (BB/J001252/1). Authors wish to thank Dr. John Taggart and Ms. Beatrix Bickes for advice and direction with the microarray analyses and Dr. Sebastian Boltaña for guidance with the microarray statistical analysis.

Author contributions

M.B.B. performed, interpreted and evaluated all biological, molecular and bioinformatic analysis. M.S. performed the biochemical analyses. Gene constructs were designed by J.A.N. and O.S. with vector construction and plant transgenesis performed by O.S. All agronomy, seed harvest and analyses was performed by S.U. The salmon trial was designed by P.J.C., D.R.T. and M.B.B. and supervised by P.J.C., D.R.T. and J.A.N. were responsible for the study design and supervised the entire work. The manuscript was written by D.R.T. and M.B.B. All authors discussed and commented on the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: None. However, this work was funded by a Biotechnology and Biological Sciences Research Council (BBSRC) Industrial Partnership Award (IPA), where 10% of overall costs are provided by an industrial partner, in this case, BioMar (UK) Ltd.

How to cite this article: Betancor, M.B. et al. A nutritionally-enhanced oil from transgenic Camelina sativa effectively replaces fish oil as a source of eicosapentaenoic acid for fish. Sci. Rep. 8, 8104; DOI:10.1038/s41598-0180814 (2015).
