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Can nutritional programming in Atlantic salmon (*Salmo salar*) be optimised with a reduced stimulus period?

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ABSTRACT

New strategies are required to enhance the efficient assimilation and bioconversion of plant-based ingredients in Atlantic salmon (*Salmo salar*) diets, especially relating to the essential long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3). Our study investigated nutritional programming and specifically evaluated the optimal duration of dietary 'stimulus' and whether it could be reduced compared to a previous study using a three- week 'stimulus'. Fish were fed an experimental 'stimulus' vegetable-based diet (V_S, 5% marine meals [MM]/0% fish oil [FO]) or a standard marine-based control (M_S, 82% MM/4% FO) for either one (V1) or two weeks (V2 and M) from first exogenous feeding. All groups were then fed a standard marine based formulation, for an 'intermediate' grow-out phase to the end of 16 weeks post-first feeding, prior to a 'challenge' phase of six weeks when all fish were fed a vegetable-based diet (V_C, 10% MM/0% FO). Compared to M, fish from both V1 and V2 groups were significantly smaller at the end of the 'stimulus' phase, but there were no statistical differences in overall growth, proximate or fatty acid compositions at the end of the trial. However, hepatosomatic and viscerosomatic indices were significantly lower in V1 compared to V2 fish and there was an overall trend of improved performance in V1 fish throughout the 'intermediate' and 'challenge' phases. During the 'challenge' phase, M fish displayed a greater net gain of DHA than V1 fish, whilst V2 was a net consumer of all *n*-3 LC-PUFA over the same period. Compared to M, *n*-3 LC-PUFA biosynthesis genes in pyloric caeca were downregulated in both experimental groups indicating possible post-transcriptional modification of this pathway in either V1 or V2, considering the differences in DHA retention levels between groups. Taken together, the results suggested that nutritional programming was not initiated by a one- or two- week 'stimulus'. However, more studies are required to elucidate the mechanism behind enhanced performance of V1 fish.

1. Introduction

Expansion of the Atlantic salmon (*Salmo salar*) industry faces many challenges, including the finite availability of traditional marine raw materials. One solution is to produce feeds with higher proportions of plant-derived oils and proteins, but these diets have lower levels of

Abbreviations: ADC, apparent digestibility coefficient; ALA, α-linolenic acid; ARA, arachidonic acid; BW, body weight; dd, degree days; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; FE, feed efficiency; FI, feed intake; FM, fish meal; FO, fish oil; HSI, hepatosomatic index; *K*, condition factor; LA, linoleic acid; M, fish fed M_s for full two-week stimulus; M_I, marine ingredient based intermediate phase diet; miRNA, microRNA; MM, marine meals; M_S, marine ingredient-based stimulus diet; NER, normalised expression ratio; NP, nutritional programming; RT-qPCR, reverse transcription quantitative polymerase chain reaction; PUFA, polyunsaturated fatty acid; SGR, specific growth rate; TL, total length; V1, fish fed V_s for 1st week of stimulus; V2, fish fed V_s for full two-week stimulus; V_c, vegetable-based challenge phase diet; V_S, vegetable-based stimulus diet; VSI, viscerosomatic index..

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essential omega-3 long-chain polyunsaturated fatty acids (*n*-3 LC-PUFA), eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3) and can lead to reduced feed efficiency (Aslaksen et al., 2007; Bell et al., 2010; Clarkson et al., 2017). Consequently, harvested fish have lower fillet EPA + DHA contents as diet is the predominant influence on tissue fatty acid profiles (Sprague et al., 2020; Sargent et al., 2003). EPA and DHA are known for their health benefits in fish including anti-inflammatory and immune responses, and DHA is an essential and major component of synaptic and retinal membranes in nervous tissues (Foroutani et al., 2018; Glencross, 2009; Martinez-Rubio et al., 2012; Tocher, 2010). Thus, an insufficient supply of these essential fatty acids during development could affect performance, tissue integrity, health and stress responses (Bou et al., 2017; Rosenlund et al., 2016; Tocher, 2010).

Both EPA and DHA have significant health benefits for humans, including anti-inflammatory properties associated with a reduction in cardiovascular and other chronic diseases (Calder and Yaqoob, 2009; Djuricic and Calder, 2021; European Food Safety Authority, 2010). Although not classed as essential nutrients in the human diet, *n*-3 LC-PUFA, especially EPA and DHA, cannot be synthesised at sufficient rates in humans, meaning that dietary sources such as fish and seafood are required for humans to gain the health benefits (Brenna et al., 2009; Pike, 2015). Atlantic salmon can endogenously biosynthesise EPA and DHA, but at an inadequate rate to reach desired levels, providing there are sufficient levels of precursor α-linolenic acid (ALA; 18:3*n*-3) in the diet (Ruyter et al., 2000a, 2000b; Ruyter et al., 2000c; Tocher et al., 1997). Furthermore, net production of *n*-3 LC-PUFA has been demonstrated in Atlantic salmon (DHA) and rainbow trout (*Oncorhynchus mykiss*, EPA and DHA) when fed vegetable-based diets, although the latter demonstrated a greater capacity to biosynthesise DHA (Berge et al., 2021; Sanden et al., 2011; Turchini et al., 2011). Therefore, it is essential to fully optimise the bioconversion process to make salmon efficient net producers of *n*-3 LC PUFA.

A novel solution to this problem is applying an early nutritional intervention or 'stimulus' where fish are fed a predominantly vegetable-based diet for a short period, to induce more efficient uptake and utilisation of nutrients when fed a similar diet later in development, a concept referred to as 'nutritional programming' (NP). This mechanism exploits periods of high developmental plasticity within an organism, usually maternally or during early development, to initiate long-term changes in function (Hou and Fuiman, 2020; Lucas, 1998). In the wild, this mechanism is responsible for phenomena such as caste differentiation in bees, and there is evidence that it can be harnessed to control pests such as Drosophila flies (Sario et al., 2022; Zhu et al., 2017). In fish, a successful NP response has been demonstrated in several commercially important aquaculture species following a dietary stimulus at, or close to, first exogenous feeding, including gilthead sea bream (*Sparus aurata*) (Perera and Yufera, 2017), rainbow trout (Geurden et al., 2007) and sea bass (*Dicentrarchus labrax*) (Vagner et al., 2007). For a more comprehensive review on this topic see Hou and Fuiman (2020).

Previously, it was demonstrated that Atlantic salmon fed vegetable-based diets low in *n*-3 LC-PUFA during a three-week stimulus phase from first exogenous feeding, resulted in significantly greater growth and nutrient retention efficiency, including EPA and DHA, during freshwater growth stage when fed a similar vegetable-based 'challenge' diet (Clarkson et al., 2017). Associated with this improved growth and feed efficiency during the challenge phase was upregulation in key pathways of intermediary metabolism (Vera et al., 2017). However, fish fed the vegetable-based diet were significantly smaller at the end of the stimulus phase compared to those fed a standard marine based diet (Clarkson et al., 2017; Vera et al., 2017). Smaller fish, stemming from a vegetable-based stimulus can be a confounding factor and a bottleneck for a commercial trial, because fish size itself affects subsequent fish performance (Clarkson et al., 2017; Geurden et al., 2013). Although the time required to stimulate a NP response may be species and target nutrient specific, a stimulus length of three days has produced a positive response at a later developmental stage in zebrafish (*Danio rerio*)(Perera and Yufera, 2016) and rainbow trout (Geurden et al., 2007) stimulated with soy, with or without saponins, and glucose respectively.

Therefore, the aim of the present study was to investigate whether a shortened nutritional stimulus length in Atlantic salmon can still trigger an improved response to the vegetable-based diet at a later stage in development. To this end, two groups of Atlantic salmon were fed the experimental vegetable-based diet for one- or two-weeks, whilst a third group was fed a standard marine based diet, starting at first exogenous feeding. Comparison of the performance between the two stimulus groups was used to predict the optimal first feeding time with respect to a stronger response when fed a similar diet later in development.

2. Methods

Unless otherwise stated, all chemicals and reagents were purchased from Fisher Scientific (Loughborough, UK).

2.1. Ethics

All experimental procedures were implemented in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice, HMSO, London, January 1997), in accordance with EU regulation (EC Directive 86/609/EEC). All experimentation was performed in a temperate freshwater recirculation facility at the Institute of Aquaculture, University of Stirling (UoS), and was subject to ethical review approval carried out by the UoS Animal Welfare and Ethical Review Board (AWERB) and covered by AWERB/1819/045/New ASPA.

2.2. Experimental diets

All diets were formulated by BioMar AS (Myre, Norway) and manufactured at the BioMar Tech Centre (Brande, Denmark). Diets used during 'stimulus' (M_S, V_S), 'intermediate' (M_I) and 'challenge' (V_C) phases were formulated to meet the principal nutritional requirements of Atlantic salmon (National Research Council, 2011) for the duration of the trial (Table 1). Therefore, pellet size (0.5 mm to 2 mm) and protein and lipid contents were adapted according to growth stage. Briefly, marine-based diets (M_s/M_I) were based largely on marine ingredients including fishmeal (FM; 66%/49%), crustacean and fish peptones (15%/15%), and fish oil (4.4%/9%) as protein and lipid sources, respectively. Vegetable-based diets (V_S/V_c) were formulated with a mixture of plant proteins, including soya protein concentrate, pea protein concentrate, wheat gluten and a low proportion of marine derived protein (5% MM), with rapeseed oil (10%) as the sole lipid source. The stimulus diets (M_s and V_S) had very similar proximate compositions and were isonitrogenous and isocaloric. However, there was considerable variation between the fatty acid profiles of the marine (M_S/ M_I) and vegetable (V_S/ V_c) diets, with higher levels of EPA and DHA in marine diets compared to their vegetable counterparts (M_s 26% EPA/DHA vs. V_s 5.9% EPA/DHA; Table 1). Thus, the EPA + DHA content was 4.4-fold higher in the M_S diet compared with the V_S diet.

2.2.1. Experimental fish and culture conditions

Atlantic salmon eggs (spawning date 18th October 2018) at 388 degree-days (dd) post fertilization were acquired from Mowi (Tveitevåg, Norway) on 20th December 2018. A total of 2700 eggs were divided equally between nine 150 L tanks. All eggs hatched by 6 January 2019 after 500 dd with the fish remaining in the alevin stage until 850 dd. Water temperature was maintained at 7.0 ± 0.4 °C throughout egg incubation and the alevin stage. Once larvae reached fry stage (850 dd), following complete absorption of the yolk sac, water volume was increased to 300 L per tank. Survival to this stage was 97.7 ± 0.6%.

Throughout the trial, all tanks were exposed to 24 h artificial light. Prior to start of feeding, water temperature was gradually increased over

Table 1
Formulation, proximate and fatty acid compositions of standard marine diets (M_S and M_I) and low fishmeal/fish oil vegetable-based diets (V_S and V_C) used in respective feeding phases.

Experimental phase:	Stimulus		Intermediate	Challenge
Diet:	M_S	V_S	M_I	V_C
Ingredients (g/kg)				
Marine meals				
Fishmeal*	667	0	490	50
Krill meal†	100	25	0	0
Fish peptones†	50	25	0	50
Vegetable meals				
SPC†	0	155	161	90
Wheat products§	70	271	175	281
PPC‖	0	300	20	250
Other vegetable sources	30	20	0	60
Fish oil**	44	0	86	0
Rapeseed oil§	0	67	51	141
Lecithin¶	5.2	36.5	5	5
Vitamins and minerals††	28.7	72.5	23.8	65.7
Amino acids‡‡	1.3	36.4	1.7	21.45
Analysed proximate composition				
Ash crude (%)	12.2	8.8	10.1	7.3
Lipid crude (%)	13.3	13.4	17.8	16.3
Protein crude (%)	60.0	56.0	52.6	51.8
Energy gross (MJ/kg)	20.6	21.1	21.8	22.3
All fatty acids (% total fatty acids)				
SFA	29.0	17.0	20.9	10.4
OA (18:1*n*-9)	38.7	12.8	26.3	52.6
MUFA	28.8	45.9	41.8	55.4
LA (18:2*n*-6)	5.7	21.5	10.3	23.6
ARA (20:4*n*-6)	0.1	0.7	0.5	0.1
ALA (18:3*n*-3)	1.8	7.0	4.2	8.9
EPA (20:5*n*-3)	11.9	3.4	8.1	0.5
DHA (22:6*n*-3)	14.1	2.5	8.2	0.7
PUFA	42.2	37.1	37.3	34.2
n-3 LC-PUFA	27.7	6.1	17.7	1.2

ALA, α-linolenic acid; ARA, arachidonic acid; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acid; MUFA, monoenes; OA, Oleic acid; PPC, pea protein concentrate; SFA, saturated fatty acid; SPC, soya protein concentrate.
* Feed Services Bremen, Bremen, Germany.
† Aker BioMarine, Lysaker, Norway.
‡ Caramuru, Itumbiara, Brazil.
§ Cargill, Minnesota, US.
‖ Agrident, Amsterdam, Netherlands.
** ED&F Man, London, UK.
¶ Nova Nutriway GmbH, Hamburg, Germany.
†† DSM, Heerlen, Netherlands.
‡‡ Evonik, Essen, Germany.

a period of one week and then held at 13.1 ± 0.5 °C for the remainder of the trial. Water parameters including temperature, oxygen level (86% saturation, 8–9 mg/L), pH (7.16 ± 0.2), nitrogen NO_2 (0.32 ± 0.2 mg/L), total ammonia nitrogen (0.13 ± 0.7 mg/L) and chloride (135 ± 13 mg/L) were recorded and controlled daily. After a baseline sampling, 278 ± 2 fish remained per tank and feeding trial was initiated on 21st February 2019 (875 dd). From this point onwards the experiment was divided into three phases: 'stimulus', 'intermediate' and 'challenge' (Fig. 1).

2.2.2. Feeding trial

Fish were fed by automatic feeders (Arvo-tec TD2000; Huutokoski, Finland) with user interface (ArvoPRO) for 22 h/day, with a pause for 1 h at 09:00 and 16:00 for feed collection. Daily feeding rate was based on feeding tables (BioMar Inicio Plus), plus 10% to ensure excess feed was provided to enable accurate estimates of feed consumed. During the first two-week exogenous feeding 'stimulus' phase there were three dietary regimes termed M, V1 and V2, each represented by triplicate tanks (n = 3). Groups were then named according to their stimulus diet regime for the reminder of trial and in this manuscript. M and V2 fish were fed diets M_S or V_S respectively, for the full two weeks. V1 fish were fed V_S diet for the first week before being switched to the M_S diet for the remainder of stimulus phase. All fish were then transferred to the M_I diet for a 14-week 'intermediate' grow out phase. For the final six-week 'challenge' phase, all fish were switched to the V_C diet. Following sampling at the end of 'challenge' phase, feeding of V_C diet resumed for a further week for the remaining fish, to enable faeces sampling for digestibility analysis.

2.2.3. Sampling procedures

Fish were starved 24 h prior to bulk weighing of tank populations and collection of samples. Individuals were sampled randomly and euthanised with an overdose of anaesthetic (Tricaine, 1000 ppm; MS-222, Pharmaq, Norway) followed by manual severance of spinal cord. Prior to first feeding (13 fish/tank), and at the end of 'stimulus' (40 fish/tank), mid-'intermediate' (10 fish/tank), start- (10 fish/tank) and end-'challenge' (14 fish/tank) phases, body weight (BW) and total length (TL) were recorded from the above mentioned number of fish before freezing whole (−20 °C) for subsequent composition analysis. The number of harvested fish depended on their size at sampling point and the amount of material required for compositional analysis. For gene expression, at the end of the 'challenge' phase, pyloric caeca was dissected from 6 fish/tank and preserved in RNALater®, then refrigerated for 24 h to allow preservative to penetrate tissues, before freezing (-–20 °C). For digestibility, faeces were stripped and pooled from a minimum of 50 fish post mortem following the method of Austreng (Austreng, 1978) and frozen (−20 °C) prior to analysis.

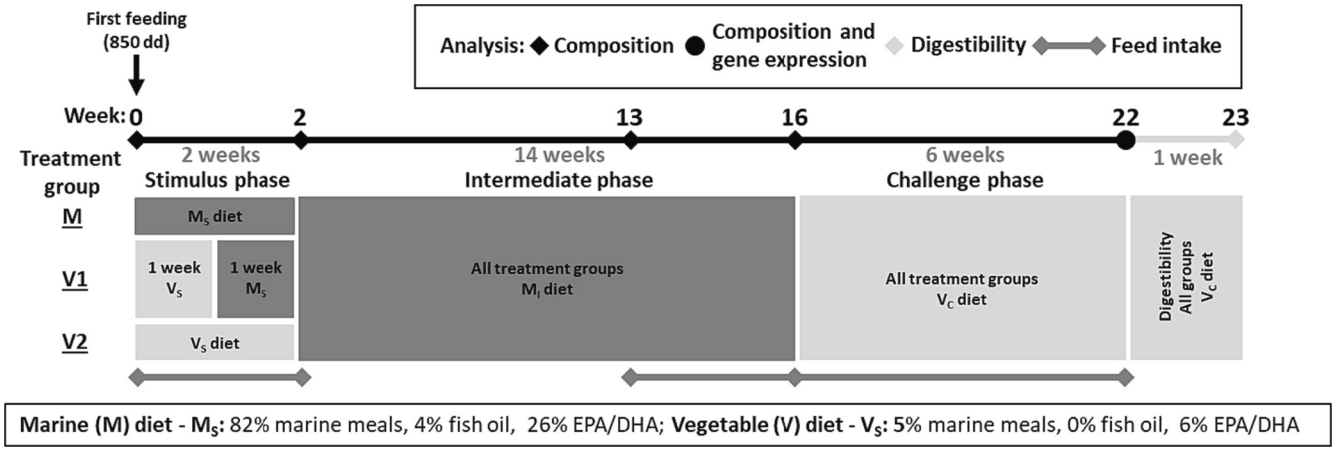

Fig. 1. Schematic representation of feeding trial.

2.3. Feed consumption

To calculate daily feed intake (FI), excess feed was collected twice daily throughout the 'stimulus' phase and 'challenge' phase and during the final three weeks of the 'intermediate' phase (Fig. 1). Excess feed and faeces were manually syphoned from the bottom of tanks. Feed was collected on a 300 μm mesh, which faeces passed through, and then dried (24 h, 110 °C). To calculate a nutrient dissolution factor of feeds in water 10 g of each diet was incubated in system water in duplicates for 6 h and 16 h before drying (as above) and weighing. Excess dry feed weight was corrected for nutrient dissolution and standardised to FI per 100 g average body weight (% BW/d).

2.4. Biochemical composition

For analysis of body composition, fish were pooled before first feeding (13 fish/tank), and at the end of 'stimulus' (40 fish/tank), mid-'intermediate' (5 fish/tank), start- (5 fish/tank) and end-'challenge' (3 fish/tank). Samples of whole fish or faeces were freeze-dried (Christ Alpha 1–4 LSC; Osterode am Harz, Germany), and analyses were corrected to wet weight based on moisture loss during this step. All samples, including diets, were blended and homogenised (Waring Laboratory Science, Stamford, CT, USA) before processing. Proximate composition of experimental diets, whole fish and faecal samples was completed following the Association of Official Analytical Chemists (AOAC) Official Methods of Analysis (2000) standard procedures. Moisture and ash contents were established by heating (110 °C) or incineration (600 °C) for 16 h. For all samples except those collected at the end of the 'stimulus' phase, energy content was determined using bomb calorimetry (Autobomb; Gallenkamp & Co. Ltd., Cambridge, UK). Protein was measured based on analysed N content (N × 6.25), following sulphuric acid digestion, with an Opsis AB LiquidLINE KjelROC Analyser (Furulund, Sweden).

Inductively coupled plasma mass spectrometry (ICP-MS) utilising collision cell technology was used to determine total mineral trace elements and yttrium marker following Sprague et al. (2020). Briefly, approximately 100 mg of freeze-dried sample was microwave digested (20 min at 190 °C; MARS™ 2, CEM Microwave Technology Ltd., Buckingham, UK) with 5 mL of 69% nitric acid (Aristar® analytical grade; VWR Chemicals, Poole, UK). Cooled sample digest was made up to 10 mL with deionised water, then 0.4 mL of this diluted sample was added to 0.2 mL methanol and made up to 10 mL with deionised water. Samples were analysed using an X Series 2 ICP-MS (Thermo Fisher Scientific, MA, USA) running on kinetic energy discrimination (KED) mode, where helium and argon were used as a collision gas and plasma, respectively. To ensure procedural integrity, a certified reference material (Fish Muscle ERM-BB42; Institute for Reference Materials and Measurements [IRMM], Geel, Belgium) was included in all analysis runs.

Total lipid was extracted from samples homogenised and incubated (−20 °C, 16 h) in chloroform/methanol (2:1, *v*/v), following the method of Folch (Folch et al., 1957). Fatty acid methyl esters (FAME) were produced from total lipid by acid-catalysed transesterification at 50 °C for 16 h (Christie, 2003) and then isolated and purified as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography using a Thermo Finnigan Trace GC (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm internal diameter × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injection and flame ionisation detection. Data were collected and processed using Chromcard for Windows (Version 1.19; Thermoquest Italia S.p.A., Milan, Italy). FAME identification was completed individually by comparison to known standards (Restek 20-FAME Marine Oil Standard; Thames Restek UK, Ltd., Buckinghamshire, UK) and published data (Tocher and Harvie, 1988).

2.4.1. Pyloric caeca RNA extraction and cDNA synthesis

To investigate evidence of NP at a molecular level, expression of genes involved in fatty acid metabolism were quantified by relative real time quantitative polymerase chain reaction (RT-qPCR) following reverse transcription of RNA to cDNA. Samples of pyloric caeca (end 'challenge' phase) from individual fish were added to Sigma-Aldrich TRI Reagent® (Merck Life Sciences UK Limited, Glasgow, UK) and incubated on ice for 1 h before homogenisation (3450 oscillations/m, 60 s) in a Mini-Beadbeater-24 (BioSpec Products, Bartlesville, OK, USA). Samples were centrifuged (12,000 ×*g* for 10 min at 4 °C) and supernatant retained. RNA was then isolated following TRI Reagent manufacturer's instructions, with a modified step of precipitation in 50% (*v*/v) RNA precipitation solution (1.2 M NaCl and 0.8 M sodium citrate sesquihydrate in deionised water) in absolute isopropanol and rehydrated in deionised water. RNA was quantified spectrophotometrically by Nanodrop® ND-1000. RNA integrity was assessed by agarose gel electrophoresis and Qubit 4.0 RNA IQ assay kit following manufacturer's instructions. RNA concentration was standardised, and samples were pooled in triplicate. cDNA was reverse transcribed from 2 μg of total RNA in 20 μL reactions using the Applied Biosystems High-Capacity cDNA reverse transcription kit, following the manufacturer's instructions with the addition of oligo dT primer. Synthesised cDNA generated was diluted 20-fold with deionised water.

2.4.2. RT-qPCR

Expression levels of genes involved in LC-PUFA biosynthesis (*fads2Δ6*, delta-6 fatty acyl desaturase; *fads2Δ5*, delta-5 fatty acyl desaturase; *elovl2*, fatty acyl elongase 2; *elovl5a*, fatty acyl elongase isoform a; *elovl5b*, fatty acyl elongase 5 isoform b) were determined in reactions of 10 μL, in 96-well plates, comprised of 5 μL Luminaris Color Higreen qPCR Master Mix, 1 μL of primer (Table 2) representing gene target, 2.5 μL of cDNA (aside from reference genes where only 1 μL of cDNA was required) and made up to volume with deionised water. Samples were run on a Biometra TOptical Thermocycler (Analytik Jena, Jena, Germany) using the following cycle parameters: 50 °C for 2 min, an initial activation step at 95 °C for 10 min, then 35 cycles of 15 s at 95 °C, 30 s at the annealing Tm (°C) and 30 s at 72 °C. A negative no template control (NTC) reaction with no cDNA was included. Results were normalised using the ΔCt method (Pfaffl, 2001) using reference genes hypoxanthine guanine phosphoribosyl transferase (*hprt*) and elongation factor 1 α (*ef1α*), which had been shown to be most stable using Genorm (Pattyn et al., 2003).

2.5. Calculations

2.5.1. Growth parameters

The relationship between BW and FI was measured as feed efficiency (FE) and calculated using –

$$\left(\mathrm{BW}_f - \mathrm{BW}_i\right)/\mathrm{FI}$$

where BW_f is end body weight, $\mathrm{BW_i}$ is start body weight.

Growth was determined using the specific growth rate (SGR) calculation following Ricker (1975) -

$$\mathrm{SGR}\ (\%\mathrm{BW/day}) = 100 \times (e^g - 1)$$

$$\text{where } g = \left(ln\left(\mathrm{BW_f}\right) - ln\left(\mathrm{BW_i}\right)\right)/(t_2 - t_1)$$

$$\text{or } g = \left(ln\left(\mathrm{BW_f}\right) - ln\left(\mathrm{BW_i}\right)\right) \times (t_2 - t_1)^{-1}$$

where e^g - 1 is the proportional rate of growth per unit of time, BW_f is end body weight, $\mathrm{BW_i}$ is start body weight, t_2 is finish time and t_1 is start time.

Biochemical compositions (proximate and FAME) of fish whole body were used to calculate retention efficiency of protein, lipid, energy and fatty acids, with the influence of BW gain, as follows –

$$\%\mathrm{retention} = 100 \times \left(\mathrm{BW}_f \times \mathrm{nut}_f - \mathrm{BW}_i \times \mathrm{nut}_i\right)/\mathrm{FI} \times \mathrm{nut}_d$$

Table 2
Primers used in the study.

Gene target	Primer sequence (5′ → 3′)	Amplicon length (bp)	Ta (°C)	Accession No.[a]	Reference
fads2Δ6	F:TCCTCTGGTGCGTACTTTGT R:AAATCCCGTCCAGAGTCAGG	163	59	NM_001123575.2	Betancor et al., 2018
fads2Δ5	F:GCCACTGGTTTGTATGGGTG R:TTGAGGTGTCCACTGAACCA	148	59	NM_001123542.2	Betancor et al., 2018
elovl2	F:GGTGCTGTGGTGGTACTACT R:ACTGTTAAGAGTCGGCCCAA	190	59	NM_001136553.1	Betancor et al., 2018
elovl5a	F:TGTTGCTTCATTGAATGGCCA R:TCCCATCTCTCCTAGCGACA	150	59	GU238431.1	Betancor et al., 2018
elovl5b	F:CTGTGCAGTCATTTGGCCAT R:GGTGTCACCCCATTTGCATG	192	59	NM_001136552.1	Betancor et al., 2018
hprt	F:GATGATGAGCAGGGATATGAC R:GCAGAGAGCCACGATATGG	165	60	BT043501	Anderson and Elizur, 2012
ef1α	F:CTGCCCCTCCAGGACGTTTACAA R:CACCGGGCATAGCCGATTCC	175	60	AF321836	Betancor et al., 2018

bp, base pair; *ef1α*, elongation factor 1 alpha; *elovl2*, fatty acyl elongase 2; *elovl5a*, fatty acyl elongase isoform a; *elovl5b*, fatty acyl elongase 5 isoform b; *fads2Δ5,* delta-5 fatty acyl desaturase; *fads2Δ6*, delta-6 fatty acyl desaturase; *hprt*, hypoxanthine-guanine phosphoribosyl transferase; Ta, annealing temperature.
[a] GenBank (http://www.ncbi.nlm.nih.gov/).

where *nut* is the % nutrient or energy content (kj/g) at the start (nut_f), end (nut_i) and in feed (nut_d), respectively (Geurden et al., 2013).

Hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (*K*) were calculated as follows -

$$\mathrm{HSI} = (\text{liver W}/\text{fish W}) \times 100$$

$$\mathrm{VSI} = (\text{viscera W}/\text{fish W}) \times 100$$

$$\mathrm{K} = \left(\text{fish W}/(\text{total L})^3\right) \times 100$$

where *W* is weight (g) and *L* is length (mm).

2.5.2. Energy

As insufficient sample material was available to determine energy experimentally at the end of 'stimulus' phase, energy was calculated according to Henken et al. (Henken et al., 1986) –

$$\mathrm{kj}/g = (((p \times 5.65) + (f \times 9.45) + (\mathrm{carb} \times 4.2)) \times 4.18)/100$$

where p represents protein (%), f represents lipid (%) and carb represents carbohydrate (%).

2.5.3. Digestibility

Apparent digestibility coefficient (ADC) was calculated according to Aas et al. (2015) as follows -.

$$\mathrm{ADC}\ (\%) = 100 \times ((\mathrm{a} - \mathrm{b})/\mathrm{a})$$

where *a* represents the nutrient to marker ratio in feed and *b* represents the nutrient to marker ratio in faeces.

2.6. Statistical analysis

All statistical analysis was completed using IBM SPSS version 28 (Edinburgh, UK) considering 'stimulus' diet regimes as an independent variable. Data was subjected to a Shapiro-Wilk test to evaluate normal distribution and Levene's test to confirm homogeneity of variance. Datasets without a normal distribution were separately log and square root transformed before re-evaluation. Proportional (%) data was analysed following arcsine transformation. Growth and feed intake parameters were analysed by general linear model using phase starting weight as a co-factor. All other data, except data not normally distributed, were analysed by one-way ANOVA before significantly different means were compared with Tukey's *post-hoc* test. A non-parametric Kruskall-Wallis test with Dunn's *post-hoc* test and Bonferroni correction was used in the event that data was not normally distributed. Significance was accepted at $P < 0.05$.

3. Results

3.1. Fish performance

Whole body weight at the end of 'stimulus' phase was significantly higher in fish from the M group (fed the M_S diet for the full 2-week phase) compared to V2 (fed the V_S diet for the full 2-week phase; $P = 0.048$) or V1 (fed the V_S diet for one week only before the switch to M_S;

Table 3
Growth parameters, feed intake (FI) and feed utilisation during stimulus feeding phase* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	Mean	SEM	Mean	SEM	Mean	SEM
Growth parameters (× Ind.)						
Initial body weight (g)	**0.15**	0.0	**0.15**	0.0	**0.15**	0.0
Final body weight (g)	**0.36**$^{(A)}$	0.0	**0.33**$^{(B)}$	0.0	**0.34**$^{(B)}$	0.0
Condition factor (*K*)	**1.07**$^{(A, B)}$	0.0	**0.94**$^{(B)}$	0.0	**1.19**$^{(A)}$	0.1
Protein gain (mg)	**25.6**$^{(A)}$	0.6	**22.4**$^{(B)}$	0.3	**23.6**$^{(A, B)}$	0.7
Lipid gain (mg)	**3.6**	0.5	**2.5**	0.4	**3.0**	0.2
Energy gain (kJ)	**0.83**$^{(A)}$	0.0	**0.70**$^{(B)}$	0.0	**0.77**$^{(A, B)}$	0.0
Voluntary feed intake (FI)						
FI (% BW/day)	**3.2**	0.2	**2.3**	0.1	**3.2**	0.4
Nutrient and energy retentions (% intake)						
Protein	**32.9**	2.6	**42.7**	1.2	**34.1**	5.6
Lipid	**20.2**	1.7	**20.8**	2.5	**18.4**	3.3
Energy	**31.0**	1.8	**37.9**	0.8	**29.4**	4.9
18:2*n*-6 (LA)	**15.6**	2.9	**14.6**	1.9	**23.8**	3.4
20:4*n*-6 (ARA)	**−6.6**$^{(A)}$	4.3	**−39.3**$^{(A, B)}$	14.4	**−213.2**$^{(B)}$	21.6
18:3*n*-3 (ALA)	**−1.1**	5.1	**−1.6**	3.4	**10.9**	1.4
20:5*n*-3 (EPA)	**5.3**$^{(A)}$	0.5	**−1.1**$^{(A)}$	2.6	**−37.3**$^{(B)}$	3.4
22:5*n*-3 (DPA)	**−27.5**$^{(A)}$	10.7	**−66.2**$^{(A)}$	18.2	**−388.0**$^{(B)}$	28.1
22:6*n*-3 (DHA)	**29.6**$^{(A)}$	2.9	**40.0**$^{(A)}$	5.4	**−5.2**$^{(B)}$	6.4
n-3 LC-PUFA	**16.5**$^{(A)}$	1.9	**16.2**$^{(A)}$	4.9	**−31.9**$^{(B)}$	2.2
TOTAL						

ALA, α-linolenic acid; ARA, Arachidonic acid; BW, body weight; LA, linolenic acid; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acid.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed MS for full two weeks; V1 fed VS for 1st week then MS for 2nd week; V2 fed VS for full two weeks). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between NH and was accepted at $P < 0{\cdot}05$.

$P = 0.021$) (Table 3). No significant differences in mean individual body weight were found at the end of 'intermediate' or 'challenge' phases (Tables 4 and 5). During the 'stimulus' phase, M fish had significantly higher growth than V2 ($P = 0.018$) or V1 fish ($P = 0.046$) (Table 6). FI (% BW/day) was not significantly different during any feeding phase, although it was notably lower in the V1 group during 'stimulus' phase (Table 3). The reduction in V1 feed intake was similar during the first (fed the V_S diet) and second (fed the M_S diet) weeks of 'stimulus', at 25% and 26% respectively (measured as % of BW), compared to consumption by M fish. In contrast V2 fish consumed slightly more feed than the M fed groups during both weeks of the 'stimulus' phase. A slight significant decrease ($P = 0.025$) in survival at the end of 'challenge' was found in fish in the V1 group compared to M fish (Table 6). There were no other statistical differences in survival (%) or feed efficiency (FE) during any other phase of the trial (Table 6), although FE during the 'stimulus' phase was slightly, although not significantly, higher in V1 fish, associated with lower overall feed intake in that group (Tables 3 and 6). Although there was no difference in HSI or VSI during the 'intermediate' phase, both ratios in V1 were significantly lower than those in V2 fish (HSI, $P = 0.01$; VSI, $P = 0.019$) at the end of 'challenge' phase (Tables 4 and 5). At the end of the 'stimulus' phase, condition factor (K) was significantly higher in V2 ($P = 0.012$) than V1 (Table 3), but there were no differences between groups by this measure at the end of 'intermediate' or 'challenge' phases (Tables 4 and 5).

3.2. Fish biochemical and fatty acid compositions

Ash content of whole body at the end of 'stimulus' phase was significantly greater in M than V2 ($P = 0.019$). However, no other differences were found in proximate composition of fish at the end of the 'stimulus' phase, or the 'intermediate' and 'challenge' phases (Table 7).

Whole body fatty acid compositions, as a proportion of total lipid, largely mirrored the corresponding dietary fatty acid profiles. Thus, levels of total saturated fatty acids (SFA; $P = 0.022$), EPA ($P = 0.022$) and 22:5*n*-3 ($P = 0.022$) were significantly higher in M compared to V2 fish (Table 8 and Supplementary Table 2). Furthermore, levels of DHA and total *n*-3 LC-PUFA followed the same trend and were numerically higher, although not significantly, in M compared to V2 fish (Table 8 and Supplementary Table 2). Correspondingly, proportions of 18:1*n*-9 ($P = 0.022$), 18:2*n*-6 ($P = 0.022$), total *n*-6 PUFA ($P = 0.022$) and 18:3*n*-3 ($P = 0.022$) were significantly higher in V2 than M fish (Table 8 and Supplementary Table 2). Again, correlating with diet content, absolute whole body (mg/100 g) 18: 1*n*-9 ($P = 0.034$), 18:2*n*-6 ($P = 0.022$), total *n*-6 PUFA ($P = 0.022$) and 18:3*n*-3 ($P = 0.034$) levels were all significantly higher in V2 fish compared to the M group (Supplementary Table 2). There were no other statistically significant differences in whole body fatty acid compositions at the end of the 'stimulus' phase, either as a proportion of lipid or in absolute terms (mg/100 g). Indeed, absolute levels of *n*-3 LC-PUFA, EPA, docosapentaenoic acid (DPA) and DHA, and total *n*-3 PUFA in V1 and M fish were generally similar, despite the former being fed the low *n*-3 LC-PUFA V_S diet for the first week of this phase (Supplementary Table 2). There were no significant differences, as a proportion of total lipid or absolute whole body, in fatty

Table 4
Growth parameters, feed intake (FI), hepatosomatic and viscerosomatic indices, and feed utilisation during final three weeks of intermediate feeding phase* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	Mean	SEM	Mean	SEM	Mean	SEM
Growth parameters (× in/d)						
Initial body weight (g)	**9.5**	0.1	**9.6**	0.2	**9.2**	0.2
Final body weight (g)	**15.2**	0.1	**15.7**	0.3	**14.5**	0.4
VSI (%/bw)	**9.8**	0.2	**9.6**	0.1	**9.7**	0.1
HSI (%/bw)	**1.2**	0.1	**1.1**	0.0	**1.2**	0.0
Condition factor (*K*)	**0.9**	0.0	**0.9**	0.0	**0.9**	0.0
Protein gain (g)	**1.0**	0.0	**1.1**	0.1	**0.9**	0.0
Lipid gain (g)	**0.6**$^{(A, B)}$	0.0	**0.6**$^{(A)}$	0.0	**0.5**$^{(B)}$	0.0
Energy gain (kJ)	**46.9**$^{(A, B)}$	2.2	**49.1**$^{(A)}$	1.7	**41.0**$^{(B)}$	1.6
Voluntary feed intake (FI)						
FI (% BW/day)	**1.6**	0.0	**1.6**	0.0	**1.6**	0.0
Nutrient and energy retentions (% intake)						
Protein	**46.5**	0.5	**47.5**	4.2	**42.4**	1.4
Lipid	**82.7**	4.5	**82.7**	1.6	**70.9**	3.1
Energy	**51.9**	2.4	**53.1**	2.2	**46.1**	1.0
18:2*n*-6 (LA)	**73.4**	5.8	**73.0**	1.5	**65.1**	2.4
20:4*n*-6 (ARA)	**67.1**	12.0	**79.3**	4.4	**68.6**	2.1
18:3*n*-3 (ALA)	**65.5**	3.7	**65.3**	2.0	**58.2**	2.9
20:5*n*-3 (EPA)	**24.2**	6.4	**30.3**	1.3	**27.3**	2.7
22:5*n*-3 (DPA)	**86.4**	18.2	**102.8**	5.1	**91.8**	5.8
22:6*n*-3 (DHA)	**75.7**	18.7	**102.2**	6.6	**86.4**	6.2
n-3 LC-PUFA TOTAL	**53.6**	13.0	**69.9**	4.3	**60.2**	4.7

ALA, α-linolenic acid; ARA, Arachidonic acid; BW, body weight; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; HSI, hepatosomatic index; LA, linolenic acid; LC-PUFA, long-chain polyunsaturated fatty acid; VSI, viscerosomatic index.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed M_S for full two weeks; V1 fed V_S for 1st week then M_S for 2nd week; V2 fed V_S for full two weeks). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between NH and was accepted at $P < 0{\cdot}05$.

Table 5
Growth parameters, feed intake (FI), hepatosomatic and viscerosomatic indices, and feed utilisation during the challenge feeding phase* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	Mean	SEM	Mean	SEM	Mean	SEM
Growth parameters (× in/d)						
Initial body weight (g)	**15.24**	0.1	**15.73**	0.3	**14.46**	0.4
Final body weight (g)	**33.12**	1.2	**35.88**	1.5	**31.78**	2.5
VSI (%/bw)	**8.1**$^{(A, B)}$	0.2	**7.6**$^{(B)}$	0.1	**8.9**$^{(A)}$	0.3
HSI (%/bw)	**1.1**$^{(A, B)}$	0.0	**0.9**$^{(B)}$	0.0	**1.2**$^{(A)}$	0.1
Condition factor (*K*)	**1.03**	0.0	**1.01**	0.0	**0.99**	0.0
Protein gain (g)	**3.1**	0.3	**3.6**	0.3	**3.0**	0.4
Lipid gain (g)	**2.0**	0.2	**2.2**	0.1	**1.9**	0.3
Energy gain (kJ)	**157.8**	11.0	**178.3**	9.8	**150.5**	21.2
Voluntary feed intake (FI)						
FI (% BW/day)	**1.8**	0.0	**1.7**	0.0	**1.8**	0.1
Nutrient and energy retentions (% intake)						
Protein	**34.9**	2.8	**39.1**	2.9	**35.0**	3.9
Lipid	**68.6**	5.8	**74.6**	2.0	**66.8**	9.3
Energy	**40.7**	2.8	**44.7**	2.2	**40.0**	5.1
18:2*n*-6 (LA)	**52.0**	3.2	**56.3**	2.4	**51.8**	6.1
20:4*n*-6 (ARA)	**1320**	128.9	**1340**	34.2	**1230**	171.8
18:3*n*-3 (ALA)	**32.3**	2.5	**34.2**	2.4	**31.7**	3.3
20:5*n*-3 (EPA)	**35.5**	7.1	**18.81**	16.5	**−24.48**	15.9
22:5*n*-3 (DPA)	**275.3**$^{(A)}$	32.2	**172.5**$^{(A, B)}$	60.1	**4.6**$^{(B)}$	50.7
22:6*n*-3 (DHA)	**205.7**$^{(A)}$	16.2	**142.3**$^{(A, B)}$	30.1	**85.7**$^{(B)}$	25.2
n-3 LC-PUFA TOTAL	**153.1**$^{(A)}$	14.0	**104.5**$^{(A, B)}$	26.8	**47.0**$^{(B)}$	22.6

ALA, α-linolenic acid; ARA, Arachidonic acid; BW, body weight; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; HSI, hepatosomatic index; LA, linolenic acid; LC-PUFA, long-chain polyunsaturated fatty acid; VSI, viscerosomatic index.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed M_S for full two weeks; V1 fed V_S for 1st week then M_S for 2nd week; V2 fed V_S for full two weeks). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between NH and was accepted at $P < 0{\cdot}05$.

Table 6
Survival, growth rate and feed efficiency of fish during each of the three nutritional phases; stimulus, marine and challenge* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	Mean	SEM	Mean	SEM	Mean	SEM
Survival (%)						
Stimulus phase	**99.3**	0.2	**99.6**	0.2	**99.6**	0.2
Intermediate phase	**97.9**	0.4	**96.5**	0.3	**97.3**	0.6
Challenge phase	**100**$^{(A)}$	0.0	**99.3**$^{(B)}$	0.0	**99.6**$^{(A, B)}$	0.2
Growth rate (SGR %/day)						
Stimulus phase	**6.3**$^{(A)}$	0.1	**6.0**$^{(B)}$	0.2	**5.9**$^{(B)}$	0.0
Intermediate phase	**4.0**	0.0	**4.1**	0.0	**4.0**	0.0
Challenge phase	**1.8**	0.1	**1.9**	0.1	**1.8**	0.1
Feed efficiency (FE)						
Stimulus phase	**1.6**	0.1	**2.0**	0.1	**1.5**	0.2
Intermediate phase	**1.4**	0.0	**1.4**	0.1	**1.3**	0.0
Challenge phase	**1.0**	0.1	**1.1**	0.1	**1.0**	0.1

BW, body weight; NH, nutritional history; SGR, specific growth rate.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed M_S for full two weeks; V1 fed V_S for 1st week then M_S for 2nd week; V2 fed V_S for full two weeks). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between NH (with weight at start of phase a cofactor in growth analyses) and was accepted at $P < 0{\cdot}05$.

Table 7
Whole fish proximate composition at the end of each of three nutritional phases; stimulus, marine and challenge* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	Mean	SEM	Mean	SEM	Mean	SEM
Post-stimulus phase						
DM (%)	**18.75**	0.1	**18.45**	0.1	**18.85**	0.1
Lipid (%)	**2.7**	0.1	**2.6**	0.1	**2.7**	0.0
Protein (%)	**12.87**	0.0	**12.79**	0.1	**12.94**	0.0
Ash (%)	**1.77**$^{(A)}$	0.0	**1.68**$^{(A,B)}$	0.0	**1.56**$^{(B)}$	0.0
Energy gross (kJ/g)	**4.35**	**0.05**	**4.30**	**0.03**	**4.42**	**0.01**
Pre-challenge phase						
DM (%)	**28.5**	0.3	**28.5**	0.2	**28.1**	0.2
Lipid (%)	**10.0**	0.2	**10.0**	0.2	**9.9**	0.2
Protein (%)	**16.0**	0.2	**16.0**	0.1	**15.9**	0.2
Ash (%)	**2.3**	0.0	**2.3**	0.0	**2.3**	0.0
Energy gross (kJ/g)	**7.8**	0.1	**7.8**	0.1	**7.7**	0.1
Post-challenge phase						
DM (%)	**30.2**	0.1	**30.3**	0.1	**30.0**	0.4
Lipid (%)	**10.6**	0.1	**10.6**	0.2	**10.4**	0.2
Protein (%)	**16.8**	0.2	**17.1**	0.1	**16.8**	0.2
Ash (%)	**2.17**	0.0	**2.14**	0.0	**2.17**	0.0
Energy gross (kJ/g)	**8.33**	0.1	**8.39**	0.1	**8.24**	0.1

DM, dry matter.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed M_S for full two weeks; V1 fed V_S for 1st week then M_S for 2nd week; V2 fed V_S for full two weeks). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between NH and was accepted at $P < 0{\cdot}05$.

acid compositions of fish at the end of the 'intermediate' and 'challenge' phases (Table 8 and Supplementary Table 2).

Whole body samples were analysed for total mineral and trace elements at the start and end of 'challenge' phase. This revealed few differences or patterns in profile at either timepoint, although nickel levels were significantly lower in M ($P = 0.03$) and V1 ($P = 0.02$) fish at the start of the 'challenge' phase compared to the V2 group (Supplementary Table 1). At the end of 'challenge' phase, phosphorus levels were significantly higher ($P = 0.045$) in M fish compared to the V1 group (Supplementary Table 1).

Table 8
Whole fish fatty acid compositions (% of total lipid) composition at the end of each of three nutritional phases; stimulus, marine and challenge* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	Mean	SEM	Mean	SEM	Mean	SEM
Post-stimulus phase						
Total SFA	27$^{(A)}$	0.4	26$^{(A, B)}$	0.3	20$^{(B)}$	0.4
18:1*n*-9	17$^{(B)}$	1.0	19$^{(A, B)}$	0.2	28$^{(A)}$	0.3
Total MUFA	31	1.2	31	0.1	38	0.2
18:2*n*-6	6.3$^{(B)}$	0.2	7.5$^{(A, B)}$	0.1	14.1$^{(A)}$	0.1
20:4*n*-6	1.4	0.1	1.4	0.0	1.2	0.0
Total *n*-6 PUFA	9$^{(B)}$	0.3	10$^{(A, B)}$	0.1	18$^{(A)}$	0.1
18:3*n*-3	2$^{(B)}$	0.1	2.2$^{(A, B)}$	0.1	3.6$^{(A)}$	0.2
20:5*n*-3 (EPA)	**7.4**$^{(A)}$	0.3	**6.9**$^{(A, B)}$	0.1	**4.5**$^{(B)}$	0.1
22:5*n*-3 (DPA)	3.0$^{(A)}$	0.1	3.1$^{(A, B)}$	0.1	2.6$^{(B)}$	0.0
22:6*n*-3 (DHA)	**17.7**	1.2	**17.1**	0.2	**10.6**	0.1
Total *n*-3 PUFA	33	1.6	32	0.4	24	0.4
Total *n*-3 LC-PUFA	30	1.7	29	0.4	20	0.3
Pre-challenge phase						
Total SFA	24	0.3	24	0.1	24	0.2
18:1n-9	27	0.4	26	0.3	27	0.2
Total MUFA	44	0.6	43	0.1	44	0.2
18:2*n*-6	8.5	0.0	8.5	0.1	8.5	0.0
20:4*n*-6	0.5	0.0	0.5	0.0	0.5	0.0
Total *n*-6 PUFA	10	0.1	10	0.0	10	0.1
18:3*n*-3	2.7	0.0	2.8	0.0	2.8	0.0
20:5*n*-3 (EPA)	**3.2**	0.2	**3.4**	0.0	**3.4**	0.1
22:5*n*-3 (DPA)	1.3	0.1	1.4	0.0	1.4	0.0
22:6*n*-3 (DHA)	**10.0**	0.6	**11.0**	0.1	**10.9**	0.2
Total *n*-3 PUFA	20	0.9	21	0.1	21	0.4
Total *n*-3 LC-PUFA	16	0.8	17	0.1	17	0.3
Post-challenge phase						
Total SFA	18	0.2	18	0.3	18	0.3
18:1*n*-9	40	0.3	40	0.7	40	0.6
Total MUFA	50	0.3	51	0.1	51	0.4
18:2*n*-6	13.7	0.0	13.9	0.2	14.0	0.2
20:4*n*-6	0.8	0.0	0.7	0.0	0.7	0.0
Total *n*-6 PUFA	18	0.1	18	0.3	18	0.3
18:3*n*-3	3.6	0.0	3.5	0.1	3.6	0.1
20:5*n*-3 (EPA)	**1.5**	0.0	**1.5**	0.1	**1.4**	0.1
22:5*n*-3 (DPA)	0.7	0.0	0.6	0.0	0.6	0.0
22:6*n*-3 (DHA)	**5.5**	0.1	**5.3**	0.1	**5.3**	0.3
Total *n*-3 PUFA	14	0.2	13	0.2	13	0.5
Total *n*-3 LC-PUFA	8	0.2	8	0.2	8	0.5

DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acid; MUFA, monoenes; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed M_S for full two weeks; V1 fed V_S for 1st week then M_S for 2nd week; V2 fed V_S for full two weeks). Significance was calculated between NH and was accepted at $P < 0{\cdot}05$.

3.3. Nutrient efficiency and retention

'Stimulus' phase protein ($P = 0.043$) and energy ($P = 0.001$) gain were significantly greater in M fish compared to V1 (Table 3). In contrast, during the 'intermediate' phase, V1 fish lipid ($P = 0.049$) and energy ($P = 0.047$) gain were significantly greater than in the V2 group (Table 4), whilst there was no difference in nutrient gain at the end of the 'challenge' phase (Table 5). Retentions of several *n*-3 LC-PUFA during the 'stimulus' phase were significantly higher in M fish compared to the other two groups, including EPA (both $P \leq 0.001$), DPA (both $P \leq 0.001$), DHA (V1 $P = 0.007$; V2 $P = 0.002$) and total *n*-3 LC-PUFA (both $P \leq 0.001$) (Table 3), with the general trend indicating poorer nutrient efficiency in the V2 salmon during that phase. Whilst there were no significant differences in nutrient utilisation during 'intermediate' phase, retentions of DPA ($P = 0.019$), DHA ($P = 0.031$) and total *n*-3 LC-PUFA ($P = 0.032$) were significantly greater in M fish over the 'challenge' phase compared with the V2 group (Tables 4 and 5).

3.4. Digestibility

Whilst there were largely no changes relating to the 'stimulus' regime in digestibility of the V_c diet at the end of 'challenge' phase, a slight but significant ($P = 0.034$) reduction was identified in DHA digestibility in the V2 group, compared to the V1 fish (Table 9).

3.5. Gene expression

Over the 'challenge' phase, expression levels of all fatty acid biosynthesis genes in the evaluated tissues appeared to be lower in V1 and V2 compared to the M group (Fig. 2). Analysis of RNA extracted from pyloric caeca revealed that, compared to M fish, expression levels of *fads2Δ6* ($P = 0.027$) and *elovl5b* ($P = 0.038$) were significantly lower in V1 fish, whilst *elovl5a* expression was significantly lower in both V1 ($P = 0.04$) and V2 ($P = 0.031$) groups (Fig. 2).

4. Discussion

4.1. Fish fed the V_S diet were smaller at the end of the 'stimulus' phase

The aim of the present study was to investigate short-term NP responses in Atlantic salmon. In a previous trial, where fish were fed a first feeding vegetable-based 'stimulus' diet compared to a-marine based control for three weeks, the fish were smaller at the end of both the 'stimulus' and 'intermediate' phases, and it was speculated that subsequent performance in the 'challenge' phase was impacted by animal size (Clarkson et al., 2017). Despite a shorter 'stimulus' duration of 1 or 2 weeks being fed the V_S diet, growth of both V1 and V2 fish was significantly less than in the M fish with correspondingly lower individual weights (8% and 6% lower, respectively; Tables 3 and 6) at the end of this phase, although no differences were found in survival. While it was hypothesised that a 'stimulus' phase of shorter duration would abolish size differences at the end of the phase, the present result was still a substantial improvement compared to the 30% reduction in size of fish fed the vegetable-based 'stimulus' diet obtained in the previous study (Clarkson et al., 2017).

Intriguingly, FI during the 'stimulus' phase was the same in V2 and M fish (Table 3) suggesting that the reduced growth was the result of less efficient nutrient utilisation and/or assimilation, an outcome reported previously in salmon fed diets with alternative plant-based ingredients (Overland et al., 2009; Pratoomyot et al., 2010; Refstie et al., 1998). In contrast, although not statistically significant, V1 fish consumed an average of 26% less feed daily (measured as % of BW) compared to M fish, indicating reduced intake of both diets (V_s and M_s) as a reason for decreased growth compared to M fish. This reduction in feed consumption was similar over both weeks (25% and 26% respectively) suggesting this was not simply a negative response to switching feeds after such a short period of 1 week. Perhaps, the slightly lower average initial weight (0.146 g of V1 compared to 0.152 g of M fish) of V1 fish contributed to the lower feed intake during this phase. Higher (although not significant) FE of V1 fish counteracted the FI data resulting in no changes in growth or end phase weight compared to the V2 group (Table 6). Previous studies have shown either reduced FI (Espe et al., 2006; Pratoomyot et al., 2010; Torstensen et al., 2008) or FE (Refstie et al., 2000; Refstie et al., 1998) for vegetable-based feeds in Atlantic salmon compared to a standard marine control diet. As with other fish species, Atlantic salmon possess considerable phenotypic plasticity in response to environmental factors, including diet (Glover et al., 2018; Harvey et al., 2016; Solberg et al., 2013). Accordingly, the FE of V1 could indicate an underlying mechanism, related to organismal plasticity, which could be driving the more efficient utilisation of nutrients, possibly associated with the change of diet or mixture of diets over the early feeding period.

Table 9
Digestibility of V_c diet nutrients in fish at the end of challenge phase* (Mean values with their standard errors, n 3).

Nutritional history	M		V1		V2	
	%	SEM	%	SEM	%	SEM
Protein	**90.5**	0.0	**90.1**	0.4	**89.9**	0.5
Ash	**41.7**	1.7	**43.8**	0.5	**42.0**	0.8
Lipid	**86.6**	0.4	**87.1**	0.7	**86.6**	1.3
FAMES						
Total saturated	**94.0**	0.3	**94.8**	0.3	**93.9**	0.5
18:1*n*-9	**98.7**	0.1	**99.0**	0.1	**98.8**	0.1
Total monounsaturated	**98.5**	0.1	**98.8**	0.1	**98.5**	0.2
18:2*n*-6	**98.0**	0.1	**98.2**	0.1	**97.9**	0.2
20:4*n*-6	**66.3**	4.2	**72.5**	0.6	**61.6**	2.9
Total *n*-6 PUFA	**97.7**	0.1	**97.9**	0.1	**97.6**	0.2
18:3*n*-3	**99.3**	0.1	**99.5**	0.0	**99.3**	0.1
20:5*n*-3 (EPA)	**95.9**	0.3	**96.7**	0.2	**95.4**	0.9
22:5n-3	**88.8**	1.3	**91.3**	0.6	**88.8**	1.9
22:6*n*-3 (DHA)	**86.6**$^{(A, B)}$	0.87	**88.8**$^{(A)}$	0.28	**84.9**$^{(B)}$	1.14
Total *n*-3 PUFA	**97.8**	0.3	**98.3**	0.1	**97.8**	0.3
Total PUFA	**97.7**	0.2	**98.1**	0.1	**97.7**	0.2

DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acid; PUFA, polyunsaturated fatty acid.
A or B indicate significant differences between nutritional history (NH).
* Based on NH during the 2-week stimulus phase (M fed M_S for full two weeks; V1 fed V_S for 1st week then M_S for 2nd week; V2 fed V_S for full two weeks). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between NH and was accepted at $P < 0{\cdot}05$.

4.2. Decreased hepatosomatic and viscerosomatic indices in V1 fish at end of 'challenge'

During 'intermediate and 'challenge' phases there were no significant differences in FI, growth or FE between groups (Tables 4, 5 and 6). These data contradict both Clarkson et al. (2017) where salmon demonstrated the same FI but greater FE, and a similar study in rainbow trout that reported both FI and FE increased after receiving a vegetable-based 'stimulus' (Geurden et al., 2013). However, in the present study at the end of the 'challenge' phase, HSI and VSI were significantly lower in V1 compared to V2 (Table 5). This correlated negatively with the higher protein, lipid and energy gains and retention values in the V1 group (Table 5). Higher VSI and HSI have been associated with higher hepatic and intraperitoneal fat (lipid) contents, respectively, in Atlantic salmon and marine reared brown trout (*Salmo trutta*) (Lock et al., 2016; Mikolajczak et al., 2020; Thompson et al., 1996). It has also been suggested that decreased catabolic activity could lead to higher HSI (Whitesel, 1992).

Moreover, rainbow trout showed lower VSI when fed a diet with high mealworm (*Tenebrio molitor*) inclusion, compared to a standard FM control diet, which was related to increased oxidative lipid metabolism, possibly leading to reduced visceral lipid deposition (Melenchon et al., 2021). Taken together these previous observations could indicate a NP effect of sorts in V1 as the lower HSI and VSI would suggest that fat deposition was elsewhere in these fish, possibly in muscle, likely due to increased catabolism and oxidative lipid metabolism in the liver and viscera. Future investigations should include individual tissue compositions to test this theory.

4.3. No evidence of n-3 LC-PUFA biosynthesis triggered during 'stimulus' phase

The higher 'stimulus' protein, lipid and energy retentions in V1 and accompanying inverse correlations with the lower (significantly so for protein and energy) gains was potentially linked with the low FI but high FE during the 'stimulus' phase (Tables 3 and 6). However, there was no indication, based on the *n*-3 LC-PUFA retention values, to suggest LC-PUFA biosynthesis had been triggered by the end of the 'stimulus' phase in any treatment group. On the contrary, V2 experienced a net loss of *n*-3 LC-PUFA, including a 388% drop in DHA, likely related to the

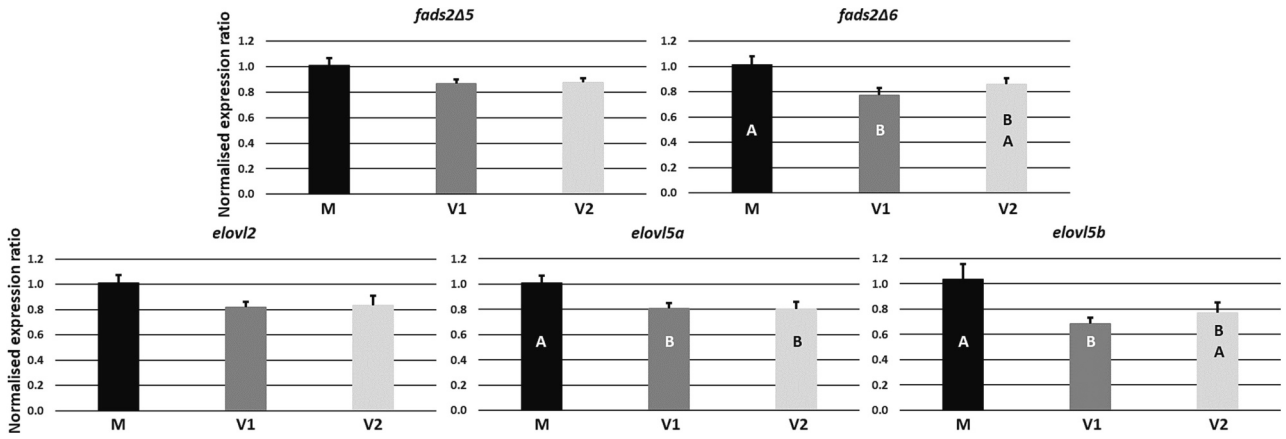

Fig. 2. Expression of target genes involved in LC-PUFA biosynthesis (Table 2), measured in pyloric caeca at the end of the challenge phase as determined by RT-qPCR. Data are presented as the normalised expression ratio (NER) in salmon fed the one- or two-week VS diet (V1 and V2) compared to the marine MS diet (M) control. A and B indicate NER changes that are statistically significant (Mean values with their standard errors, $P < 0.05$, n 6).

longer period of feeding on the low EPA + DHA V_S diet. The lack of LC-PUFA biosynthesis could have been because the 'stimulus' was simply too short. However, inducing a successful NP response later in development can be achieved with a first feeding 'stimulus' of seven days in Nile tilapia (*Oreochromis niloticus*), and as little as three days in both zebrafish and rainbow trout (Geurden et al., 2007; Perera and Yufera, 2016; Srisakultiew et al., 2022). These previous studies highlight that the required 'stimulus' duration will likely vary between species and be potentially related to exactly what nutritional mechanism is being stimulated such as glucose metabolism in two of the previous studies (Geurden et al., 2007; Srisakultiew et al., 2022).

4.4. Fatty acid levels and nutritional programming

Another reason for the lack of LC-PUFA biosynthesis at the end of 'stimulus' phase is that the level of EPA + DHA in the present trial's V_S diet (as a % of total feed) was 0.59% (Table 1), which was greater than the minimum level of 0.5% required by Atlantic salmon juveniles in freshwater suggested in a recent study (Qian et al., 2020), potentially preventing induction of *n*-3 LC-PUFA biosynthesis and NP mechanisms. Furthermore, a previous NP trial using high- and low-level *n*-3 LC-PUFA diets in sea bass demonstrated that levels of LC-PUFA biosynthesis gene *fads2Δ6* expression were dependent on the severity of EPA + DHA reduction, with fish fed the lowest proportion of these two fatty acids having the highest expression (Vagner et al., 2009).

There was a trend of higher protein, lipid and energy gains and retentions in V1 fish during the 'intermediate' phase, with lipid and energy gain being significantly greater than V2 (Table 4), suggesting compensatory growth. This mechanism has been reported previously in Nile tilapia (Kumkhong et al., 2020) and Siberian sturgeon (*Acipenser baerii*) (Luo et al., 2019) or sea bream following a first feeding or broodstock 'stimulus', respectively (Izquierdo et al., 2015). Furthermore, 'Intermediate' nutrient gains and retentions in the present trial were obtained from a 3-week period at the end of the phase, suggesting the changes in V1 were not just short term, and the overall pattern of stronger gains and proximate retentions continues through the 'challenge' phase.

In contrast to the 'stimulus' phase, there was net production of *n*-3 LC-PUFA in M and V1 during the 'challenge' phase (Table 5), following the switch from a marine based diet to a predominately vegetable-based feed, a phenomenon previously reported in Atlantic salmon (Sanden et al., 2011; Torstensen et al., 2008) following a similar dietary switch (vegetable meal and vegetable oil inclusion at 80 and 70% replacement of FM and FO, respectively). Sanden et al. (2011) also demonstrated very similar DHA retention (142%) compared to M and V1. Nonetheless, our previous successful NP trial in Atlantic salmon showed the vegetable diet 'challenge' induced a 235% increase in DHA retention in fish previously stimulated for three-weeks with a similar vegetable diet, compared to fish previously fed only marine diets (Clarkson et al., 2017). In the present trial, the V1 and V2 groups retained 31% and 58% less DHA, respectively, than M fish, with a similar pattern in other *n*-3 LC-PUFA and no evidence of any net gain in fish receiving the two-week V_S diet (Table 5). Of note was the very low retention (4.5%) of DPA in V2, as this LC-PUFA is an intermediate between EPA and DHA and a good indicator of biosynthetic activity (Betancor et al., 2014; Linderborg et al., 2013).

The above data would indicate that in the present study there was no NP response in either experimental group to the initial V_S diet in terms of increased net production of *n*-3 LC-PUFA, with the opposite being true for V2 fish. Although V_S EPA + DHA proportion exceeded the 0.5% requirement suggested by Qian et al. (2020), levels in the V_C diet were 0.2–0.25% of total diet, suggesting that biosynthesis could have been triggered during the 'challenge' phase in all treatments, so it is not clear why this was not apparent in V2 fish. A further potential factor is the ratio of *n*-3 α-linolenic acid to *n*-6 linoleic acid (ALA:LA) within each diet. In the present trial there was 3-fold reduction in ALA levels, compared to LA, in both V_S and V_C diets (Table 1). Studies have shown that a higher ALA:LA ratio is required for optimal EPA + DHA production, as opposed to the production of arachidonic acid (ARA, 20:4 *n*-6) from substrate LA (Jalali et al., 2021; Sprague et al., 2019; Xu et al., 2021). ARA is the primary precursor for eicosanoid production and an essential LC-PUFA in fish (Bell et al., 2003). However, there is a competing relationship between ARA and EPA for specific metabolic and enzymatic pathways and increased ARA can lead to decreased EPA production (Mazorra et al., 2003; Sissener et al., 2020). Experiments in rainbow trout have suggested that low levels of EPA may negatively affect the *n*-6 synthesis pathway without increasing *n*-3 biosynthesis (Mellery et al., 2017). High net production of ARA has been reported previously and, when in excess, it appears not to be β-oxidized to the same extent as EPA and DHA (Sanden et al., 2011). Thus, biosynthesis of *n*-3 LC-PUFA could have been inhibited in favour of ARA, which had net gains of >1200% in all experimental groups.

Aside from a significantly higher (13%) ash content in M fish compared to V2, there were no differences in proximate and calorific compositions in any phase of trial (Table 7). The higher ash content could be due to the higher proportion of ash in the M diet due to the presence of fishmeal, but an alternative explanation may be the greater weight of fish at the end of this phase, because the proportion of skeleton increases with fish size, with corresponding higher mineral content due to this (Metcalfe et al., 2002; Rottiers, 1993). Levels of several fatty acids were also different at the end of the 'stimulus' phase, notably

significantly lower EPA and DHA in V2 compared to M fish (Table 8), which would be expected as fish fatty acid profile reflects that of diet (Bell et al., 2010; Clarkson et al., 2017). There were no significant differences in fatty acid levels after the 'challenge' phase, despite the increased retention of *n*-3 LC-PUFA in all but the V2 fish. This was likely related to the slightly larger size of M and V1 fish by the end of the trial, indicating that the net gain of *n*-3 LC-PUFA was simply reflecting the additional body tissue rather than an increase in proportion (Table 5).

4.5. LC-PUFA biosynthesis gene expression down regulated during 'challenge' in V 'stimulus' groups

In view of the net accumulation of *n*-3 LC-PUFA, notably DHA, by V1 fish during the 'challenge' phase, it was both surprising and unexpected that expression of biosynthesis genes *fads2Δ6*, *elovl5a* and *elovl5b* were apparently downregulated in pyloric caeca of V1 compared to M fish (Fig. 2). Furthermore, in comparison to M fish, there was a general trend of downregulation in all five biosynthesis genes measured in both V1 and V2 fish, although only *elovl5a* was significant in the latter. This contradicts a previous study which found upregulation of Δ6 desaturase gene in whole body of sea bream juveniles fed a partial vegetable oil replacement 'challenge' diet following maternal 'stimulus' of a similar diet, compared to broodstock fish fed a 100% fish oil diet (Izquierdo et al., 2015). Furthermore, within the pyloric caeca of Atlantic salmon an upregulation of LC-PUFA biosynthesis genes has been demonstrated after feeding with wild-type and transgenic Camelina oil-based diets, compared to standard FO diet (Betancor et al., 2015). Although not the main site of LC-PUFA biosynthesis, pyloric caeca is a highly relevant tissue for measuring gene expression because the gastrointestinal tract has first contact with dietary nutrients and is highly adaptable to changing environmental conditions, including nutrition, via numerous endocrine-regulated pathways (Buddington and Krogdahl, 2004; Kwasek et al., 2020). Moreover, a previous study revealed higher intestinal copy numbers of fads2Δ5 and fads2Δ6 in Atlantic salmon compared to nine other tissues tested (Zheng et al., 2005).

The reduction in LC-PUFA biosynthesis gene expression in V2 fish was consistent with this group having the lowest retentions of all *n*-3 LC-PUFAs, all of which were below 100%, suggesting limited or no biosynthesis capacity (Fig. 2 and Table 5). However, post 'challenge' *n*-3 LC-PUFA retentions in V1 fish indicated a net production of 22:5*n*-3, DHA and total *n*-3 LC-PUFA which was recorded despite downregulation of biosynthesis genes compared to M fish. Furthermore, expression profiles in V1 fish were similar to V2 fish despite the evidence suggesting net production of *n*-3 LC-PUFA only in the former. The significantly higher digestibility of DHA in V1 than V2 may have played a limited role in the increased retention between these groups, but it does not fully explain the net gain of *n*-3 LC-PUFA. One possible explanation may be post-transcriptional modification triggered by low DHA or other *n*-3 LC-PUFA. It is believed that miRNAs may alter translational regulation by repression or mRNA degradation via exogenous triggers (Flowers et al., 2013; Szabo and Bala, 2013). Upregulation of miRNA miR-146a in rabbitfish (*Siganus canaliculatus*), a model species for the study post-transcriptional modification of LC-PUFA biosynthesis, has been found to have a negative correlation with expression of Elovl5 and was shown to bind to the 3′UTR of *elovl5* mRNA, preventing translation (Chen et al., 2018; Xie et al., 2021). Accordingly, one explanation for the disparity in *n*-3 LC-PUFA net production in the two V 'stimulus' groups, despite the similar *elovl5* gene expression, is that miR-146a could have repressed translation of the protein maintaining *n*-3 LC-PUFA levels in V2 fish, in turn preserving levels of *n*-3 LC-PUFA between groups. An alternative explanation could be changes in the microbiome relating to the different 'stimulus' diets. Previous studies have demonstrated that feeding vegetable-based diets, compared to FM controls, can lead to a different gut microbial composition in Atlantic salmon (Gajardo et al., 2017) and zebrafish (Patula et al., 2021). Gut microbiota can modulate growth, metabolism and immune response in fish. Consequently, a microbial imbalance can have a negative impact on fish health (Ghanbari et al., 2015; Rimoldi et al., 2018) and there is an increasing need to study the functionality of gut microbial populations to predict potential impacts (Lorgen-Ritchie et al., 2021; Rimoldi et al., 2019). Work is ongoing to elucidate the influence gut microbiota has played in the present study (Tawfik et al., In preparation).

4.6. Conclusion

Results from the present study do not suggest positive NP effects in either V1 or V2 group compared to their M counterparts at the end of the 'challenge' phase, following the one- or two- week 'stimulus' of this trial. This could have been related to this study's shorter stimulus duration or an elevated proportion of EPA and DHA in the V_s diet, compared to Clarkson et al. (2017). Further work is required to elucidate the best levels of *n*-3 LC-PUFA and other critical fatty acid inclusion in low marine ingredient 'stimulus' diets to optimise the NP mechanism, leading to efficient utilisation and assimilation of these and other nutrients later in development. In addition, the present study has uncovered evidence of post-transcriptional regulation of the *n*-3 LC-PUFA biosynthesis pathway, and future investigation is required on the potential role of this mechanism in NP, and lipid metabolism in Atlantic salmon more broadly. Finally, it is clear that *n*-3 LC-PUFA biosynthesis alone will not entirely bridge the gap between tissue EPA + DHA in fish fed standard marine-based versus alternative vegetable-based diets (Sissener et al., 2017). Thus, from a commercial perspective, implementation of optimised NP approaches would need to be incorporated with other novel feeding strategies and ingredients, for continued sustainable industry growth whilst maintaining product quality and nutritional benefits to human consumers.

CRediT authorship contribution statement

Stuart McMillan: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **Samuel A.M. Martin:** Methodology, Visualization, Writing – review & editing. **Elżbieta Król:** Methodology, Resources, Writing – review & editing. **Fernando Norambuena:** Data curation, Resources, Writing – review & editing. **Simon Baumgärtner:** Formal analysis, Investigation, Writing – review & editing. **Xu Gong:** Formal analysis, Investigation, Writing – review & editing. **Marwa Mamdouh Tawfik:** Formal analysis, Investigation, Writing – review & editing. **Brett Glencross:** Funding acquisition, Methodology, Writing – review & editing. **John F. Taylor:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Douglas R. Tocher:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Mónica B. Betancor:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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