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Isolation of intake mediated effects demonstrate that the phenomic benefits of dietary omega-3 are nominal to Atlantic salmon when reared in a challenging (hypoxic) environment

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ABSTRACT

The present study was designed to examine whether challenging environmental conditions (such as hypoxia) influence a fish's response to diet by changing responses to nutrient utilisation or down-regulation of appetite. To examine this, post-smolt Atlantic salmon (138.8 ± 0.65 g/fish; mean ± SD) were fed sub- (2.6% TFA; Ln3) and optimal (6.0% TFA; Hn3) levels of long-chain polyunsaturated omega-3 fatty acids (n-3 LC-PUFA) when maintained under either normoxic (8.1 ± 0.6 mg/L) or hypoxic (6.8 ± 0.5 mg/L) environmental conditions. To control for the anticipated effects of hypoxia on appetite and food intake, two additional treatments included the two diets that were pair-fed to the same intake levels as in the hypoxic treatments. The reduction in dissolved oxygen was accompanied by an increase in dissolved CO_2 and decline in seawater pH, with no change in total alkalinity. After 138 days of the study the fish increased their average weight to between 616 and 720 g/fish subject to treatment. Hypoxia had a significant negative impact on final weight and weight gain, and these were the result primarily of effects on feed intake. No effects of treatments on feed conversion were observed. Significant diet x oxygen interactions were observed on whole-body lipid levels, but no other proximate compositional parameters. Sub-clinical effects of hypoxia and ration on several plasma chemistry parameters were observed, but few responses were diet related. Hypoxia affected plasma total protein, cholesterol, and red blood cell counts. Diet digestibility was typically not affected by hypoxia or ration, though a range of diet related effects were evident. Nutrient retention data showed evidence of elongation and desaturation of dietary short-chain PUFA, with clear effects of diet affecting the deposition of both omega-6 and omega-3 fatty acids. This study builds on findings from earlier studies, but crucially now include controls for the feed intake effects to clarify the roles of hypoxia on the nutrient and intake mediated effects. As a result of these controls, it is now clearer that many of the phenomic responses to long-chain omega-3 by Atlantic salmon are nominal and are largely unaffected by challenging environmental conditions like hypoxia. The inclusion of a series of pair-fed treatments, matching the feed intake levels of corresponding hypoxic treatments, shows that the effects of n-3 LC-PUFA appear to be largely intake mediated effects for Atlantic salmon post-smolts.

1. Introduction

Feed intake by salmonids has been shown to respond to a range of environmental and nutritional variables, with two such variables being low dissolved oxygen levels (hypoxia) and the level of dietary omega-3 fatty acids (Huyben et al., 2021a, 2021b). Studies on both Atlantic salmon and rainbow trout have shown that dietary omega-3 triggers a clear feed intake response, leading to improved growth but usually no improvements in feed efficiency (Geurden et al., 2007; Roy et al., 2020; Huyben et al., 2021a). Such observations raise the question of whether

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the effects of such "essential" nutrients are on improvements in dietary utilisation or merely intake responses, with recent evidence suggesting the latter. While omega-3 have significant effects on developmental and immunological responses, it has been difficult to separate growth from intake effects in most studies (Glencross, 2009a; Hundal et al., 2022; Huyben et al., 2023; Carr et al., 2023).

Studies examining fish responses to hypoxia have noted that the animals down-regulate their appetite, and there are suggestions that maintenance metabolic pathways are down-regulated during these conditions (Bernier and Craig, 2005; Saravanan et al., 2013; Remen et al., 2014; Green et al., 2016; Magnoni et al., 2019). However, studies specifically examining the marginal utilisation efficiencies of protein and energy, and the maintenance demands for both protein and energy under both normoxia and hypoxia have found no such alterations in either maintenance or utilisation efficiency (van Raaij et al., 1996; Glencross, 2009b; Saravanan et al., 2013). This response suggests that such down-regulation is not due in any part to changes in utilisation of any nutrients per se, but rather are consistent with down-regulation of metabolic demands of the animal (Pouliot and De la Noue, 1989; Vikeså et al., 2017; Mosberian-Tanha et al., 2018). Clearly the responses to both omega-3 and hypoxia both appear to be linked to feed intake. This raises the question as to whether an interaction effect exists between both feed intake effectors (Huyben et al., 2021b).

Earlier studies examining the interaction of hypoxia and omega-3 requirements have shown that effects of energy demand outweigh omega-3 effects, but those studies lacked the appropriate control of a pair-fed series of treatments (Huyben et al., 2021a). Consequently, it wasn't possible to separate intake from nutrient dependent effects. To follow up from those earlier studies, the aim of the present study was to challenge Atlantic salmon when fed sub- (low) and supra-(high) optimal levels of omega-3 when maintained under either normoxic or hypoxic environmental conditions. To control for the intake effects expected with hypoxia, two additional treatments were included that were pair-fed at the same intake levels of the hypoxic treatments, thus allowing for the effective isolation of intake versus diet and environment effects. This study builds on earlier work of the group (Huyben et al., 2021b), by controlling for intake related effects to enable further exploration of the mechanisms by which omega-3 affect Atlantic salmon performance.

2. Methods

2.1. Diet development and fish management

Two diets were formulated with low (2.6% TFA) and high (6.0% TFA) levels of n-3 LC-PUFA (Ln3 and Hn3) (Table 1). The two n-3 LC-PUFA levels were defined as sub- and supra-optimal based on previous studies (Glencross et al., 2014; Huyben et al., 2021a). Diets were formulated to be isoenergetic and isoproteic, with 460 g/kg of protein, 240 g/kg of lipid and 100 g/kg of starch. The diets were manufactured as 4 mm pellets by SPAROS I&D (Olhão, Portugal) using twin-screw extrusion, vacuum lipid coating, and stored at 4 °C. The composition of both diets is reported in Table 2.

Post-smolt Atlantic salmon were acquired from a hatchery (MOWI, Loch Ailort, Scotland) and transferred to Machrihanish Environmental Research Laboratory (MERL, Machrihanish, Argyll, Scotland) where they were allowed to acclimate to coastal seawater in 5000 L tanks for a 14-day period. Prior to allocation to the experimental tanks the fish were sedated with unbuffered MS222, weighed (138.8 ± 0.65, mean ± SD) and sorted into 18 tanks (1000*L*) to achieve 40 fish per tank, with each of the six treatments being replicated three times. Each tank was equipped with lighting on a 16:8 light:dark cycle and an automated feeding system (Arvotec, Finland), that was programmed to deliver a feed ration slightly above the expected intake over two three-hour periods (0600–0900, and 1900–2200) each day. Uneaten feed was collected each morning and weighed to allow estimation of the daily intakes by each tank. The pair-fed rations were set based on the mean of the previous day's measured

Table 1
Diet formulations.

Formulation:	Ln3	Hn3
Fishmeal LT70	18.0%	18.0%
Soy protein concentrate	16.0%	16.0%
Wheat gluten	11.8%	11.8%
Corn gluten	6.0%	6.0%
Wheat meal	14.5%	14.5%
Soy protein isolate	6.0%	6.0%
Fish oil (Anchoveta)	1.6%	4.5%
Rapeseed oil	3.0%	3.0%
Linseed oil	5.0%	5.0%
Olive oil	11.7%	8.8%
Rape lecithin	1.0%	1.0%
Vitamin and Mineral Premix (DSM OVN™)	0.5%	0.5%
Choline chloride	0.1%	0.1%
Natural antioxidant (Verdilox)	0.1%	0.1%
Monocalcium phosphate	3.0%	3.0%
Astaxanthin (Carophyll Pink 10%)	0.1%	0.1%
L-Histidine	0.8%	0.8%
L-Lysine	0.4%	0.4%
L-Taurine	0.3%	0.3%
DL-Methionine	0.1%	0.1%
Yttrium oxide	0.1%	0.1%

All ingredients provided by SPAROS Lda from commercial suppliers.

Table 2
Diet composition. All values g/kg (as fed) unless otherwise stated.

Nutrient	Ln3		Hn3	
Dry matter	954		954	
Crude Protein	459		463	
Lipid	243		235	
Carbohydrates	180		185	
Starch	92		93	
Ash	72		71	
Energy (MJ/kg)	23.3		23.1	
All fatty acid values are mg/g diet and %TFA				
14:0	135	0.7%	291	1.5%
16:0	1999	9.8%	2113	10.8%
18:0	684	3.4%	658	3.4%
20:0	68	0.3%	60	0.3%
22:0	34	0.2%	32	0.2%
24:0	14	0.1%	14	0.1%
16:1n-7	248	1.2%	431	2.2%
18:1n-9	10,865	53.2%	9024	46.0%
18:1n-7	402	2.0%	426	2.2%
20:1n-11	28	0.1%	45	0.2%
20:1n-9	157	0.8%	173	0.9%
22:1n-11	110	0.5%	130	0.7%
22:1n-9	25	0.1%	29	0.1%
24:1n-9	29	0.1%	34	0.2%
18:2n-6	2601	12.7%	2449	12.5%
20:2n-6	19	0.1%	7	0.0%
20:4n-6	21	0.1%	45	0.2%
18:3n-3	2356	11.5%	2329	11.9%
18:4n-3	48	0.2%	98	0.5%
20:4n-3	14	0.1%	30	0.2%
20:5n-3	267	1.3%	635	3.2%
22:5n-3	32	0.2%	76	0.4%
22:6n-3	185	0.9%	357	1.8%
Σ SFA	2950	14.5%	3194	16.3%
Σ MUFA	11,913	58.4%	10,362	52.8%
Σ scPUFA	5005	24.5%	4890	24.9%
Σ lcPUFA	538	2.6%	1176	6.0%
Σ n-3	2902	14.2%	3550	18.1%
Σ n-6	2641	12.9%	2516	12.8%
Σ Fatty Acids	20,407	100.0%	19,621	100.0%

SFA: saturated fatty acids. MUFA: monosaturated fatty acids. scPUFA: short-chain polyunsaturated fatty acids. lcPUFA: long-chain polyunsaturated fatty acids. %TFA: percent of total fatty acids.

intakes from the two hypoxia (low flow) treatments, with this pre-weighed ration fed out entirely during the same feeding period as the other tanks.

Dissolved oxygen and temperature were recorded continuously (Oxyguard A/S, Farum, Denmark). Over the 138-day experiment, water was supplied in an ambient flow through basis, with water temperature averaging 10.7 ± 2.0 °C (mean ± SD) across all tanks. The level of dissolved oxygen was regulated in the tanks by both maintaining high water flow (>5 L/min) or restricting water flow (<2 L/min) and minimising aeration. Oxygen levels in the high flow (normoxia) treatments averaged 8.1 ± 0.6 mg/L (89.6 ± 2.7% air-saturation, ~18.7 kPa), which was within the bounds considered for normoxia for this species (Oldham et al., 2019). Whereas in the low flow (hypoxia) treatments the oxygen level averaged 6.8 ± 0.5 mg/L (75.4 ± 3.4% air-saturation, ~15.8 kPa), over the duration of the study. The experiment was approved by the University of Stirling Animal Welfare and Ethical Review Body (reference AWERB-18/19–008) in accordance with the UK Home Office under the Animals Scientific Procedures Act 2013.

At the end of the study the fish were netted from the experimental tanks and sedated with MS222 and then weighed 24 h post-prandial. Faecal samples were collected using stripping techniques and pooled per tank and stored at −20 °C. At each time point, four fish per tank were euthanised by an overdose of MS222 and cervical dislocation for whole-body composition analysis. An additional four fish were similarly euthanised and a blood sample was collected via caudal vein puncture by a heparinised syringe, pooled, and split into a whole blood and centrifuged to collect a plasma sample (n = 3 tanks, and 12 fish per treatment). Fish were further dissected with liver, heart, brain, and head-kidney samples collected. Each sample was frozen in cryotubes on dry ice and stored at −80 °C for subsequent analysis. Hepatosomatic index (HSI), cardiosomatic index (CSI), and viscerosomatic index (VSI) were calculated according to the following equations:

$$\text{HSI}\ (\%) = (\text{liver weight/final weight})\ \text{x}\ 100.$$

$$\text{CSI}\ (\%) = (\text{heart weight/final weight})\ \text{x}\ 100.$$

$$\text{VSI}\ (\%) = (\text{viscera weight/final weight})\ \text{x}\ 100.$$

2.2. Water chemistry analysis

The pO_2 values used were obtained from the continuous logging records based on the times that coincided with when water samples were taken for CO_2 analysis. The pCO_2 values calculated from measured pH, salinity, temperature, and total CO_2 levels.

Water samples (15 mL) were collected from each fish tank on 12 different dates over the study and preserved by filtration (0.22 μm syringe filters) and addition of mercuric (II) chloride ($HgCl_2$) as described in Dickson et al. (2007) and stored at 4 °C. Dissolved inorganic carbon (DIC) analysis was carried out at the University of Exeter using a custom-built DIC Analyser system as described by Lewis et al. (2013). The pH and salinity were also measured using a combination pH electrode (Hach Intellical PHC735 RedRod pH electrode) and a conductivity cell (Hach CDC401 Laboratory 4-poles conductivity) connected to a digital pH meter (Hach multi HQ40d meter) at room temperature (sample temperature measured using aforementioned pH electrode). Carbonate chemistry parameters were then calculated utilising CO2Sys_v2.3 (K1, K2 from Lutfi et al., 2023, $KHSO_4$ from Dickson, 1990, KHF from Perez and Fraga, 1987) using the salinity and temperature of the tank at time of water sampling, together with pH and DIC measured in the lab.

To understand whether there was any obvious diel cycle in water chemistry, perhaps related to time of day or feeding, on two additional occasions (14–15 November 2019 & 27–28 January 2020), water chemistry parameters were measured at 6-h intervals (00:00, 06:00, 12:00, and 18:00 h). On the second occasion both the 12:00 and 18:00 sample times were omitted as all the fish had been removed from the tanks and terminally sampled by then.

2.3. Biochemical, haematological and digestibility analysis

Analysis of the proximate and fatty acid composition of the diets, carcasses and faeces were undertaken at the Institute of Aquaculture (Nutrition Analytical Services, University of Stirling, Stirling, Scotland). Whole fish carcass samples were minced, with a sample taken directly for moisture measurement, and another sample was freeze dried for further analysis. All faecal samples were freeze dried prior to analysis. All samples were milled to a fine powder and analysed as an air-dry powder. Each of the samples were analysed for dry matter, protein, lipid, ash, yttrium, and fatty acids. Moisture and ash were analysed using ovens held at 105 and 550 °C for approximately 24 and 12 h, respectively. Total yttrium concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-MS). Protein was analysed as nitrogen x 6.25 using the Kjeldahl method following digestion of a sample in sulphuric acid at 400 °C for one hour followed by titration against sodium hydroxide by a Tecator Kjeltec system to determine the specific level of nitrogen in the sample (FOSS A/S, Hillerød, Denmark). Total lipids were determined gravimetrically following extraction of the lipids using the chloroform: methanol solubilisation method. Gross energy was determined by ballistic bomb calorimetry (Parr 6200 bomb calorimeter; Parr Instrument Co., Moline, IL, USA). Samples were analysed for fatty acid composition in addition to their proximate analysis. Fatty acid methyl esters (FAME) were produced by acid-catalysed esterification of 1 mg of total lipid by overnight incubation at 50 °C with an internal standard of 17:0, sulphuric acid, methanol, and toluene. A solution of 1:1 isohexane/diethyl ether was added and then centrifuged. The upper layer was purified through a silica cartridge, redissolved in isohexane, and then injected using an on-column injector onto a gas liquid chromatograph (GLC) 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), equipped with a flame ionisation detector. Individual FAMEs were identified by MD800 mass spectrometer (ThermoFisher Scientific, Hempstead, UK) and compared to external standards of a marine oil. Data were collected and processed using Chromcard software version 2.01 (Thermoquest Italia S.p.A., Milan, Italy).

Clinical haematological analysis was performed on heparinised blood and frozen plasma samples on an automated chemistry analyser (AU400, Olympus Optical Co. Ltd., Tokyo, Japan) using standard assay kits developed for the auto-analyser (Scottish Agricultural College Veterinary Services, Penicuik, Scotland).

Retention efficiency of the various nutrients analysed was calculated based on final weights (FW) and initial weights (IW) of the fish, feed intake (FI), and the concentration of nutrient of interest (C) in each case based on:

$$\text{Nutrient retention}\ (\%) = [(\text{FW x C}/100)–(\text{IW x C}/100)]/(\text{FI x C}/100)\ \text{x}\ 100.$$

Diet apparent digestibility coefficients (DADC) for each of the nutritional parameters examined in each diet were determined based on the following formula (Huyben et al., 2021b):

$$\text{DADC}_{\text{Nutr}} = 1 - \left(\text{Y}_{\text{diet}}.\text{Nutr}_{\text{faeces}}\right)/\left(\text{Y}_{\text{faeces}}.\text{Nutr}_{\text{diet}}\right).$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Nutr_{diet}$ and $Nutr_{faeces}$ represent the nutritional parameter of concern (protein, lipid, energy, or fatty acids) content of the diet and faeces respectively.

2.4. Statistical analysis

All data are presented as means ± SEM unless otherwise specified. All analyses were undertaken using RStudio version 4.2.2. Normality

and heterogeneity of the datasets were determined using Shapiro-Wilk and Levene tests, with data normalized using a log transformation when required. A one-way ANOVA was initially performed on all parameters to compared for simple effects. For the more complex elements of the design an incomplete three-way ANOVA (MANOVA) was performed to determine effects of Diet (D), Oxygen (O), Ration (R), D x O, D x R interactions, but was not able to assess the O x R, and D x O x R interactions due to the incomplete nature of the design. Significant effects ($P < 0.05$) were examined using Tukey's HSD post-hoc test. Regression analyses were undertaken using the data analysis package of Microsoft Excel.

3. Results

3.1. Water chemistry

Seawater pO_2 was significantly lower ($P < 0.001$, df = 141, $t = -21.1$) by around 23% in the low O_2/high CO_2 treatment (14.1 ± 0.2 kPa; $n = 70$; i.e., 68.3% air-saturation) compared to the normoxic satiety fed Hn3 diet control treatment (18.3 ± 0.1 kPa; $n = 144$; i.e., 88.1% air-saturation; Fig. 1). Seawater pCO_2 was significantly higher ($P < 0.001$, df = 80, $t = 10.8$), by ~73% in the low O_2/ high CO_2 treatment (0.085 ± 0.003 kPa $n = 71$) compared to the control treatment (0.049 ± 0.001 kPa; $n = 144$; Fig. 2). For reference, seawater that is in perfect equilibrium with atmospheric air would have a pCO_2 of 0.041 kPa. In accordance with the elevated CO_2, pH was significantly lower ($P < 0.001$, df = 101, $t = -13.3$) by around 0.3 pH units in the low O_2/ high CO_2 treatment (7.87 ± 0.01; $n = 71$) than the control treatment (8.07 ± 0.01; $n = 144$; Fig. 3).

Total alkalinity (TA) was not significantly different between the two treatments ($P = 0.18$, df = 213, $t = 1.35$), with a mean of 2173 ± 5 (n = 144) and 2184 ± 6 μmol/kg (n = 71) for the control and low O_2/high CO_2 treatment, respectively (Fig. 4). Salinity was temporally stable throughout the study and did not differ between treatments.

Bicarbonate was significantly higher ($P < 0.001$, df = 141, $t = 14.4$) by about 5% in the low O_2/high CO_2 treatment (2013.5 ± 5.5 μmol/kg; n = 71), compared to the control treatment (1915.0 ± 3.9 μmol/kg; n = 144; Fig. 5). Carbonate was correspondingly lower ($P < 0.001$, df = 144, $t = -14.2$), by about 33% in the low O_2/high CO_2 treatment (70.2 ± 2.0 μmol/kg; n = 71) compared to the control treatment (105.0 ± 1.4 μmol/kg; n = 144; Fig. 6).

3.2. Phenomic responses

The primary phenomic responses of each treatment are presented in Table 3. Feed intake was affected by oxygen level ($P = 0.014$), and ration ($P = 0.013$), but not diet ($P = 0.402$). There were no significant interaction effects on feed intake. The final weight of the fish was significantly affected by oxygen level ($P = 0.001$), and ration (P = 0.001), but not diet ($P = 0.148$). Final weight was largest for fish fed the diet with high n-3 LC-PUFA and high oxygen (720.3 ± 7.37 g/fish), which was significantly higher by about 20% than those fish in the pair-fed and hypoxic treatments, but not the Ln3 diet fed to satiety at normoxia. Based on the MANOVA analysis no interaction effects were significant. Results for weight gain followed a similar pattern to those for final weight, with fish in the high PUFA and high oxygen increasing weight by 581.9 ± 7.41 g/fish, an increase of >420% in body weight. A regression analysis of the weight gain responses relative to feed intake show a relationship of $R^2 = 0.936$, $P < 0.001$, indicating a very strong linkage between the two parameters (Fig. 7). Notably, there were no significant effects on feed conversion ratio (FCR). There were no significant effects of any treatment variables on survival, viscerosomatic index, or cardiosomatic index. Hepatosomatic index was significantly (P = 0.001) affected by oxygen level, but not by ration or diet, or any interaction.

3.3. Biochemical responses

Analysis of the haematological effects revealed few significant differences among the treatments (Table 4). Total plasma protein ($P < 0.001$), and cholesterol ($P = 0.002$) levels were significantly reduced in response to hypoxia, but not by diet or ration level, or any interaction term. Red blood cell counts, and mean cell volume (MCV) were affected by ration, with the former also affected by oxygen level. No other effects on any other haematological parameter were evident.

No body composition effects on dry matter, protein or ash levels were observed among the treatments (Table 3). However, a significant effect ($P = 0.042$) of diet x oxygen observed on lipid levels, and trends ($P < 0.1$) were observed for effects of oxygen and ration on whole-body lipid levels.

Whole-body fatty acids (Table 5) are presented on a live-weight basis

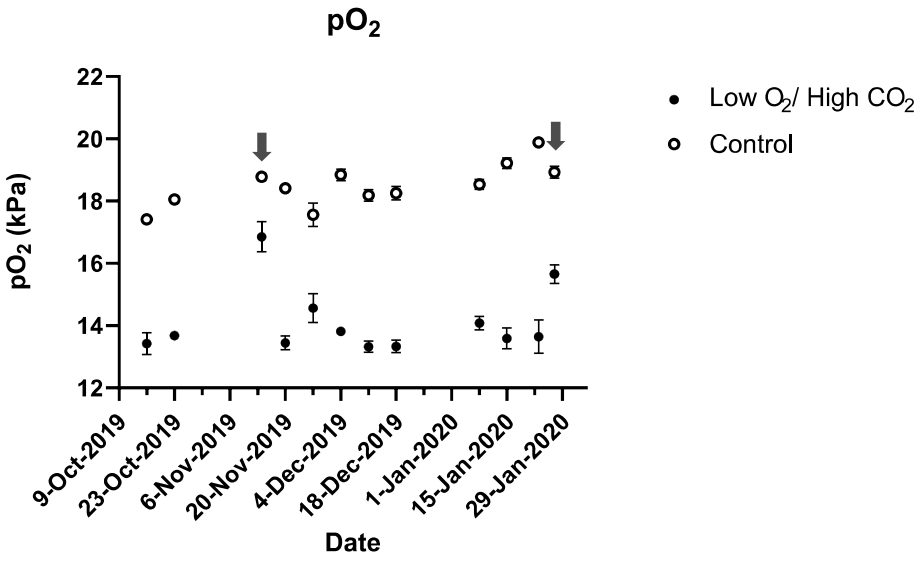

Fig. 1. The effect of oxygen treatment on pO_2 (kPa) at 12 timepoints across the study. The pO_2 values used were extracted from the continuous logging records for times that coincided with when water samples were taken for CO_2 analysis. Red arrows indicate dates on which fish were removed from tanks and water chemistry was sampled more than once per day. Data are shown as mean ± standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

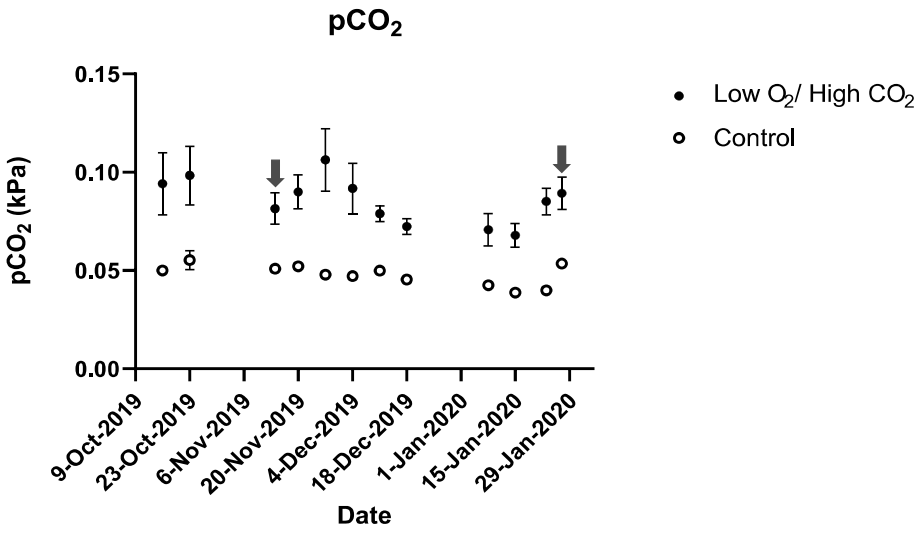

Fig. 2. The effect of oxygen treatment on pCO_2 (kPa) at 12 timepoints across the study. The pCO_2 values calculated from measured pH, salinity, temperature and total CO_2. Red arrows indicate dates on which fish were removed from tanks and water chemistry was sampled more than once per day. Data are shown as mean ± standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

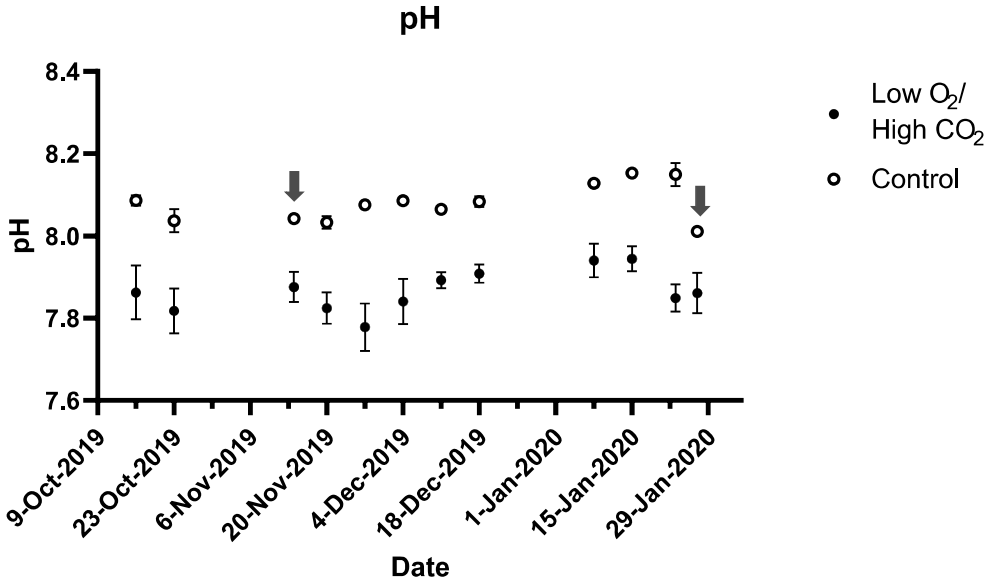

Fig. 3. The effect of oxygen treatment on pH at 12 timepoints across the study. Red arrows indicate dates on which fish were removed from tanks and water chemistry was sampled more than once per day. Data are shown as mean ± standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and show a variety of significant effects. At a fatty acid class level, significant one-way ANOVA effects were observed on Σ MUFA, Σ LC-PUFA, and Σ n-3, due to effect of diet but none of the other fatty acid classes had any effects. There were some significant interaction terms for diet x oxygen observed when analysed as a MANOVA for Σ MUFA, Σ SC-PUFA, Σ n-6, and Σ Fatty acids.

Among the saturates, significant effects of treatment were only noted for 14:0, which was attributed to a diet effect, and an interaction effect for diet x oxygen was observed on 16:0. Among the monounsatrates a range of significant effects were observed. Notable were significant effects of diet, on the levels of 16:1n-7, 16:1n-9, 18:1n-9, 20:1n-7, 20:1n-11, and 24:1n-11. Interaction terms between diet and oxygen were also observed for 16:1n-9, 18:1n-9, 22:1n-9 and 24:1n-11. Among the omega-6 fatty acids a range of significant effects were observed. Most notable were the effects of diet on the levels of 18:3n-6, 20:3n-6, and 20:4n-6. There were no significant effects on 18:2n-6 and only a single interaction term was observed for diet x oxygen on 20:2n-6. Among the omega-3 fatty acids an even greater number of significant effects were observed, with effects of diet on the levels of 18:3n-3, 18:4n-3, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3. There were effects of oxygen on the levels of 18:3n-3, and 20:3n-3. There were additional interaction terms between diet and oxygen were observed for 18:3n-3, 20:3n-3, and 20:4n-3.

3.4. Nutrient utilisation responses

Effects of treatments on digestibility of macronutrients and fatty acids were evident for several parameters (Table 6). For the most part the effects were limited to diet effects only, with only a few effects of oxygen, ration or interactions observed across any of the parameters. Clear diet effects on the digestibility of protein, lipid and energy were observed, with effects of oxygen also evident on digestibility of protein and energy, but not lipid. An effect of ration was observed on energy

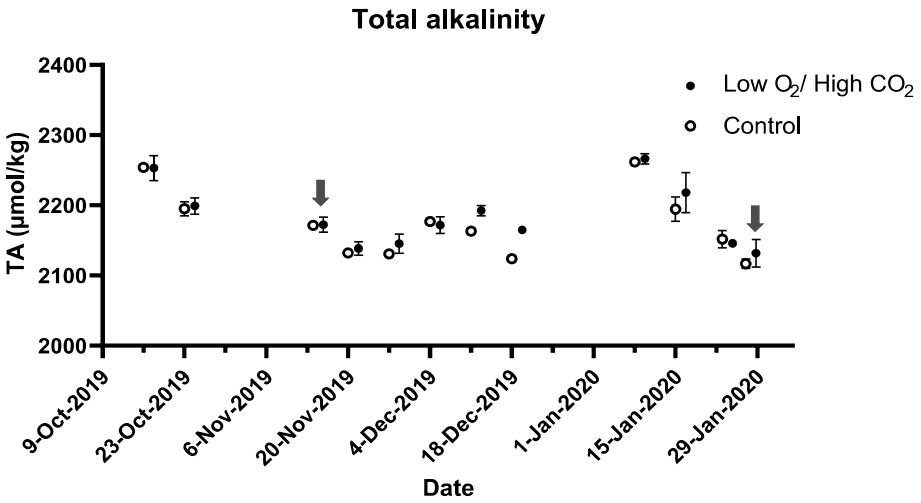

Fig. 4. The effect of oxygen treatment on total alkalinity (µmol/kg) at 12 timepoints across the study. Total alkalinity values were calculated from measured pH, salinity, temperature, and total CO_2. Red arrows indicate dates on which fish were removed from tanks and water chemistry was sampled more than once per day. Data are shown as mean ± standard error. Data for the two treatments are plotted slightly to the left or right of their true time point, to avoid visual overlap of means and error bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

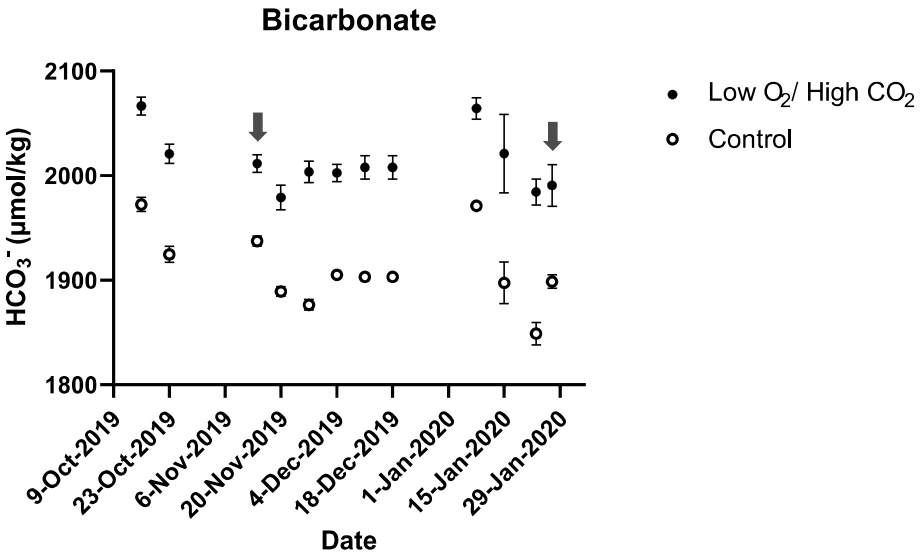

Fig. 5. The effect of oxygen treatment on bicarbonate concentration (µmol/kg) at 12 timepoints across the study. The bicarbonate values calculated from measured pH, salinity, temperature and total CO_2. Red arrows indicate dates on which fish were removed from tanks and water chemistry was sampled more than once per day. Data are shown as mean ± standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

digestibility, and an interaction effect was observed for protein between diet and oxygen. Digestibility of the fatty acids shows a variety of significant effects. At a fatty acid class level, significant MANOVA effects were observed on Σ SFA, Σ MUFA, and Σ LC-PUFA, but none of the other fatty acid classes had any effects, nor where there any interaction terms when analysed as a MANOVA. Among the saturates, significant effects of diet were noted for 16:0, 18:0, 20:0 and 22:0. Among the mono-unsatrates significant effects of diet, on the digestibility of 16:1n-7, 18:1n-9, and 20:1n-11, were observed. Among the omega-6 fatty acids significant effects of diet were observed on the digestibility of 20:3n-6, and 20:4n-6, and an effect of oxygen on 20:4n-6, but notably there were no significant effects on 18:2n-6 nor where there any interaction terms observed. Among the omega-3 fatty acids significant effects were observed for most fatty acids, with effects of diet observed on the digestibility of 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3. Although there was no discrete diet effect, there were additional interaction terms between diet and oxygen, and diet and ration observed for 18:3n-3. The only interaction terms found for the digestibility of any of the fatty acids.

Experimental effects on nutrient retention by the fish were generally more complex than those seen on other parameters (Table 7). A variety of variable and interaction effects were noted on many of the parameters. No effects were seen on retention efficiencies of protein, ash, or energy. Effects of oxygen, ration and interaction terms on diet x oxygen were observed for lipid retention. There were a variety of significant effects on fatty acid retention due to treatment variables. At a fatty acid class level, significant effects were only observed on the Σ LC-PUFA, but none of the other fatty acid classes. While there were trends ($P < 0.1$) on the interaction terms for diet x oxygen for the retention efficiencies of most of the other fatty acid classes, notably the Σ LC-PUFA and the Σ n-3 were not among these. Among the individual fatty acids, no significant effects of diet, oxygen or ration were noted for any of the saturates, although a diet x oxygen interaction effect was observed for 16:0. Effects of diet on the retention efficiency of 20:1n-9, and 22:1n-9 were observed. There were no significant effects of oxygen or ration on the

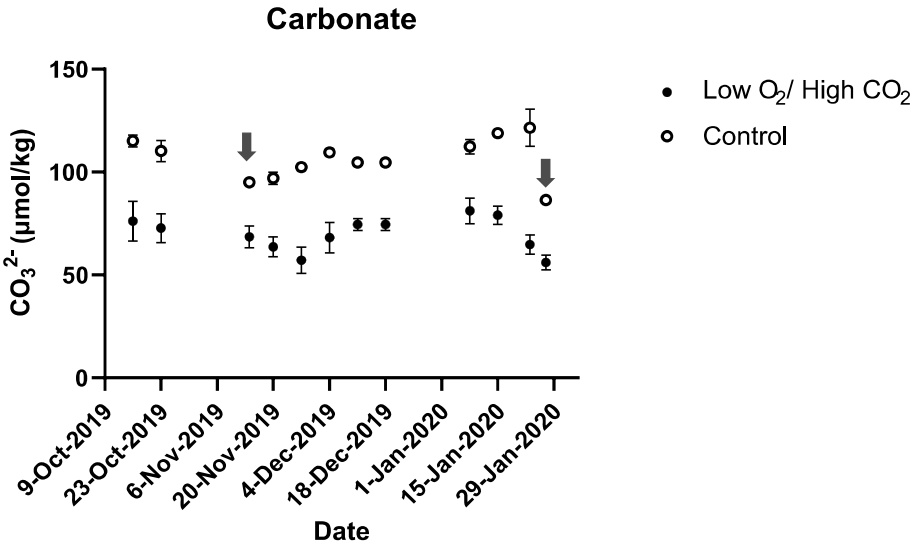

Fig. 6. The effect of oxygen treatment on carbonate concentration (µmol/kg) at 12 timepoints across the study. The carbonate values calculated from measured pH, salinity, temperature and total CO_2. Red arrows indicate dates on which fish were removed from tanks and water chemistry was sampled more than once per day. Data are shown as mean ± standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Phenomic responses to diet, hypoxia, and feed ration.

Ration	S	S	P	P	S	S		*P-values*				
Oxygen	90%	90%	90%	90%	70%	70%	Pooled	Diet	Oxygen	Ration	D x O	D x R
Diet	Ln3	Hn3	Ln3	Hn3	Ln3	Hn3	SEM					
Performance												
Initial (g/fish)	139.4	138.4	138.6	139.3	138.4	138.7	0.13	0.903	0.802	0.368	0.257	0.672
Final (g/fish)	686.4	720.3	616.0	621.6	594.9	632.6	15.54	0.148	**0.001**	**0.001**	0.927	0.500
Gain (g/fish)	547.0	581.9	477.4	482.3	456.5	493.8	15.45	0.146	**0.001**	**0.001**	0.960	0.477
Intake (g/fish)	476.2	504.3	413.7	416.6	404.8	428.6	12.52	0.402	**0.014**	**0.013**	0.937	0.633
FCR (feed/gain)	0.87	0.87	0.87	0.86	0.89	0.87	0.006	0.619	0.721	0.834	0.741	0.996
Survival (%)	100	100	98.6	100	100	98.6	0.32	1.000	0.404	0.404	0.404	0.404
Somatic Indices (% LW)												
Cardiac	0.13	0.11	0.13	0.13	0.13	0.13	0.003	0.313	0.117	0.084	0.477	0.450
Hepatic	1.04	1.02	1.08	1.08	1.16	1.18	0.018	0.954	**0.001**	0.135	0.518	0.795
Viscera	8.52	8.38	8.61	8.99	8.66	9.09	0.112	0.331	0.145	0.220	0.314	0.359
Wholebody Proximate Composition (% LW)												
Dry Matter	32.8	31.3	31.2	30.7	31.2	31.8	1.17	0.422	0.405	0.120	0.131	0.413
Protein	17.6	16.9	17.0	16.8	17.4	17.2	0.16	0.371	0.970	0.357	0.546	0.590
Lipid	12.4	11.2	11.3	10.8	10.9	11.3	0.17	0.150	0.067	0.058	**0.042**	0.311
Ash	1.8	1.8	1.8	1.9	1.9	1.8	0.02	0.998	0.444	0.403	0.474	0.181
Energy (kJ/g)	8.5	7.9	8.0	7.7	7.9	8.0	0.10	0.148	0.193	0.093	0.073	0.378

S: Satiety fed, P: Pair fed against corresponding low oxygen treatments. P-values shown are those for the one-way ANOVA. FCR: Feed Conversion Ratio. LW: *live weight* P-values shown are those for the incomplete MANOVA analysis. Bolded are those values significant at $P < 0.05$.

retention efficiency of any of the monounsatrates, though diet x oxygen interaction effects were observed for of 16:1n-7, 22:1n-9, and 24:1n-9. Among the omega-6 fatty acids significant effects of diet were observed on the retention efficiencies of 18:3n-6, 20:2n-6 and 20:4n-6, but notably there were no significant effects on 18:2n-6 nor where there any interaction terms observed. Among the omega-3 fatty acids significant effects were observed for several of the fatty acids. Effects of diet were observed on the retention efficiency of 18:3n-3, 18:4n-3, 20:4n-3, 22:5n-3 and 22:6n-3, though notably not 20:5n-3. There was no discrete effects of oxygen or ration for any n-3 fatty acid, and an interaction term between diet and oxygen, was observed only for 20:4n-3.

4. Discussion

Previous examinations of the interaction between hypoxia and omega-3 requirements have shown that energy demand effects outweigh omega-3 effects (Huyben et al., 2021a). However, the lack of the appropriate controls for the feed intake effects of hypoxia mean that it wasn't possible to separate intake from nutrient dependent effects in that study. To control for the intake effects, the inclusion of two additional treatments whose feeding was matched to the same intake levels of the hypoxic treatments (pair-fed), allowed for the effective isolation of intake versus diet and environment effects in the present study. By effectively controlling for changes in food intake between treatments, we have been able to further define the mechanisms by which omega-3 affect Atlantic salmon performance.

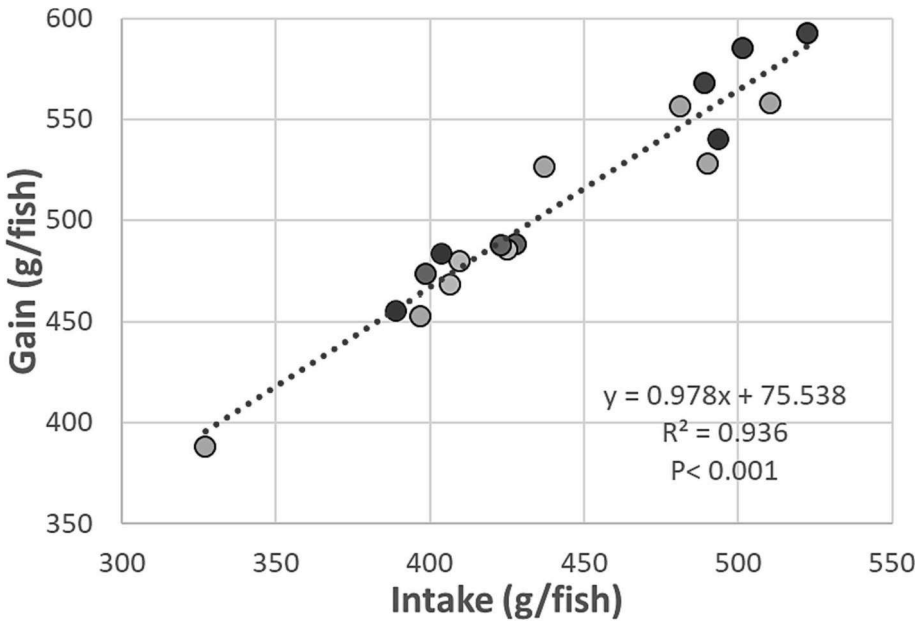

Fig. 7. Regression analysis of weight gain (g/fish) against feed intake (g/fish) of each tank in the experiment showing highly significant linearity in the response. Blue data points are the normoxia satietal fed treatments. Green data points are the pair-fed treatments at normoxia. Red data points are the hypoxia satietal fed treatments, Darker colour shades represent the Hn3 diet, and the lighter colour shades the Ln3 diets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Haematological responses to diet, hypoxia, and feed ration.

Ration		S	S	P	P	S	S		*P-values*				
Oxygen	Units	90%	90%	90%	90%	70%	70%	Pooled	Diet	Oxygen	Ration	D x O	D x R
Diet		Ln3	Hn3	Ln3	Hn3	Ln3	Hn3	SEM					
ALA aminotransferase		3.0	1.7	6.3	1.3	2.3	1.3	0.9	0.207	0.828	0.517	0.942	0.430
ASP aminotransferase		334	197	905	248	206	187	128.5	0.332	0.837	0.362	0.860	0.444
Creatine kinase		34,757	33,873	37,187	27,083	18,630	29,083	3766.7	0.983	0.320	0.832	0.584	0.656
Creatinine		23.7	22.3	21.3	25.0	20.7	20.7	0.6	0.505	0.118	0.906	0.639	0.096
Total protein		44.7	42.3	43.3	46.0	37.0	37.3	0.9	0.837	**0.000**	0.386	0.324	0.078
Cholesterol		10.0	8.9	9.0	9.3	8.0	7.6	0.2	0.299	**0.002**	0.533	0.373	0.111
Red blood cell counts		1.1	1.1	0.9	1.0	1.0	0.9	0.0	0.464	**0.039**	**0.026**	0.652	0.501
Haemoglobin		189.3	187.3	195.0	167.0	181.3	180.0	6.3	0.469	0.662	0.676	0.985	0.462
Packed cell volume		0.7	0.7	0.7	0.7	0.6	0.7	0.0	0.444	0.934	0.651	0.622	0.651
MCV		606	589	797	739	679	745	32.0	0.962	0.143	**0.039**	0.575	0.783
MCHC		287	289	297	239	286	271	13.3	0.437	0.805	0.591	0.825	0.424
WBC counts		10.5	9.7	9.8	7.6	13.2	18.7	1.8	0.830	0.232	0.768	0.515	0.872
Heterophils		7.3	5.7	7.0	5.5	8.0	10.8	0.9	0.948	0.264	0.921	0.393	0.995
Lymphocytes		1.5	2.2	1.1	1.5	1.4	2.6	0.2	0.166	0.789	0.384	0.708	0.768
Monocytes		1.7	1.8	1.7	0.7	3.8	5.3	0.7	0.892	0.156	0.772	0.707	0.772

S: Satiety fed, P: Pair fed. ALA: Alanine. ASP: Aspartate. MCV: mean cell volume. MCHC: Mean corpuscular haemoglobin concentration. WBC: White blood cell. P-values shown are those for the incomplete MANOVA analysis. Bolded are those values significant at $P < 0.05$.

4.1. Responses to hypoxia

The experimental design essentially relied on the metabolism of the fish in each tank to reduce dissolved oxygen levels in the hypoxic treatments (−23% compared to controls). As a result, hypoxia was associated with a simultaneous reduction in seawater pH caused by the reciprocal but larger relative change (+58%) in dissolved CO_2 in the same treatments. The molar ratio of excreted CO_2 to consumed O_2 is close to 1, but it is the physical chemistry of O_2 and CO_2 in seawater that explains the difference in their relative change between treatments. Firstly, CO_2 is about 30 times more soluble than O_2 in water, meaning it is harder to remove from seawater once excreted by the fish. Secondly, >90% of the excreted CO_2 ends up being converted to bicarbonate (HCO_3^-). Whilst aeration helps to remove some CO_2, it is gradually replaced by the much larger pool of bicarbonate reacting with H^+ ions to form more CO_2:

$$HCO_3^- + H^+ \rightleftharpoons CO_2 + H_2O.$$

This reaction is slow without a catalyst, which delays the opportunity for aeration to remove the replacement CO_2 molecules. This results in a greater increase pCO_2 relative to the reduction in pO_2.

The simultaneous changes in seawater O_2, CO_2 and pH reflect what occurs both in nature and in aquaculture in response to intensive respiration relative to the re-equilibration with atmospheric gases. Our experimental design therefore mimics hypoxia experienced both in the wild and in farm settings better than when using nitrogen gas to generate hypoxic seawater. However, it also means we cannot categorically attribute any effects of the hypoxia treatment specifically to low O_2, as opposed to low pH or elevated CO_2. There is clear evidence that low O_2 (at levels similar to those used here) reduces appetite and growth in salmon (Vikeså et al., 2017; Huyben et al., 2021a, 2021b). However, whilst reduced appetite and growth are documented for exposure to

Table 5
Whole-body fatty acid profile (mg/100 g LW) in response to diet, hypoxia, and ration.

Ration	S	S	P	P	S	S		*P-values*				
Oxygen	90%	90%	90%	90%	70%	70%	Pooled	Diet	Oxygen	Ration	D x O	D x R
Diet	Ln3	Hn3	Ln3	Hn3	Ln3	Hn3	SEM					
14:0	91	136	86	135	82	136	6.2	**0.000**	0.343	0.516	0.320	0.676
16:0	998	961	928	948	836	974	18.1	0.218	0.072	0.292	**0.039**	0.465
18:0	355	318	323	312	302	326	6.5	0.549	0.161	0.233	0.064	0.413
20:0	23	20	21	20	20	20	0.4	0.083	0.217	0.371	0.054	0.305
22:0	11	10	10	10	10	10	0.2	0.631	0.452	0.636	0.069	0.231
16:1n-9	35	27	32	26	29	27	0.9	**0.000**	**0.039**	0.204	**0.045**	0.623
16:1n-7	145	185	133	186	124	190	7.0	**0.000**	0.250	0.415	0.062	0.325
18:1n-9	4593	3619	4229	3614	3870	3709	104.0	**0.001**	0.079	0.285	**0.029**	0.296
18:1n-7	199	197	184	198	172	201	3.6	**0.048**	0.148	0.369	0.061	0.321
20:1n-11	18	21	17	22	17	22	0.7	**0.000**	0.882	1.000	0.337	0.215
20:1n-9	219	185	198	192	190	192	4.2	0.122	0.271	0.507	0.070	0.153
20:1n-7	8	10	8	11	8	10	0.4	**0.000**	0.383	0.998	0.473	0.257
22:1n-11	84	85	82	94	87	90	1.6	0.110	0.330	0.346	0.658	0.151
22:1n-9	26	23	24	25	23	24	0.4	0.687	0.235	0.963	**0.033**	0.073
24:1n-9	25	24	23	26	22	26	0.4	**0.004**	0.500	0.953	**0.041**	**0.041**
18:2n-6	1011	913	943	915	876	929	15.7	0.427	0.118	0.370	0.054	0.341
18:3n-6	24	15	24	15	15	16	1.5	**0.042**	0.254	0.984	0.165	0.988
20:2n-6	71	61	62	66	55	64	1.7	0.737	0.113	0.523	**0.024**	0.086
20:3n-6	41	24	40	25	38	26	1.9	**0.000**	0.741	0.915	0.059	0.392
20:4n-6	16	19	16	19	16	20	0.4	**0.000**	0.908	0.441	0.679	0.957
18:3n-3	667	675	617	661	557	675	13.1	**0.012**	**0.035**	0.190	**0.037**	0.449
18:4n-3	91	66	89	66	86	70	2.9	**0.000**	0.969	0.762	0.172	0.765
20:3n-3	43	45	37	46	32	46	1.5	**0.001**	**0.040**	0.196	**0.029**	0.156
20:4n-3	62	54	57	56	52	57	1.2	0.637	0.210	0.617	**0.013**	0.126
20:5n-3	63	137	82	136	81	134	8.7	**0.000**	0.555	0.469	0.434	0.437
22:5n-3	38	57	36	58	35	58	2.6	**0.000**	0.486	0.590	0.359	0.393
22:6n-3	218	249	206	251	204	257	5.8	**0.000**	0.627	0.477	0.135	0.304
Σ SFA	1489	1458	1378	1438	1258	1481	27.9	0.107	0.103	0.289	0.052	0.457
Σ MUFA	5364	4389	4943	4408	4552	4506	108	**0.007**	0.100	0.323	**0.035**	0.282
Σ scPUFA	1793	1669	1672	1657	1534	1690	27.7	0.919	0.074	0.296	**0.040**	0.388
Σ lcPUFA	553	655	536	665	517	670	18.3	**0.000**	0.724	0.911	0.365	0.628
Σ n-3	1183	1292	1124	1283	1051	1305	28.3	**0.001**	0.241	0.495	0.156	0.615
Σ n-6	1163	1032	1084	1039	1000	1055	18.4	0.230	0.099	0.378	**0.035**	0.291
Σ Fatty Acids	9225	8213	8554	8208	7884	8389	154.2	0.326	0.112	0.340	**0.045**	0.345

S: Satiety fed, P: Pair fed. SFA: saturated fatty acids. MUFA: monosaturated fatty acids. scPUFA: short-chain polyunsaturated fatty acids. lcPUFA: long-chain polyunsaturated fatty acids. P-values shown are those for the incomplete MANOVA analysis. Bolded are those values significant at $P < 0.05$.

high CO_2, the level used here (0.085 kPa or ~ 1.7 mg/L) is much lower than the threshold documented to reduce specific growth rate in Atlantic salmon (Skov, 2019). This suggests that the effects observed in the present study (at least those linked to reduced food intake) are most likely attributed to the reduced O_2 level in the hypoxia treatment. We have therefore discussed effects observed on responses to "hypoxia", but it should be noted that some effects of high CO_2 cannot be ruled out.

A variety of phenomic responses to hypoxia were observed in the present experiment. Among the most notable responses to hypoxia was a significant reduction in feed intake by the fish, which is consistent with much of the previous literature on the effects of hypoxia on salmonids (Glencross, 2009b; Saravanan et al., 2013; Vikeså et al., 2017; Huyben et al., 2021a, 2021b). Differences in the weight changes seen in this study (e.g., a main effect contrast of 342% cf. 376% gain between hypoxic and normoxic conditions, respectively) follow as a clear response to the feed intake effects. The further observation in the present study that there was no change in FCR with the exposure to hypoxia is also consistent with other studies examining hypoxia in salmonids (Glencross, 2009b; Saravanan et al., 2013; Huyben et al., 2021a, 2021b). Given that no effect on FCR was observed, but that growth was significantly affected clearly indicates that the response is almost entirely driven by feed intake, and again those responses are further confirmed by the regression analysis of weight gain against intake in the present study where the R^2 was >0.95. While a significant effect of hypoxia was observed on energy digestibility of the diets, the observation that this effect was also significant for ration indicates that the effect on digestibility is mostly intake linked.

There were few whole-body composition effects of note due to hypoxia, with only whole-body lipid levels showing a non-significant decline, and there were no other macronutrient compositional parameters affected by this variable at all (Table 5). Other studies, like the present one, have also only reported trends in this decline in body lipid levels under hypoxic conditions (Huyben et al., 2021b; Aksakal et al., 2023), as well as similarly few effects on nutrient retention. Other than a significant effect on lipid retention, no other effects of hypoxia on the retention of any of the nutrients assessed was evident. This supports the other observations that hypoxia is not affecting the utilisation of any discrete nutrient other than the overall level of lipid, and apparently at a consistent rate across all fatty acids, hence the absence of any individual fatty acid effects (Huyben et al., 2021b). Had there been any differential utilisation of nutrients, we should have seen impacts of hypoxia on their retention independent of ration effects.

Only a few of the haematological effects were affected by oxygen level, including plasma total protein, cholesterol, and red blood cell counts. Interestingly, no effects of oxygen level on haemoglobin or packed cell volume were evident, suggesting that chronic hypoxia does not lead to any upregulation in the oxygen carrying capacity of Atlantic salmon. An alternative interpretation is that any increase in blood haemoglobin in response to the hypoxia treatment was small compared to the increase caused by the acute stress and subsequent adrenergic responses associated with the netting, anaesthesia, and blood sampling process (e.g., Axelsson, 2005; Caldwell et al., 2006; Davison et al., 2023).

4.2. Responses to omega-3

Despite that the dietary treatments in this study were designed to be sub- and supra optimal based on the results of several quantitative

Table 6
Diet macronutrient and fatty acid digestibilities (%).

Ration	S	S	P	P	S	S		*P-values*				
Oxygen	90%	90%	90%	90%	70%	70%	Pooled	Diet	Oxygen	Ration	D x O	D x R
Diet	Ln3	Hn3	Ln3	Hn3	Ln3	Hn3	SEM					
Dry matter	66%	63%	71%	70%	69%	71%	1.4%	0.127	0.450	0.131	0.540	0.909
Protein	88%	88%	89%	88%	90%	88%	0.2%	**0.007**	**0.039**	0.124	**0.014**	0.365
Lipid	97%	96%	97%	97%	97%	96%	0.1%	**0.015**	0.338	0.229	0.412	0.905
Energy	83%	83%	85%	84%	85%	84%	0.2%	**0.027**	**0.010**	**0.010**	0.133	0.238
14:0	93%	93%	94%	94%	91%	92%	0.5%	0.912	0.209	0.378	0.463	0.725
16:0	93%	91%	94%	92%	93%	89%	0.4%	**0.002**	0.208	0.142	0.319	0.672
18:0	92%	90%	93%	91%	92%	87%	0.6%	**0.001**	0.198	0.213	0.169	0.847
20:0	89%	86%	90%	88%	90%	82%	0.8%	**0.000**	0.147	0.292	0.093	0.893
22:0	84%	81%	85%	84%	84%	76%	1.0%	**0.039**	0.254	0.318	0.222	0.652
24:0	75%	72%	75%	76%	72%	65%	1.1%	0.074	0.054	0.224	0.331	0.366
16:1n-9	92%	91%	93%	94%	90%	91%	0.6%	0.544	0.382	0.252	0.424	0.376
16:1n-7	97%	98%	97%	98%	97%	97%	0.1%	**0.042**	0.395	0.602	0.985	1.000
18:1n-9	98%	98%	98%	98%	98%	98%	0.1%	**0.006**	0.990	0.612	0.185	0.198
18:1n-7	97%	96%	97%	96%	96%	96%	0.2%	0.109	0.460	0.342	0.937	0.632
20:1n-11	94%	95%	94%	95%	93%	94%	0.3%	0.028	0.358	0.362	0.781	0.884
20:1n-9	92%	91%	92%	93%	90%	90%	0.5%	0.941	0.324	0.420	0.474	0.334
22:1n-11	92%	92%	92%	93%	90%	91%	0.4%	0.738	0.359	0.415	0.662	0.734
22:1n-9	87%	86%	87%	90%	84%	85%	0.9%	0.498	0.398	0.385	0.611	0.440
24:1n-9	66%	62%	71%	79%	51%	63%	3.8%	0.529	0.482	0.255	0.413	0.538
18:2n-6	97%	97%	97%	97%	97%	96%	0.1%	0.936	0.570	0.350	0.701	0.808
20:2n-6	87%	55%	85%	77%	81%	45%	5.3%	**0.013**	0.446	0.367	0.868	0.291
20:4n-6	92%	95%	89%	98%	86%	94%	1.0%	**0.000**	**0.015**	0.774	0.105	0.065
18:3n-3	99%	99%	99%	99%	99%	99%	0.0%	0.113	0.307	0.591	0.042	**0.033**
18:4n-3	98%	99%	98%	99%	98%	99%	0.1%	**0.000**	0.853	0.673	0.933	0.391
20:4n-3	95%	99%	92%	97%	94%	96%	0.8%	**0.025**	0.248	0.182	0.811	0.556
20:5n-3	98%	99%	97%	99%	97%	98%	0.2%	**0.000**	0.075	0.507	0.271	0.089
22:5n-3	93%	95%	92%	97%	89%	95%	0.7%	**0.001**	0.131	0.669	0.181	0.262
22:6n-3	89%	93%	88%	96%	84%	91%	1.0%	**0.001**	0.089	0.555	0.443	0.217
Σ SFA	93%	91%	93%	92%	92%	89%	0.5%	**0.004**	0.198	0.162	0.319	0.704
Σ MUFA	98%	97%	98%	97%	98%	97%	0.1%	**0.012**	0.750	0.402	0.431	0.537
Σ scPUFA	98%	98%	98%	98%	98%	98%	0.1%	0.970	0.694	0.397	0.446	0.865
Σ lcPUFA	93%	96%	92%	98%	90%	95%	0.7%	**0.000**	0.058	0.812	0.282	0.159
Σ n-3	98%	98%	98%	99%	98%	98%	0.1%	0.202	0.249	0.596	0.902	0.389
Σ n-6	96%	96%	97%	97%	96%	96%	0.1%	0.933	0.426	0.352	0.808	0.652
Σ Fatty Acids	97%	96%	97%	97%	97%	96%	0.1%	**0.012**	0.335	0.235	0.398	0.912

S: Satiety fed, P: Pair fed. SFA: saturated fatty acids. MUFA: monosaturated fatty acids. scPUFA: short-chain polyunsaturated fatty acids. lcPUFA: long-chain polyunsaturated fatty acids. P-values shown are those for the incomplete MANOVA analysis. Bolded are those values significant at $P < 0.05$.

studies on the requirements for omega-3 LC-PUFA by Atlantic salmon (Glencross et al., 2015; Rosenlund et al., 2016; Bou et al., 2017), there were no significant differences in growth or feed conversion between the two diets. This lack of growth response to omega-3 LC-PUFA is however consistent with earlier observations on the impacts of these nutrients on Atlantic salmon when intake effects are accounted for (Glencross et al., 2014). It has been noted in other studies that growth in Atlantic salmon appears relatively insensitive to subtle changes in the levels of omega-3 LC-PUFA, especially when SC-PUFA like 18:3n-3 are prevalent in the diet (Glencross et al., 2014; Mock et al., 2019; Ruyter et al., 2022). This is mostly likely because the animal maintains some capacity to elongate and desaturate 18:3n-3 to compensate for any (minor) short-fall in physiological demands for EPA and/or DHA (Zheng et al., 2005; Sprague et al., 2019; Huyben et al., 2021a, 2021b).

Of the few body composition effects observed in the present study, most were those that were diet-linked, which is a well-known effect of dietary fatty acid variation (Mock et al., 2020; Glencross et al., 2023). One was the observation that when the fish were fed to apparent satiety that those fed the Ln3 diet had higher levels of lipid deposition. This effect is also seen with higher levels of 18:1n-9 (the most abundant fatty acid in the fish) and is consistent with other observations on the effects of low levels of omega-3 LC-PUFA fed to Atlantic salmon (Hundal et al., 2022). Otherwise, most of the whole-body levels of the various fatty acids are clearly diet related effects, which is generally typical for this fish species (Glencross et al., 2014; Mock et al., 2020; Ruyter et al., 2022).

Nutrient retention effects due to diet were the most influenced results in the present study (Table 7). Although no dietary effects on the retention of any of the proximates were observed, retention of many of the fatty acids were affected. Most notable of the whole-body retention of the various fatty acids linked to diet were those of the total LC-PUFA. However, even within that nutrient class it was notable that the retention of DHA was affected by diet, but not EPA. This is consistent with observations of other studies (Glencross et al., 2014; Mock et al., 2019). In fact, of all the n-3 PUFA the only fatty acids to not be affected by diet were EPA and 20:3n-3. Retention of the n-6 PUFA were also affected, with 20:4n-6 a notable case. Retention of many of the elongation and desaturation products (e.g., 18:3n-6 and 20:2n-6) from 18:2n-6 were affected, but notably not 18:2n-6 itself. These observations are consistent with a significant level of elongation and desaturation activity in this species (Mock et al., 2019).

Interestingly retentions of some of the MUFA were also affected by diet, with 20:1n-9 and 22:1n-9 being two notable examples. Both fatty acids were better retained in the Ln3 diet fed fish. Interestingly both fatty acids are elongation products from 18:1n9, the most abundant fatty acid supplied in the diets, despite that the levels in each of the experimental diets were relatively similar. This seems to suggest that in the absence of n-3 LC-PUFA the fish are elongating not just other n-3 and n-6 fatty acids, but also MUFA.

4.3. Responses to the interaction

An important element to the design of the present study is its factorial nature, thus allowing for exploration of interaction effects between key parameters. However, it should be noted here that this factorial nature only extends to the capacity to determine interactions

Table 7
Proximate and fatty acid retentions (%).

Ration	S	S	P	P	S	S		*P-values*				
Oxygen	90%	90%	90%	90%	70%	70%	Pooled	Diet	Oxygen	Ration	D x O	D x R
Diet	Ln3	Hn3	Ln3	Hn3	Ln3	Hn3	SEM					
Protein	44.3%	42.0%	42.3%	41.6%	42.9%	42.8%	0.6%	0.451	0.997	0.615	0.543	0.653
Lipid	63.0%	57.7%	57.0%	56.1%	53.6%	58.6%	1.0%	0.802	**0.050**	0.073	**0.020**	0.274
Energy	43.1%	39.4%	39.7%	38.4%	38.2%	40.2%	0.6%	0.387	0.204	0.173	0.070	0.425
14:0	55%	48%	47%	47%	40%	47%	1.7%	0.893	0.051	0.211	0.079	0.379
16:0	58%	53%	54%	52%	46%	54%	1.3%	0.882	0.083	0.382	**0.044**	0.499
18:0	65%	59%	59%	59%	54%	62%	1.5%	0.783	0.280	0.407	0.093	0.482
20:0	41%	38%	38%	39%	35%	41%	0.9%	0.353	0.364	0.578	0.090	0.402
22:0	39%	36%	36%	39%	34%	39%	0.9%	0.301	0.637	0.871	0.103	0.299
16:1n-7	59%	47%	50%	47%	44%	49%	1.6%	0.288	0.060	0.240	0.026	0.226
18:1n-9	55%	50%	51%	51%	46%	53%	1.1%	0.672	0.246	0.613	0.060	0.397
18:1n-7	57%	54%	52%	54%	47%	55%	1.3%	0.365	0.146	0.438	0.064	0.353
20:1n-11	51%	44%	42%	46%	42%	46%	1.5%	0.902	0.375	0.382	0.214	0.185
20:1n-9	143%	103%	122%	106%	111%	106%	4.4%	**0.010**	0.099	0.292	0.053	0.175
22:1n-11	38%	35%	28%	38%	30%	34%	1.6%	0.249	0.276	0.405	0.441	0.141
22:1n-9	109%	79%	98%	86%	86%	82%	3.0%	**0.003**	0.058	0.646	**0.020**	0.086
24:1n-9	82%	70%	70%	75%	65%	73%	1.8%	0.965	0.108	0.450	**0.025**	**0.049**
18:2n-6	47%	45%	44%	45%	40%	46%	1.0%	0.428	0.202	0.575	0.080	0.417
20:2n-6	448%	983%	382%	1078%	327%	1048%	81.3%	**0.000**	0.573	0.760	0.072	0.114
20:4n-6	85%	48%	81%	47%	82%	49%	4.4%	**0.000**	0.759	0.527	0.607	0.763
18:3n-3	38%	39%	36%	39%	32%	40%	0.9%	**0.016**	0.170	0.607	0.063	0.468
18:4n-3	230%	76%	227%	76%	216%	83%	18.1%	**0.000**	0.733	0.894	0.350	0.891
20:4n-3	545%	217%	497%	233%	438%	238%	34.0%	**0.000**	0.106	0.528	**0.023**	0.214
20:5n-3	21%	26%	32%	26%	30%	25%	2.5%	0.777	0.521	0.427	0.485	0.438
22:5n-3	116%	86%	103%	87%	99%	87%	3.4%	**0.003**	0.212	0.383	0.175	0.289
22:6n-3	102%	67%	88%	65%	82%	67%	3.7%	**0.000**	0.062	0.120	0.053	0.214
Σ SFA	59%	53%	54%	53%	47%	54%	1.4%	0.940	0.113	0.372	0.058	0.485
Σ MUFA	56%	51%	52%	52%	47%	54%	1.2%	0.778	0.220	0.573	0.060	0.371
Σ scPUFA	45%	43%	43%	43%	38%	44%	0.9%	0.458	0.182	0.597	0.063	0.447
Σ lcPUFA	104%	61%	99%	62%	91%	63%	5.0%	**0.000**	0.398	0.749	0.296	0.649
Σ n-3	54%	49%	50%	50%	45%	51%	1.1%	0.630	0.274	0.687	0.138	0.594
Σ n-6	49%	45%	47%	45%	42%	46%	1.0%	0.847	0.166	0.583	0.052	0.363
Σ Fatty Acids	55%	50%	51%	51%	46%	52%	1.1%	0.980	0.188	0.538	0.064	0.412

S: Satiety fed, P: Pair fed. SFA: saturated fatty acids. MUFA: monosaturated fatty acids. scPUFA: short-chain polyunsaturated fatty acids. lcPUFA: long-chain polyunsaturated fatty acids. P-values shown are those for the incomplete MANOVA analysis. Bolded are those values significant at $P < 0.05$.

between hypoxia and diet, and ration and diet, because it was illogical to include treatments that were pair-fed and hypoxic, and therefore it is not possible to examine effects of hypoxia and ration.

When we examine for interaction effects across the study, we note that there were no significant interaction effects observed for any of the phenomic response parameters like feed intake, growth, or FCR (Table 4). Only a single significant effect of diet x oxygen on lipid levels in the whole body of the fish was noted among those primary physical response parameters. However, interaction effects were far more prominent for their impact on whole body fatty acid composition. Among the fatty acid classes, both MUFA, and n-6 classes had significant interaction effects but not the SFA, LC-PUFA or n-3 fatty acid classes. The lack of a n-3 and LC-PUFA class effect is interesting, because at an individual fatty acid level, several of the n-3 fatty acids did register with interaction (D x O) effects. Notably interaction effects were seen for 18:3n-3, 20:3n-3 and 20:4n-3. The interaction effect within the MUFA was also interesting with an interaction effect on 18:1n-9, 22:1n-9 and 24:1n-9 also reported significant interactions, but not 20:1n-9. This appears to implicate a greater effect on the elongation MUFA, and n-3 fatty acids being influenced by the interaction between diet and oxygen level. These observations are consistent with those of Einen et al. (1999), who noted that during short-term starvation an increase in the levels of MUFA occurred in the muscle, although not the liver.

Lipid retention efficiency was strongly affected by the interaction between diet and oxygen, with a range of specific fatty acids underpinning that effect. While trends were seen for interaction effects for SFA, MUFA, SC-PUFA and n-6 classes, significant interaction effects were seen for 16:0, 16:1n-7 and 22:1n-9. Given what was observed with the whole-body fatty acids, these effects further support the notion that the elongation of MUFA, and some other fatty acids, is influenced by the interaction between diet and oxygen level, and that this is seen as not only an effect of total levels, but also the retention efficiency.

4.4. What controlling for intake tells us

As anticipated a range of phenomic responses were affected by ration. Indeed, this pairing of the ration related treatments to the anticipated feed intake suppression due to the hypoxia effect, provides a clear strength in the present study as it allows for clear isolation of the observed intake and diet composition related effects, which has been absent from most of the other studies examining hypoxia related effects (Huyben et al., 2021a). However, the observation that growth in the present study responded to feed intake in such a strong and direct linear fashion with an R^2 value >0.95 implies that virtually all the observed growth effects were related to feed intake. This is further reaffirmed by the observation that the clear sub- and supra-optimal level differences in omega-3 LC-PUFA had no impact on feed conversion when feed intake was controlled. A further notable issue is that when the feeds were pair-fed to the same level as the low-oxygen treatments virtually no growth or conversion effects were seen between the two diets. This lack of growth response to omega-3 LC-PUFA is consistent with earlier observations on the impacts of these nutrients on Atlantic salmon when intake effects are accounted for, where other studies have shown that growth in this species is relatively insensitive to omega-3 LC-PUFA, especially when SC-PUFA are optimised (Glencross et al., 2014; Mock et al., 2019; Ruyter et al., 2022). Other studies have also shown that salmonids exhibit some degree of neural response to feed intake with the presence of certain fatty acids in their diet (Velasco et al., 2016, 2017). The specific relationship between LC-PUFA and feed intake and its neural responses requires further exploration.

Earlier studies had claimed that omega-3 fatty acids provided significant benefits to Atlantic salmon when the fish were reared under challenging conditions in cages (Bou et al., 2017). A more detailed consideration of that study does shows that the fish in the cage fed the higher level of omega-3 performed better, with fewer mortalities, but the effect of diet cannot be isolated from cage effects due to the lack of replication. More recently, additional studies have demonstrated that higher levels of omega-3 LC-PUFA are consistent with a range of production benefits in Atlantic salmon, including growth, welfare, robustness, and fillet quality among others (Bou et al., 2020; Lutfi et al., 2023). However, consistent with the present study, those studies also suggest that these effects are intake linked.

5. Conclusion

In conclusion, the results from this study builds on the findings from Huyben et al. (2021b), but crucially now includes controls for the feed intake effects to clarify the roles of hypoxia on the nutrient and intake mediated effects. As a result of these controls, it is now clearer that the majority of the phenomic responses to omega-3 LC-PUFA in Atlantic salmon are intake-linked and their benefits when used in diets in challenging environmental conditions, like hypoxia, are mostly driven by those effects on feed intake.

Ethical approval

This experiment was approved by the Animal Welfare and Ethical Review Body (reference AWERB-18/19–008) of the University of Stirling in accordance with the UK Home Office regulations under the Animals (Scientific Procedures) Act 2013.

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Consent for publication

All co-authors have seen and agreed to publish this manuscript.

CRediT authorship contribution statement

Brett D. Glencross: Conceptualization, Writing – original draft, Investigation, Formal analysis, Data curation, Project administration. **Alex Berry:** Writing – review & editing, Investigation, Formal analysis. **Ben Clokie:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Ernst Hevroy:** Writing – review & editing. **David Huyben:** Writing – review & editing, Investigation, Formal analysis. **Laura Martinez-Rubio:** Writing – review & editing. **Chessor A. Mathew:** Writing – review & editing. **Pedro Munoz:** Writing – review & editing, Investigation, Data curation. **Simon MacKenzie:** Conceptualization, Writing – review & editing, Investigation, Project administration. **Rod W. Wilson:** Conceptualization, Writing – original draft, Investigation, Formal analysis.

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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