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Can improved nutrition for Atlantic salmon in freshwater increase fish robustness, survival and growth after seawater transfer?

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ABSTRACT

The loss of fish in the seawater (SW) phase of Atlantic salmon farming is high, and a major proportion of this loss occurs in the period just after SW transfer. In the current study, we hypothesize that improvements made to the diet during the freshwater (FW) stage affect fish growth, survival and robustness later in the SW stage. To test this, salmon parr were fed five experimental diets in FW at 12 °C. In addition to a commercial-like control diet, fish were fed a diet with changed FA composition aimed to be more like the natural feed of salmon in FW, a diet with increased concentrations of selected AA/N-compounds (methionine, lysine, threonine and taurine), a diet with increased concentrations of methionine and certain B-vitamins (folate, B_{12} and B_6) and a final diet combining all of these potential improvements. At the time of SW transfer, the robustness of fish fed the different diets was tested by direct transfer to SW at three different temperatures (8, 12 and 16 °C, without prior acclimation), as well as transfer into open net pens, while fed on a common commercial diet. Growth and proximate composition of the fish did not differ between the diet groups. All diet groups seemed to handle transfer to SW well, and while SW transfer elicited a stress response in the fish, this was not significantly different between diet groups. Fish transferred to SW at 8 °C had higher mortality, reduced mucus layer and increased prevalence of scale loss and wounds, but this applied to all diet groups. Hence, direct transfer to SW at a lower temperature than the fish has been acclimated to cannot be recommended. At the two highest temperatures, there were some differences between the groups in the severity of cataracts. Apart from this, none of the health- or welfare related parameters measured showed any difference between the diet groups, indicating that the control diet was already sufficient.

1. Introduction

Atlantic salmon (*Salmo salar* L.) has a life-history pattern with spawning and juvenile stages in freshwater (FW), followed by the parr-smolt transformation and migration to sea. In Norwegian salmon aquaculture, the total loss during the seawater (SW) phase is about 16–17% (Sommerset et al., 2020). A high proportion of this loss occurs just after SW transfer, indicating poor smolt quality and sub-optimal conditions in the FW phase (Hjeltnes et al., 2016). Improving the nutritional status of the smolt by optimizing the feed in the FW phase could result in a more robust smolt that handles the transfer to SW better. Compared to SW, relatively small amounts of feed are required in the FW phase. Hence, minor investments made in enhancing the feed in the FW phase could potentially result in improved fish welfare, reflected in increased growth and survival in later production stages.

Nutrient requirement studies have generally been conducted under highly controlled experimental conditions, where stress for the fish is reduced as much as possible, while environmental factors are kept constant and optimal. This is very different from what the fish experiences in a commercial aquaculture setting, with stress related to transport and handling, fluctuating environmental conditions, disease pressure etc. As has been shown for EPA and DHA requirement for Atlantic salmon in the SW phase, suboptimal nutrient levels may not be revealed under highly controlled experimental conditions, but can lead

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to high levels of mortality when such fish are challenged by stressful conditions (Sissener et al., 2016; Bou et al., 2017). Hence, the published nutrient requirements for salmonids may not always be sufficient in farming conditions. SW transfer per se is a stressful event for the fish, and early mortalities in the SW phase may be related to problems with the adaptation to SW (Hjeltnes et al., 2016). It has been shown that the FW diets could affect stress response during SW transfer (Jutfelt et al., 2007). Brain serotonin (5-HT) is central in stress coping (Puglisi-Allegra and Andolina, 2015) and changes in 5-HTergic neurochemistry is often used as an indicator of stress in fishes and other vertebrates (Winberg and Nilsson, 1993), in addition to more classical stress markers such as plasma cortisol. Furthermore, in response to stress and disease, organisms often induce redox regulation, get lowered total glutathione (GSH) concentration and become more oxidized (Estensoro et al., 2011; Lygren et al., 2000; Larsson et al., 2012). Therefore, GSH metabolism and redox signaling was used for studying dietary effects of health and welfare in the current study. Together with other factors, suboptimal nutrition may cause production related health disorders, including cataracts and bone deformities (Hansen et al., 2010; Bjerkås et al., 2006). Previous studies indicate that FW diet may predispose the fish for later cataract development (Waagbø et al., 2004; Sissener et al., 2016), while bone deformities can occur at all life stages of the fish (Fjelldal et al., 2012). All of these factors go into what we consider to be a robust fish; healthy and able to handle various physical and environmental stressors and disease challenges.

There are a number of mechanisms by which the FW diet may affect the parr-smolt transformation and SW performance. Successful accomplishment of the parr-smolt transformation depends on fish nutritional status and energy turnover above a certain level (Thorpe et al., 1998), and specific nutrients and dietary constituents can affect osmoregulatory ability after SW transfer (Bell et al., 1997; Tocher et al., 2000; Perry et al., 2006). This could, in turn, affect how long it takes for the fish to regain appetite and resume growth after transfer. Furthermore, nutrient status of the fish will reflect the FW diets for quite some time after SW transfer, as a 7-fold increase in body weight was necessary to equilibrate fish tissues to the dietary composition of certain fatty acids (FA) (Rosenlund et al., 2016).

Commercial diets used in the FW phase are generally high in fish meal and fish oil and hence in the long-chain n-3 FA eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). While this has been common practice for many years, it is very different from the natural diet of wild salmon at this stage consisting of FW invertebrates, with the main differences being higher arachidonic acid (ARA, 20:4n-6), higher 18:3n-3 and less DHA in the diet of wild fish (Bell et al., 1994; Ghioni et al., 1996). ARA, in particular, may play an important role in the parr-smolt transformation, as the content of this FA nearly doubled in liver and gills of salmon in the weeks leading up to SW transfer (Bell et al., 1997). Using vegetable oils (which share some of the characteristics of FW invertebrates in comparison to fish oil) has given some positive results, including reduced plasma chloride in SW challenge tests in salmon smolt fed rapeseed and linseed oil compared to fish oil, indicating an improved ability to osmoregulate (Bell et al., 1997; Tocher et al., 2000).

As building blocks for peptides and proteins, and taking part in the nucleotide synthesis and DNA methylation, vitamins and the amino acid (AA) composition of the feed plays an important role during periods of rapid growth (Andersen et al., 2016; Wu, 2010; Xu et al., 2016). Especially methionine and the B-vitamins folate, B6 and B12, are important because they take part in biological methylation reactions including epigenetic regulation of gene expression (Saito et al., 2020). Thus, deficiencies of these dietary compounds during sensitive developmental stages, such as the parr-smolt transformation, can have long-term phenotypic effects. The main plant ingredients used in diets for cultured salmon is soy protein concentrate and pea protein concentrate. These protein ingredients are generally low in methionine, taurine, threonine, lysine and B vitamins, and these nutrients need to be added in the diet. In a recent EU-project (ARRAINA), the practical requirements of the B-vitamins folate, B12 and B6 were found to be considerably higher than what has been determined previously (Hemre et al., 2016; NRC, 2011), and adding extra methionine increased the growth potential after SW transfer (Espe et al., 2020). We hypothesize that the currently given requirements are not adequate for methionine, taurine, threonine, lysine and B-vitamins in the FW stage of Atlantic salmon.

The main aim of the current study was to investigate if a changed dietary composition compared to a commercial-like control diet during the FW stage of Atlantic salmon affected robustness, growth performance and survival in the SW stage. To achieve this, salmon parr were fed five experimental diets; a commercial-like control diet, a diet with changed FA composition aimed to be more like the natural feed of salmon in FW, a diet with increased levels of selected AA, a diet with increased levels of methionine and certain B-vitamins and a final diet combining all of these potential improvements compared to the commercial diet. The robustness of fish fed the different diets was tested by direct transfer to SW at three different temperatures (8, 12 and 16 °C, without prior acclimation), as well as transfer into open net pens where fish would be exposed to varying environmental conditions, with assessments of growth performance and markers of fish health and welfare.

2. Materials and methods

2.1. Fish stock and rearing conditions

The study was conducted with Atlantic salmon from the AquaGen strain (Aquagen Atlantic QTL InnOva Prime, AquaGen AS, Trondheim, Norway) at Matre Research Station at the Institute of Marine Research (IMR) in Matre, Bergen, Norway. The eye eggs arrived at Matre on 12 January 2018 and were 369 dg old at arrival. Hatching took place between 06 and 12 February, and first feeding was on 05 April. The eggs were incubated in trays in a flow through system, and the fry were first fed in 3 m tanks. Water temperature was <8C during egg incubation and stable around 12C during first feeding. The fish were fed commercial salmon diets (Skretting AS, Stavanger, Norway) with pellet size increasing according to fish size. On 02 August, all the fish ($n = 2475$, ~15 g) were individually tagged with microtransponders (Glass tag 2, 12 mm, TrackID AS, Stavanger, Norway) and randomly distributed between 15 fiberglass tanks (0.95 × 0.95 m, 0.4 m water depth, 360 l) and allowed to acclimatize for 3 weeks before the start of the study period. The study involved three separate experiments. First a freshwater feeding trial (Exp 1), followed by a temperature study in seawater tanks (Exp 2) and a sea-cage study (Exp 3). The two latter with the former FW feed groups in common garden. Fig. 1 shows environmental data including light regime, rearing temperature and salinity for the different parts of the experiment. The trial was conducted according to the guidelines of the Norwegian State Commission for Laboratory Animals. The National food safety authorities approved the protocol (identification number: ID 15465).

2.2. Freshwater feeding trial (exp 1)

Five experimental diets were formulated for salmon to be fed in the freshwater (FW) phase. The control diet was aimed to be as similar as possible to the average composition of a commercial diet for Atlantic salmon at this life stage, based on data available from the surveillance of Norwegian fish feeds conducted by the Institute of Marine Research (Bergen, Norway). In the four remaining diets, either the FA profile was changed (FA diet), selected AA were added at increased levels (AA diet), some B-vitamins and methionine were increased (B-vitamin diet), or all of these changes were implemented together (combined diet). Added AA and B-vitamins in the three latter diets were balanced with wheat, while the control diet and the FA diet only differed in the oil blend. Feed formulations can be found in Table 1, and are given for the 2 mm pellets.

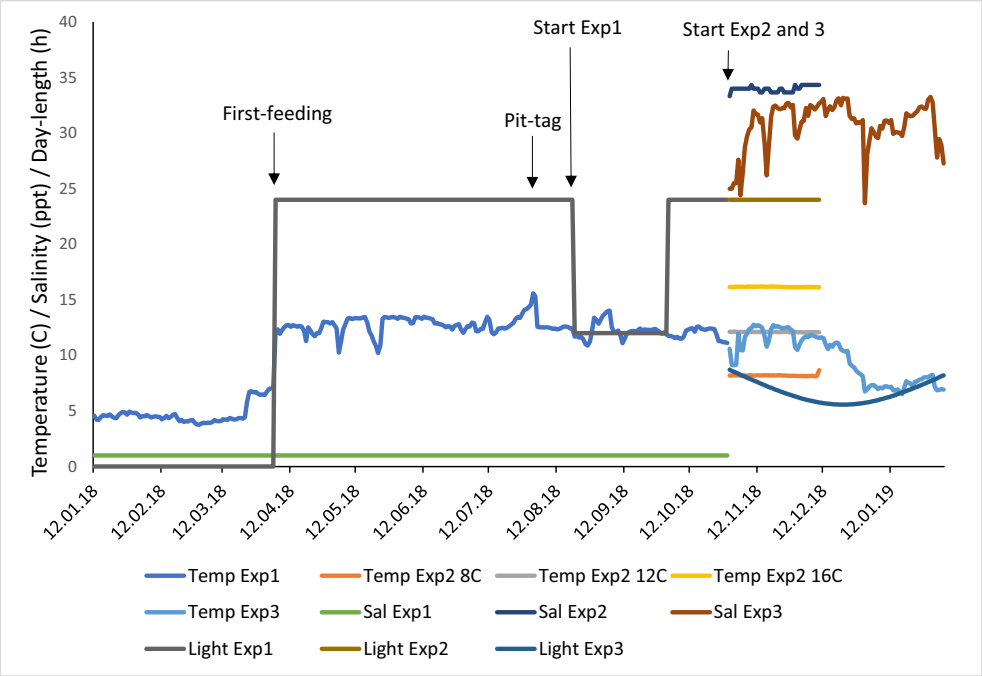

Fig. 1. Environmental conditions during the different parts of the experiment.

Table 1
Diet formulation, g 100 g^{-1} of the five experimental diets used for Atlantic salmon in the late freshwater phase leading up to seawater transfer.

	Control	FA	AA	B-vit	Combined
Wheat[1]	18.9	18.9	16.5	17.9	16.5
Wheat gluten[2]	3.5	3.5	3.4	3.7	3.4
Soy protein concentrate[3]	26.5	26.5	26.5	26.5	26.5
Pea protein concentrate[4]	9.0	9.0	9.0	9.0	9.0
Fish meal[5]	25.0	25.0	25.0	25.0	25.0
Rapeseed oil[3]	4.6	4.0	4.6	4.5	3.9
Fish oil[6]	10.0	0	10.0	10.0	0.0
Palm oil[7]	0.0	2.7	0.0	0.0	2.8
Linseed oil[8]	0.0	2.4	0.0	0.0	2.4
EPA concentrate[9]	0.0	4.3	0.0	0.0	4.3
ARA concentrate[10]	0.0	1.2	0.0	0.0	1.2
Water	0.72	0.72	1.01	0.84	1.01
Taurine[11]	0.13	0.13	0.33	0.13	0.33
DL-Methionine[12]	0.00	0.00	0.68	0.67	0.68
L-Lysine[13]	0.64	0.64	1.30	0.64	1.30
L-Threonine[14]	0.06	0.06	0.59	0.07	0.59
Vit B6[14]	0.01	0.01	0.01	0.02	0.02
Vit B12[14]	0.01	0.01	0.01	0.04	0.04
Folate[14]	0.00	0.00	0.00	0.01	0.01
NRC vitamin premix[14]	0.10	0.10	0.10	0.10	0.10
Mineral premix[14]	0.10	0.10	0.10	0.10	0.10
Yttrium premix[15]	0.10	0.10	0.10	0.10	0.10
Astaxanthin 10%[16]	0.05	0.05	0.05	0.05	0.05
Other microingredients	0.71	0.70	0.74	0.72	0.72

[1] Fiskå mølle, Forus, Norway [2]Cargill Nordic AS, Søborg, Denmark [3]European Commodity Company S.A., Luxembourg, [4]AM Nutrition, Stavanger, Norway, [5]Norsildmel, Bergen, Norway [6]P/F Havsbrun, Fuglafjørdur, Faroe Islands, [7]Aarhus Karlshamn, Sweden, [8]Linagro, Lichtervelde, Belgium, [9]Incromega, Croda Health Care, [10]Cabio Biotech, Wuhan, China, [11]Orffa, Bornem, Belgium, [12]Evonic Nutrition and Care GmbH, Essen, Germany, [13]Meihua Group International Trading, Hong Kong, [14]Trouw Nutrition Netherlands BW, Putten, Netherlands, propriety of Skretting ARC, [15]Trouw Nutrition Netherlands BW, Putten, Netherlands, the mix provides yttrium oxide in a concentration of 100 mg/kg feed, while the product also contains sepiolite (E562, 472.1 g/kg yttrium mix), calcium (109.1 g/kg), magnesium (64.9 g/kg), sodium (2.8 g/kg), potassium (4.5 g/kg), iron (6.8 g/kg), moisture (149.2 g/kg), [16]Divi's laboratories Europe B·V, Basel, Switzerland.

In addition, 3 mm pellets were also produced, and were practically the same, apart for small adjustments in protein and lipid content according to fish size following the standard for commercial feeds. All feeds were formulated and produced by Skretting ARC (Stavanger, Norway). While the control diet contained fish oil and rapeseed oil, the FA diet contained rapeseed oil, linseed oil and palm oil, and was supplemented with EPA-concentrate and ARA-concentrate. The AA diet had increased concentrations of methionine, lysine, threonine and taurine, while the B-vitamin-diet had additional vitamin B_6, vitamin B_{12}, folate and methionine. In the combined-diet, all of these changes from the control diet were implemented, including a modified FA composition, added AA and added B-vitamins. The fish meal level was kept constant at 25% of the recipe in all feeds.

Starting on 20 August, the five experimental feeds were fed to fish in triplicate tanks ($n = 165$ per tank). The fish had an initial weight of 36.0 g (SD 4.3), and the period with different feeds lasted for 10 weeks until 29 October 2018. The fish had been reared under continuous light from first feeding until 20 August, and during the feeding trial the fish were reared under a LD12:12 photoperiod for 6 weeks (20.08–01.10) followed by a LD24:0 photoperiod for 4 weeks (01.10–29.10) to induce the parr-smolt transformation. The water flow was adjusted so that the oxygen saturation of the outlet water was always above 80%. The temperature was kept constant at 12 °C. Fish were fed during the light period in LD12:12, and continuously under the LD24:0 regime.

On 29 October, at the end of the FW period, a sampling was conducted. Fish were anesthetized in a bath with tricaine methanesulfonate (FINQUEL MS-222, 0.1 g/l) before registration of the pit tag. Body weight and length was recorded for all sampled fish. For the first 6 fish sampled per tank, blood was collected from the caudal blood vessel by a heparinized syringe, and centrifuged at 13200RPM for 2 min, before collection of plasma. Furthermore, liver, heart and the whole viscera were weighted, and pooled samples of liver and muscle (NQC) were collected and flash frozen. The next 6 fish sampled per tank were homogenized for a pooled whole body fish sample. Cataract status was recorded for 8–12 fish per diet group per tank, before collection of organs/homogenization of whole fish. Cataract was investigated using a Heine HSL 150 hand-held slit lamp (HEINE Optotechnik GmbH & Co. KG, Herrsching, Germany). Each lens was given a score of 0–4,

indicating the degree of opacification, giving in a total score of 0–8 per fish, according to Wall and Bjerkas (1999). When the sampling was finished, the remaining fish were distributed between Exp 2 and Exp 3.

2.3. Seawater transfer and temperature trial (exp 2)

After the FW feeding trial, fish were transferred to SW (salinity 34 ppt) at three different temperatures, 8, 12 and 16 °C, without prior acclimatization. From each diet group 24 fish were transferred to each temperature in triplicate tanks (diet groups were mixed), and sampled after 1 h or 24 h in SW, respectively (12 fish per diet group and time point). Different tanks were used for the fish that were sampled after 1 h and the fish that were sampled after 24 h, to avoid additional stressing of the 24 h fish during the 1 h-sampling. Fish destined for each tank were transferred from FW to SW at set time intervals, to allow for sampling exactly 1 or 24 h after transfer for each tank. At the samplings, fish were anesthetized as described above, weight, length and pit tag were recorded, blood samples were taken and centrifuged for collection of plasma, and liver and muscle samples were collected, as described above. Fish were not fed during these 24 h.

Furthermore, 72 fish from each diet group were transferred to full-strength SW at three different temperatures (8, 12 and 16 °C) in duplicate (2 tanks per temperature, 12 fish from each diet group in each tank). These fish were fed a common commercial diet (Skretting Supreme) for 6 weeks. Salinity was 34 ppt and flow was adjusted so that water oxygen saturation was always above 80%, photoperiod was LD24:0. There was continuous, *ad libitum* feeding of the fish. At the final sampling from this part of the trial, 5 fish per diet group per tank were used for organ sampling (pooled samples of liver and muscle) and weighing of liver and heart, while another 5 were homogenized for pooled whole fish samples. For all ten fish, weight and length was recorded, and they were screened for cataract as described above and X-rayed for vertebrae deformities. Fish were radiographed with a Direct Radiology System (Canon CXDI-410C Wireless, CANON INC., Kawasaki, Japan) using a portable x-ray unit (Portable X-ray Unit Hiray Plus, Model Porta 100 HF, JOB Corporation, Yokohama, Japan) at 88 cm distance with 40 kV and 10 mAs. Each fish was evaluated for vertebra deformities (Witten et al., 2009), and type and location were recorded.

2.4. Sea cage trial (exp 3)

Fish destined for the sea cages were vaccinated (PharmaqMicro 6) 3 weeks prior to SW transfer. For animal welfare reasons, it is not legal to transfer unvaccinated fish to open net pens in the sea, while we wanted to avoid vaccinating all fish (pit-tags were used to separate vaccinated from unvaccinated fish when fish were distributed to the different parts of the experiment at the time of SW transfer). From each diet group, 90 fish were transferred to SW (34 ppt) in 1 × 1 m tanks (one tank per diet group) at 9 °C for 4 days, before transfer to sea cages. Three sea cages of 5 × 5 m (7 m depth) were used, each containing a mix of fish from all the original FW diet groups. The fish were fed with the same commercial diet (Skretting Supreme) as in Exp 2, but under natural photoperiod and water temperature. Environmental data in the cages were measured at 5 m depth. The water temperature decreased during the experimental period, with an average temperature of 12.1 °C (SD 0.7) in November, 10.6 °C (SD 1.2) in December and similar temperature in January and February averaging 7.3 °C (SD 0.4). Salinity was similar throughout the period, with an average value of 30.9 ppt (SD 1.9). On January 24th (two weeks before the final sampling), pumping of all fish was conducted to mimic the situation in commercial aquaculture, with frequent handling of the fish for delousing etc. A Vaki fish pump was used, and the fish was crowded in a small part of the net pen before being pumped through a 30 m hose and back into the net pen. Travelling through the hose took approximately 20 s per fish (with some individual variation depending on how much the fish tried to swim against the current), while pumping of an entire net pen took ~12 min.

The net pen trial was terminated with a final sampling February 5th 2020, 14 weeks after SW transfer. At the final sampling, pit-tag, weight and length of all fish were recorded, welfare scoring (including evaluation of skin condition, fin condition, eye status, mouth/jaw wounds and sea lice), Speilberg (scoring for vaccine side-effects) and cataract scoring was conducted on 50 fish per diet group, liver was weighed and organ samples were secured for 18 fish per diet group.

2.5. Analysis of diets

Dry matter and ash were measured gravimetrically after drying at 104 °C for 24 h and flame combustion at 550 °C for 16–18 h, respectively. Nitrogen was measured with the nitrogen analysator Vario Macro Cube, according to AOAC official methods of analysis (AOAC, 1995) and protein calculated as N x 6.25. Total fat in the feeds was measured gravimetrically after acid hydrolysis and extraction with diethyl ether. Energy was determined by a bomb calorimeter. Total amino acids were determined after hydrolysis in 6 N HCl at 22 °C using UPLC (ultra performance liquid chromatography) method as described (Andersen et al., 2013) and fatty acids by GLC (gas liquid chromatography) (Lie and Lambertsen, 1991). The B-vitamins folate and cobalamin were determined by microbiological methods (AOAC, 1996; Mæland et al., 2000), while vitamin B_6 was determined by HPLC (CEN, 2002).

2.6. Analysis of fish samples

The content of lipid, protein and dry matter in whole fish homogenates were analyzed as described for the feeds above. Liver samples were analyzed for relative and absolute amounts of lipid classes by high performance thin layer chromatography (HPTLC), identified by comparison to commercially available standards and quantified by scanning densitometry, as previously described (Torstensen et al., 2011). HPLC was used for determination of ascorbic acid according to (Mæland and Waagbø, 1998) and tocopherols according to (CEN, 1999). For the analysis of total (tGSH) and oxidized (GSSG) glutathione, supernatants were prepared from samples using a commercial kit (Prod. No. GT40, Oxford Biomedical Research, Oxford, UK), before analyzed at 405 nm in a microplate reader (iEMS Reader Ms., Labsystems, Finland) as described by Hamre et al. (2014). Plasma chloride was measured with a Sherwood Chloride Analyser 926 (Sherwood Scientific Ltd., Cambridge, UK).

2.7. Cortisol and 5-HT neurochemistry

For practical reasons, plasma cortisol was analyzed by two different laboratories with different methodology. The samples collected before SW transfer and 1 h after SW transfer were analyzed by IMR (all diet groups), while the samples collected 24 h after SW transfer from the control and combined group were analyzed by NIVA. Hence, data from 24 h after SW transfer are not directly comparable to the two other time points. At IMR, cortisol was extracted with ethyl acetate from 100 μl of plasma (Pankhurst and Carragher, 1992). Plasma cortisol was quantified by enzyme-linked immunosorbent assay (ELISA) (Cuisset et al., 1994). Anti-cortisol, acetylcholine esterase-labelled tracer and microplates precoated with monoclonal mouse antirabbit IgG were supplied by Cayman Chemicals (USA). Standard cortisol was purchased from Sigma-Aldrich (Sigma reference standards). At NIVA, plasma cortisol concentration was analyzed using a commercially available DetectX® cortisol enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) following the manufacturers protocol. The absorbance of the prepared ELISA plate was read in a plate reader at 450 nm and the concentrations were calculated using the four-parameter logistics curve.

Serotonergic activity was analyzed by NIVA. The frozen brain samples (brain stems) were homogenized in 4% (*w/v*) ice-cold perchloric acid containing 0.2% EDTA. Prior to analysis, the samples were thawed on ice, and centrifuged at 17,000 rpm for 5 min. The supernatant was

then removed and 5-HT, and its principal catabolite 5-hydroxtrindolaceticacid (5-HIAA) were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10 CE), equipped with an auto injector (Famos, Spark), a reverse phase column (4.6 × 100 mm, Hichrom, C18, 3.5 μm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at −40 and + 320 mV. A conditioning electrode (ESA 5020) with a potential of +400 mV was employed before the analytical electrodes, to oxidize possible contaminants. The mobile phase consisted of 86.25 mM/L sodium phosphate, 1.4 mM/l sodium octyl sulfate and 12.26 μM/l EDTA in deionized (resistance 18.2 MW) water containing 7% ACN brought to a pH of 3.1 with phosphoric acid. The samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd., Czech Republic). Generally, the 5-HIAA/5-HT ratio is used as a reliable proxy for determining monoamine activity/signaling (Höglund et al., 2019). In the present study, the aforementioned ratio was used for quantifying 5-HT activity i.e. synaptic release and reuptake.

Cortisol from all dietary treatment groups sampled prior to and 1 h after SW transfer were initially analyzed. Since there was no significant effect of diet at these time points (see statistical analyses and results), further analysis was limited to two diet groups (control and combined diet) and temperature (8, 12 and 16 C°) 24 h after SW transfer. Likewise, brain 5-HT neurochemistry was only analyzed in these two dietary treatment groups in samples withdrawn prior to, and 1 and 24 h after SW transfer.

2.8. Calculations

Specific growth rate (SGR, the rate of increase in growth), feed conversion ratio (FCR) and organ indices (organ weight in percentage of total body weight) were calculated according to standard formula.

2.9. Statistical analysis

Most of the statistical analyses were conducted with the software Statistica (Version 11; Statsoft, Tulsa, OK, USA). From the sampling at the end of FW, standard ANOVA was used for all data available on a tank basis/pooled samples from each tank, while nested ANOVA (with tank as the random factor) was used for data measured on individual fish. For samplings in SW, potential tanks effects would be expected to be cancelled out by all diet groups being present in each tank, but such effects were still tested for. For the temperature trial, two-way ANOVA was used, with diet and temperature as the categorical factors. Assumptions of normality and homogeneity of variance were tested. *P*-values <0.05 were considered statistically significant, and were followed up by Tukey HSD post-hoc test. Cataract scores were log transformed to meet assumptions for two-way ANOVA 6 weeks after SW transfer. Additionally, Mann-Whitney *U* test was used to compare the cataract scores of experimental diets vs. control diets within each rearing temperature six weeks after SW transfer. Analyses of plasma cortisol and brain stem 5-HT analyses were performed in R (version 3.6.2), using Rstudio (version 1.2.5033). For all models, outliers (observations with standardized residuals greater than ±2.5) were removed and checks were done to confirm that model assumptions (linearity, homoscedasticity, normally distributed and uncorrelated errors) were reasonably met. Plasma cortisol was initially analyzed by fitting a linear mixed model to the data with plasma cortisol level as log-transformed dependent variable and diet, temperature and time point (before and 1 h after SW transfer) as independent variables. There were in some cases considerable tank effects, thus tank identity was used as random variable in the linear mixed model. An additional linear mixed model was fit with plasma cortisol level as log-transformed dependent variable and diet, SW temperature (8, 12 and 16 °C) and treatment (prior to, 1 h after or 24 h after SW transfer) as independent variables. 5-HIAA/5-HT ratio was analyzed by fitting a linear model using 5-HIAA/5-HT as dependent variable, and diet, SW temperature (8, 12 and 16 °C) and treatment (prior to, 1 h after or 24 h after SW transfer) as independent variables.

3. Results

All the feeds had similar levels of macro nutrients and energy, and

Table 2
Analyses of feeds. Results from 2 mm pellets, with 3 mm pellets in parenthesis. Each number is the mean of two analytical parallels. "– "means the parameter was not analyzed, assumed to be equal to the control diet for FA, AA and B-vitamin diets (with the exception that methionine was also added to the B-vitamin diet), while the combined diet was assumed to be equal to the FA diet for fatty acids, to the AA diet for amino acids and to the B-vitamin diet for B-vitamins.

	Control	FA	AA	B-vit	Combined
Proximate composition:					
Protein	46 (46)	46 (44)	46 (46)	46 (44)	47 (46)
Lipid	19.0 (19.0)	18.3 (18.8)	18.8 (18.6)	18.8 (18.7)	19.0 (18.9)
Energy, kJ g^{-1}	21.8 (21.7)	21.7 (21.3)	21.7 (21.6)	21.8 (21.5)	21.7 (21.8)
Ash	6.2 (6.1)	6.2 (5.8)	6.1 (6.1)	6.2 (6.2)	6.2 (6.3)
Dry matter	93 (93)	93 (91)	92 (92)	93 (92)	93 (93)
Fatty acids. % of total fatty acids:					
16:0	13.1 (12.7)	11.3 (11.0)	–	–	–
Sum SFA	19.7 (19.7)	16.3 (16.3)	–	–	–
18:1n-9	27.4 (27.3)	24.4 (24.3	–	–	–
22:1n-11	6.2 (6.9)	2.1 (1.9)	–	–	–
Sum MUFA	46.9 (45.6)	32.3 (31.2)	–	–	–
18:2n-6	10.1 (10.0)	11.7 (11.7)	–	–	–
20:4n-6 (ARA)	0.4 (0.4)	3.9 (4.2)	–	–	–
Sum n-6	10.9 (10.8)	16.5 (16.9)	–	–	–
18:3n-3	4.0 (4.1)	10.2 (10.3)	–	–	–
20:5n-3 (EPA)	5.7 (5.6)	15.8 (15.9)	–	–	–
22:6n-3 (DHA)	8.3 (7.8)	5.5 (5.5)	–	–	–
Sum n-3	20.7 (21.1)	33.9 (34.6)	–	–	–
Sum PUFA	31.8 (32.2)	50.3 (51.4)	–	–	–
			–	–	–
Amino acids, mg g^{-1}feed:					
Taurine	3.5 (3.2)	–	5.2 (5.0)	–	–
Methionine	8.5 (8.1)	–	14.6 (14.0)	-*	–
Lysine	32 (33)	–	38 (37)	–	–
Threonine	18.2 (17.6)	–	22.7 (21.8)	–	–
Histidine*	11.1 (10.2)	–	10.5 (9.9)	–	–
		–		–	–
B-vitamins, mg/kg:					
Vit B6	9.3 (9.4)	–	–	17 (19)	–
Vit B12	0.22 (0.20)	–	–	0.42 (0.37)	–
Folate	3.2 (4.1)	–	–	6.9 (8.5)	–

Sum SFA also includes 14:0. 15:0. 17:0. 18:0. 20:0. 22:0. sum MUFA also includes 16:1n-9. 18:1n-1. 18:1n-7. 20:1n-11. 20:1n-9. 20:1n-7. 22:1n-11. 22:1n-9. 24:1n-9. sum n-6 also includes 18:3n-6. 20:2n-6. 20:3n-6. 22:5n-6. sum n-3 also includes 18:4n-3. 20:4n-3. 21:5n-3. 22:5n-3. 24:5n-3. *Methionine was added at the same level in the B-vitamin diet as in the AA-diet. *Histidine was not added extra in any of the diets, but is shown because it is relevant to the cataract discussion.

differed only in the aspects planned in the experimental design (Table 2). Compared to the control diet, the FA diet had slightly less saturated FAs (SFA), less monounsaturated FA (MUFA), similar 18:2n-6, ~10-fold higher ARA, >2-fold higher 18:3n-3, ~3-fold higher EPA and less DHA. In total, the FA diet contained both more n-3 FAs and more polyunsaturated FA (PUFA). The AA diet contained more taurine, methionine, lysine and threonine than the control diet, while the B-vitamin diet contained the targeted levels of B6, B12 and folate, as well as having added methionine. Both pellet sizes were similar in composition.

At the end of the FW period, fish had more than doubled their weight while being fed the experimental diets. There was a trend towards a difference in final weights ($p = 0.08$) (Table 3). The p-values both for weight gain and SGR were higher due to small, but insignificant variations in initial weights. Condition factor was significantly different between diet groups, and there was also no significant difference for fish length ($p = 0.10$). Numerically, fish fed the FA diet were the smallest at the end of the FW period, but there were strongly significant tank effects both in final weight, weight gain, SGR and length, with especially one tank from this group performing poorly (mean final weight 72.7 g). Cataract was seen in all groups, with 100% prevalence in all groups except fish given the FA diet which had 92% prevalence. The severity of cataract (mean scores) was significantly higher in fish given the AA and combined diets, compared to fish given the FA diet, while none differed from the control and B-vitamin diet.

The size of the liver relative to the whole body (hepatosomatic index, HSI) was significantly smaller for the combined diet group compared to the control group, AA group and B-vitamin group, while the FA group was intermediate. Similarly, the viscerosomatic index (VSI) was significantly smaller in the combined group compared to the FA and B-vitamin group, while the control and AA groups were intermediate. Heart index was similar in all diet groups. Analysis of liver lipid classes (Fig. 2), showed than the combined group had the lowest amount of storage lipid (triacylglycerol, TAG) in the liver of all the diet groups, but there was large variation in the data and the combined group was only significantly different from the AA group, while the other groups were intermediate.

The proximate composition of whole body at the end of the FW feeding period was similar for all five diet groups (Table 4). There were no significant differences between the diet groups, protein had a p-value of $p = 0.16$, lipid $p = 0.26$ and dry matter $p = 0.65$, respectively.

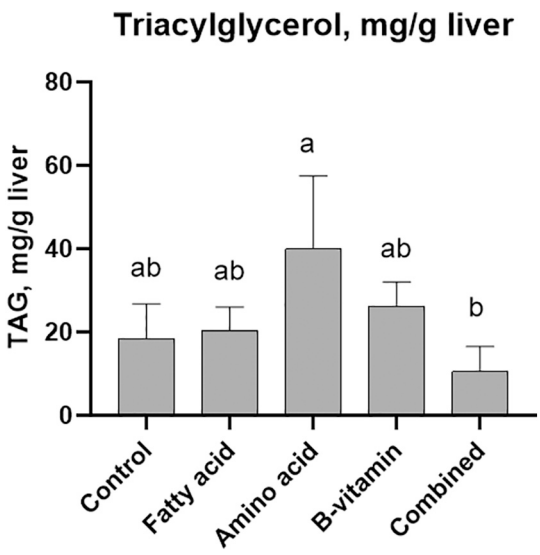

Fig. 2. Liver triacylglycerol (TAG) in Atlantic salmon from the five diet groups, at the end of the freshwater phase. The graph shows the means with standard deviation as error bars ($n = 3$, pooled samples per tank), and the letters show which groups are significantly different ($p < 0.05$).

Cortisol values before SW transfer (baseline values) displayed very high standard deviations and highly significant tank effects ($p < 0.0001$, especially two tanks from the AA diet group and one tank from the combined diet group had cortisol values that would usually be associated with acute stress). In all diet groups, there was a clear increase in plasma cortisol from before SW transfer to 1 h after SW transfer (Table 5). A linear mixed model fitted to the data revealed a significant overall effect on plasma cortisol from transfer to SW ($p < 0.0001$). There was also a significant effect of SW temperature ($p = 0.001$); fish transferred to 16 °C had significantly lower plasma cortisol 1 h after transfer than fish transferred to 8 and 12 °C. At 16 °C, the mean plasma cortisol value 1 h after SW transfer was 106 ng ml^{-1}, compared to 147 and 175 for 8 and 12 °C, respectively. After 24 h, cortisol values were no longer elevated ($p = 0.81$), and there was no difference in cortisol levels between the diets ($p = 0.96$).

There was no effects of the diet on brain 5-HIAA/5-HT ratio (Table 6), and also no effect of transfer temperature or of the transfer itself (similar values both before, 1 h after and 24 h after SW transfer).

Redox status was analyzed for all groups in the FW phase, but only in the fish fed the control and the combined diets 24 h and 6 weeks after transfer (Table 7). At the end of the FW period, none of the analyzed markers of redox status were different between the five diet groups. On the other hand, 24 h after seawater transfer there were significant differences between fish fed the two diets, with GSH being higher in muscle from fish fed the control diet in FW ($p = 0.001$). Furthermore, the redox potential (E) was lower (more reduced, $p = 0.04$) in this group. There was no effect on GSSG (Table 7) and water temperature at transfer had no impact. Muscle seemed to have similar GSH, GSSG concentrations

Table 3
Fish performance in freshwater, given as mean with standard deviation in parenthesis. Final weight, length and condition factor was measured for 90 fish per diet group, while weight gain and specific growth rate (SGR) were calculated for the same number of fish, using the individual initial weights as the fish were pit tagged. Organ indices were calculated for 18 fish per diet group. Different superscript letters show statistically significant differences ($p < 0.05$).

	Control	FA	AA	B-vit	Combined
Final weight, g	80.1 (11.2)	75.6 (10.5)	82.1 (10.9)	82.9 (11.2)	81.9 (12.0)
Final length, cm	18.5 (0.9)	18.1 (0.9)	18.6 (0.9)	18.6 (0.8)	18.6 (1.0)
Condition factor	1.26 (0.05)	1.27 (0.06)	1.27 (0.05)	1.28 (0.06)	1.27 (0.05)
Weight gain, g	43.2 (8.4)	39.7 (7.7)	46.1 (8.1)	47.0 (8.5)	46.5 (9.1)
SGR	1.15 (0.14)	1.11 (0.13)	1.23 (0.14)	1.25 (0.14)	1.25 (0.16)
HSI	0.88 (0.17)^{a}	0.77 (0.11)^{ab}	0.79 (0.15)^{a}	0.83 (0.13)^{a}	0.67 (0.08)^{b}
VSI	6.14 (0.52)^{ab}	6.41 (0.47)^{a}	6.00 (0.70)^{ab}	6.27 (0.50)^{a}	5.61 (0.61)^{b}
Heart index	0.16 (0.02)	0.15 (0.02)	0.16 (0.02)	0.15 (0.01)	0.16 (0.02)
Cataract score	2.5 (0.6)^{ab}	2.0 (0.9)^{b}	2.7 (0.9)^{a}	2.3 (0.6)^{ab}	2.7 (0.7)^{a}

Initial weight was 36.0 g (4.3) and initial length was 14.1 cm (0.5), with no significant differences between diet groups and no tank effects. SGR – specific growth rate, HSI – hepatosomatic index, VSI – viscerosomatic index.

Table 4
Whole body proximate composition at the end of the freshwater phase, in g 100 g^{-1}. Mean with standard deviation in parenthesis, $n = 3$ (pooled samples per tank). There were no statistically significant differences.

	Control	FA	AA	B-vit	Combined
Protein	17.0 (0.1)	17.7 (0.6)	17.3 (0.6)	17.0 (0.0)	17.0 (0.0)
Lipid	12.1 (0.2)	11.9 (0.2)	11.8 (0.2)	12.0 (0.3)	12.1 (0.3)
Dry matter	31.1 (0.1)	31.3 (0.5)	31.1 (0.2)	31.0 (0.2)	31.2 (0.2)

Table 5
Plasma cortisol in salmon smolt fed different freshwater diets, just prior to seawater (SW) transfer and 1 h and 24 h after SW transfer. Data are in ng ml^{-1} and are given as mean with standard deviation in parenthesis, $n = 12$ per diet group and temperature. No statistically significant differences between diet groups were seen.

	Control	FA	AA	B-vit	Combined
Before SW transfer	23 (16)	25 (18)	61 (48)	36 (31)	52 (48)
1 h after, 8°C	136 (23)	146 (75)	155 (63)	172 (93)	126 (76)
1 h after, 12°C	191 (97)	197 (118)	133 (75)	177 (100)	173 (85)
1 h after, 16°C	97 (46)	109(58)	99 (55)	121 (72)	101 (36)
24 h after, 8°C	24 (15)	–	–	–	23 (18)
24 h after, 12°C	24 (11)	–	–	–	35 (22)
24 h after, 16°C	45 (39)	–	–	–	44 (26)

Table 6
5-HIAA/5-HT ratio in the brain stem of salmon smolt fed different freshwater diets, just prior to seawater (SW) transfer and 1 h and 24 h after SW transfer. Data are in ng ml^{-1} and are given as mean with standard deviation in parenthesis, $n = 12$ per diet group and temperature.

	Control	Combined
Before SW transfer	1.30 (3.45)	0.24 (0.10)
1 h after, 8°C	0.37 (0.14)	0.32 (0.09)
1 h after, 12°C	0.33 (0.08)	0.28 (0.16)
1 h after, 16°C	1.35 (3.43)	0.36 (0.19)
24 h after, 8°C	0.38 (0.20)	0.28 (0.12)
24 h after, 12°C	0.26 (0.17)	0.39 (0.23)
24 h after, 16°C	0.25 (0.13)	0.28 (0.09)

Table 7
Redox status at the end of the freshwater phase, 24 h and 6 weeks after seawater (SW) transfer (mean ± SD in parentheses, $n = 9$–18). All groups were analyzed in freshwater, while only samples from fish fed the control and combined diets were analyzed after transfer to SW. In the table, data from the different diet groups are combined if no effects of diet were seen at that time point. Superscript letters show differences ($p < 0.05$) between the different time point (or diet in the case of muscle 24 h after SW transfer).

	GSH µmol/g	GSSG µmol/g	E mV
Liver			
Freshwater	2190 (540)^{b}	8.2 (1.8) ^{b}	−233 (7) ^{a}
24 h	2340 (470) ^{b}	9.6 (1.7) ^{b}	−233 (5) ^{a}
6 weeks	3990 (590) ^{a}	11.6 (2.1) ^{a}	−244 (5) ^{b}
Muscle			
Freshwater	375 (34)^{b}	5.3 (1.3)	−196 (3)^{b}
24 h control	490 (45)^{a}	2.7 (1.2)	−213 (11)^{a}
24 h combined	384 (67)^{b}	3.0 (1.4)	−204 (6)^{ab}
6 weeks	337 (74)^{b}	3.0 (0.5)	−199 (6)^{b}

and redox potential at SW transfer and 6 weeks after transfer. For liver, neither diet group nor temperature had significant effect at 24 h after transfer. Neither GSH, GSSG nor the redox potential in liver or muscle were affected by diet group or water temperature 6 weeks after SW transfer (Table 7). Six weeks after transfer, the liver concentrations of GSH and GSSG were higher than in the two earlier stages. Liver was also more reduced, i.e. had lowered redox potential in 6 weeks after transfer.

Before SW transfer, plasma chloride concentrations were significantly lower in fish fed the control diet compared to fish fed the AA diet ($p = 0.006$), while the other diet groups had intermediate values (Table 8). 24 h after SW transfer, there was no effect of diet on plasma chloride concentrations, only an effect of temperature, with chloride concentrations being higher in fish transferred at 16 °C compared to fish transferred 8 and 12 °C ($p < 0.0001$). Plasma chloride values before and

Table 8
Plasma chloride (mmol L^{-1}, mean with standard deviation in parenthesis) in fish sampled the day before seawater (SW) transfer, and in fish sampled 24 h after transfer at 8, 12 and 16°C, respectively. Different superscript letters indicate statistically significant differences.

	Control	FA	AA	B-vit	Combined
Before SW transfer	124.7 (4.7)^{a}	125.5 (3.4)^{ab}	129.8 (3.0)^{b}	126.8 (2.9)^{ab}	128.2 (3.2)^{ab}
24 h after, 8°C	126.2 (2.7)	127.5 (4.8)	128.3 (4.4)	124.0 (4.4)	126.1 (3.3)
24 h after, 12°C	127.7 (4.9)	128.3 (5.6)	127.1 (2.8)	124.0 (3.4)	126.0 (3.7)
24 h after, 16°C	130.7 (4.8)	131.0 (2.9)	129.7 (3.5)	130.7 (3.3)	130.3 (4.0)

24 h after SW transfer were very similar.

The final weight and length of the fish after 6 weeks in SW being fed the same commercial diet did not differ between the FW diet groups, but was higher at 12 and 16 °C compared to 8 °C (Table 9). This temperature effect was also reflected in the condition factor. Furthermore, fish from the FA diet group had significantly higher condition factor than the control group, while the other diet groups were intermediate. Temperature strongly affected both the heart- and the liver index, while there were no differences seen between fish originating from the different diet groups. There were also no effects of diet on the proximate composition, while the temperature effect was highly significant for both lipid, protein and dry matter (all $p > 0.0001$). Towards the end of the 6 week period, there were some mortalities recorded in the experiment. These were relatively evenly distributed across the different diet groups (0–3 individual fish per diet group), but all of them occurred in the tanks kept at 8 °C. At the final sampling, the fish kept at 8 °C were observed to have a highly reduced mucous layer on the skin, and also high prevalence of wounds and scale loss compared to the fish reared at higher temperatures (Fig. 3). This affected practically all fish in the 8 °C tanks, with no apparent differences between the five diet groups.

Regarding vertebra deformities, individual fish had from 0 to 5 deformed vertebra, and most fish with deformities had more than one deformed vertebra. The prevalence of deformities varied with 10% in the control group, 7% in fish fed the FA diet, 2% in fish fed the AA diet, and 5% both in fish fed the B-vitamin diet and the combined diet. The groups were not significantly different, judged with 2-way ANOVA on arcsin transformed data. Temperature after SW transfer did not have a significant impact on the prevalence or severity of deformities.

Both temperature and diet affected cataract development six weeks after SW transfer, while no interaction effects were seen (Fig. 4). The lowest scores were found for fish reared at 8 °C, then it increased at 12 °C and again at 16 °C. The overall diet effect (all temperatures combined) showed that fish given the FA diet had a lower score compared to fish given the AA, B-vitamin and combined diets, while no differences were seen compared to the control diet. The effect of diet compared to the control diet was only evident at 12 °C and 16 °C, while no differences were seen at 8 °C. At 12 °C, the B-vitamin diet had a significantly higher mean score compared to control, while at 16 °C fish given AA, B-vitamin and combined diet all had significantly higher scores compared to fish given the control diet (Fig. 4).

At the termination of the net pen trial, there were no differences in final weight between the fish originating from the different FW diet groups (Table 10). Regarding final length, fish from the AA group were significantly longer than fish from the control group, while the other groups were intermediate. As there was a similar trend also for weight, no difference was seen in the condition factor. There was also no difference in hepatosomatic index. Despite the pumping that was conducted to stress the fish 2 weeks before the final sampling, mortality was low (<1.5%) with no differences between the diet groups. No differences were seen in cataract scores between the dietary groups.

Generally, the welfare scores were very similar between diet groups

Table 9
Performance and proximate composition of whole fish after 6 weeks in seawater. Data are shown as mean with standard deviation in parenthesis. For each diet group, the data from the three temperatures are pooled, and for each temperature data from the five diet groups are pooled. *P*-values are shown for two-way ANOVA with diet and temperature as the two factors. Different superscript letters indicate statistically significant differences, with small letters for diet effects and capital letters for temperature effects. No interaction effects were seen.

	Control	FA	AA	B-vit	Combined	8 °C	12 °C	16 °C	ANOVA, diet	ANOVA, temp
Final weight, g	138.8 (22)	137.1 (24)	137.2 (22)	141.6 (25)	142.0 (23)	125.1^{A} (18)	143.5^{B} (22)	148.8^{B} (22)	n.s.	$p < 0.0001$
Final length, cm	22.8 (1.2)	22.5 (1.3)	22.4 (1.2)	22.9 (1.2)	22.8 (1.2)	22.0^{A} (1.1)	22.9^{B} (1.2)	23.1^{B} (1.2)	n.s.	$p < 0.0001$
CF	1.17^{a} (0.06)	1.22^{b} (0.08)	1.18^{ab}(0.06)	1.18^{ab} (0.04)	1.19^{ab} (0.07)	1.16^{A} (0.06)	1.19^{B}(0.06)	1.21^{B} (0.06)	$p = 0.03$	$p = 0.0003$
Liver index	1.11 (0.28)	1.08 (0.38)	1.05 (0.32)	1.12 (0.31)	1.09 (0.34)	1.46^{A} (0.16)	1.07^{B} (0.07)	0.76^{C} (0.21)	n.s.	$p < 0.0001$
Heart index	0.085 (0.011)	0.086 (0.010)	0.083 (0.009)	0.084 (0.007)	0.081 (0.011)	0.091^{A} (0.009)	0.081^{B} (0.009)	0.080^{B} (0.009)	n.s.	$p < 0.0001$
Survival, %	98.6	100	98.6	95.8	98.6	100	100	95	-*	-*
Protein, g 100 g^{-1}	18.0 (0.6)	17.6 (0.6)	17.8 (0.4)	17.5 (0.5)	17.7 (0.5)	17.3^{A} (0.5)	17.9^{B} (0.6)	18.0^{B} (0.0)	n.s.	$p < 0.0001$
Lipid, g 100 g^{-1}	7.5 (1.6)	7.4 (1.8)	7.5 (1.2)	8.6 (1.1)	8.6 (1.7)	6.7^{A} (1.5)	7.7^{A} (1.2)	9.2^{B} (0.7)	n.s., $p = 0.16$	$p < 0.0001$
Dry matter, g 100 g^{-1}	31.0 (0.8)	30.8 (0.7)	30.9 (1.1)	30.8 (0.6)	30.6 (0.8)	30.0^{A} (0.4)	30.9^{B} (0.5)	31.5^{C} (0.5)	n.s.	$p < 0.0001$

* Numbers of dead fish are too low for statistical analysis.

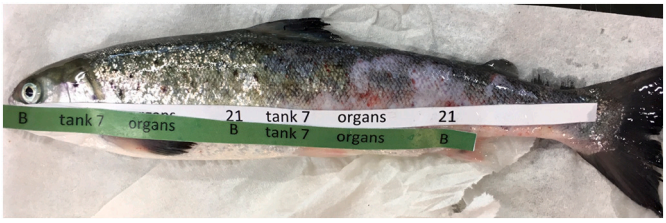

Fig. 3. Photo of fish transferred to full-strength seawater at 8 °C (from 12 °C in freshwater) without acclimatization. Across all diet groups, these fish had scale loss, abundant wounds and a visibly reduced mucus layer 6 weeks after seawater transfer, which probably would have caused a high mortality if the trial had not scheduled termination at this point.

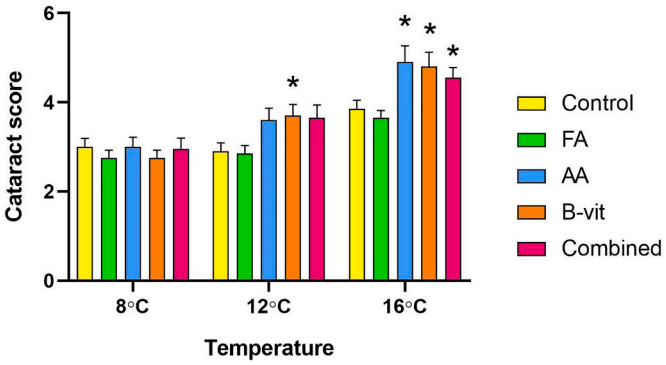

Fig. 4. Mean cataract scores (SEM as error bars) of Atlantic salmon after 6 weeks in seawater. The asterisks in the figure shows which diet groups are significantly different from the control group within that temperature (Mann-Whitney *U* test, $p < 0.05$).

(Fig. 5). For vaccine side effects (Speilberg score), the majority of the fish in all groups scored 1 and 2. For fin condition, most of the fish scored 3, with some fish scoring 1, 2 and 4 in all diet groups. For skin condition, nearly all the fish scored 3. Regarding eye status, very few fish (3–7 out of the 50 fish scored per diet group) scored above 0, with no apparent differences between the groups (data not shown). For mouth/jaw wounds, only 0–1 fish per diet group score above 0 (data not shown). The presence of sea lice on the fish was also included in the evaluation, but none of the fish had this.

Table 10
Performance of Atlantic salmon post-smolts after 3 months in net pens, fed a common commercial diet. Fish were pit-tagged to trace them back to their original diet groups from the freshwater phase. Data are shown as mean with standard deviation in parenthesis.

	Control	FA	AA	B-vit	Combined	ANOVA
Final weight, g	307 (65)	317 (42)	353 (49)	328 (66)	313 (52)	n.s.
Final length, cm	29.1 $(2.1)^{a}$	29.6 $(1.1)^{ab}$	30.8 $(1.5)^{b}$	29.9 $(2.0)^{ab}$	29.3 $(1.6)^{ab}$	p = 0.03
CF	1.24 (0.06)	1.22 (0.05)	1.26 (0.05)	1.26 (0.07)	1.23 (0.04)	n.s.
HSI	1.28 (0.13)	1.39 (0.20)	1.38 (0.21)	1.35 (0.16)	1.31 (0.14)	n.s.
Cataract score	3.2 (0.7)	3.3 (0.9)	3.5 (0.9)	3.2 (0.9)	3.2 (0.8)	n.s.

4. Discussion

In the current study, we wanted to test if dietary modifications during the FW phase could enhance fish robustness, survival and growth after SW transfer. All the experimental feeds supported rapid growth, with no significant differences between diet groups. There was a tendency of reduced growth in FW of the fish fed the modified FA composition, however, this was most likely due to the tank effects observed. Furthermore, the combined diet had the same FA composition as the FA diet, but very similar growth to the other diet groups. The FA diet group performed well regarding growth after SW transfer, as the trend seen at the end of the FW period was no longer apparent after 6 weeks in SW in the temperature trial or after 14 weeks in the net pens. Temperature affected growth after SW transfer in our trial. For Atlantic salmon post-smolt, the optimal temperature for growth was determined to be 12.8 °C for 70–150 g fish and 14 °C for 150–300 g fish (Handeland et al., 2008), fitting well with the equally high growth at 12 and 16 °C in our study. The temperature effects on growth probably also accounts for the temperature-related differences in whole body protein and lipid content. In the fish kept in the net pens, there was a tendency towards better growth in fish previously fed the AA diet in the FW phase. No tendency in the same direction was seen with the combined diet fed the same AA composition and there was no similar tendency in the temperature trial, hence our main conclusion is that the AA composition of the control diet was already sufficient, and additional levels of certain AA did not show any benefits on growth. However, it cannot be excluded that a longer feeding period would have revealed positive results on

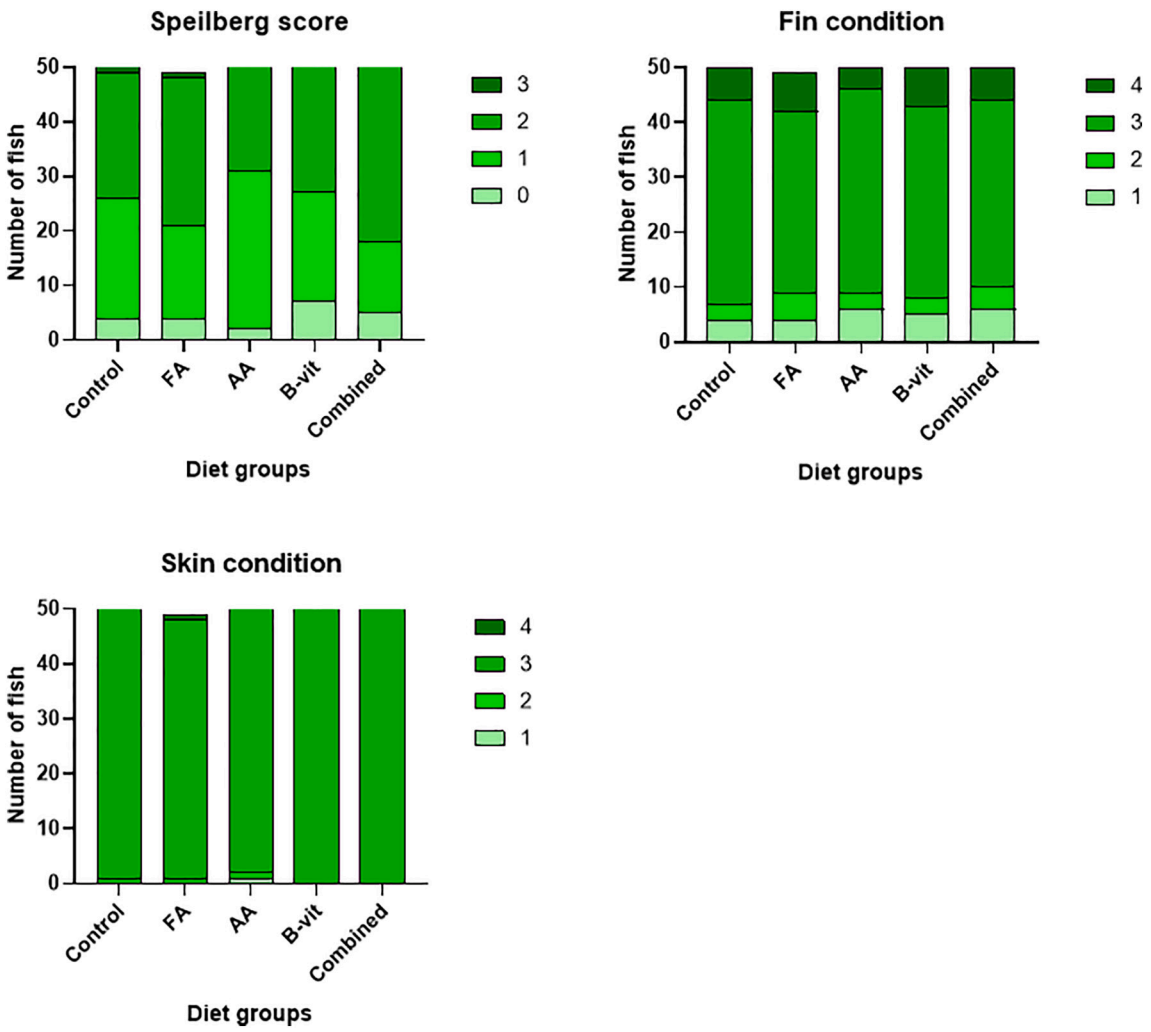

Fig. 5. Welfare scores in Atlantic salmon fed the five experimental diets during 10 weeks in the freshwater phase, after 14 weeks in open net pens in seawater on a common commercial diet.

growth from additional methionine, similar to previously published results (Espe et al., 2020).

As there were also no effects of the FW diets on survival, it seemed that all the five experimental feeds used in the current trial were sufficient to support proper parr-smolt transformation and high survival after transfer to SW. All groups seemed to handle SW transfer well, an assumption based on the similar and low plasma chloride values 24 h after SW transfer. Due to the poor state of the fish reared at 8 °C at the final sampling, including reduced mucous layer and a high prevalence of wounds and scale loss, an increasing mortality would have been expected if the trial had been continued from that point. Similar to our results, salmon kept at 6 °C in FW experienced increased mortality when transferred directly to SW at lower temperatures, although plasma chloride levels indicated well adapted smolts in all groups (Arnesen et al., 1998). In another study, smolt transferred at 6 and 8.7 °C were able to regulate plasma chloride levels, while fish transferred at 2 and 4.1 °C were not, indicating a low temperature limit for the successful transfer of smolt to SW (Sigholt and Finstad, 1990). Unlike in our study, these fish were acclimated before transfer, which indicates that transfer at lower temperatures is acceptable if the fish are acclimated to that temperature.

Maintenance of the skin and mucous layer of salmon is highly important, as the skin is the first physical and immune active barrier in fish. Previous studies have reported a sharp decline of about 50% in the number of mucous cells during smoltification (O'Byrne-Ring et al., 2003), and that the period after SW transfer is a barrier recovery phase, when skin thickness and mucus cell numbers increase together with a gradual recovery of immune activity in skin (Karlsen et al., 2018). Hence, it may be hypothesized that the fish transferred at 8 °C in the current study never managed to recover their skin barrier after SW transfer. Furthermore, wound healing in Atlantic salmon was faster at 12 than at 4 °C (Jensen et al., 2015b). Optimal nutrition plays a major role in maintaining normal skin physiology and repair mechanisms following injury (Jensen et al., 2015a). Threonine has been very little focused in salmon diets (Helland and Grisdale-Helland, 2011), but threonine is highly important in mucus as the glucoprotein mucin in the gastrointestinal tract and on the skin (Wang et al., 2009). However, we did not see an impact of the additional threonine in the AA diet in the current study. It seems that none of the nutrients varied in our experimental design were able to ameliorate the effects on skin and mucus of SW transfer at low temperature.

In the current study, we could not detect any dietary effects on brain serotonergic activity or plasma cortisol, suggesting that neither of the dietary supplementations affected the welfare of the fish compared to the control diet. Fish responded to the SW transfer with a temperature dependent elevation of plasma cortisol values, with the highest response in 12 °C. Considering that fish were acclimated to 12 °C in FW in the current study, and that abrupt temperature changes can result in stress induced elevations of plasma cortisol, one could expect a higher cortisol response in fish transferred to 8 and 16 °C SW. However, the handling of

the fish and transfer to SW in itself might be much more potent stressors. Furthermore, Madaro et al. (2018) demonstrated that the production of cortisol in salmon is temperature dependent with a higher production rate and peak values at higher temperatures. Thus, the lower cortisol values in fishes transferred to 16 °C compared to 12 °C in the current study might reflect that cortisol have reached peak values and have started to sink back to basal levels at 16 °C. Moreover, the lower cortisol values in fish reared at 8 °C could be an effect of the smolt not having reached their peak in cortisol production yet. This corresponds well with the study performed by Madaro et al. (2018), showing that cortisol production in smolts exposed to acute confinement stress reached the peak around 30, 60 and 80 min when acclimated to 17, 12 and 8 °C, respectively.

While sub-optimal levels of several micronutrients have previously been linked to cataract development in salmonids (Bjerkås et al., 2006; Waagbø et al., 2003), nutrition related cataract development in the last two decades has mainly been associated with histidine deficient diets (Breck et al., 2005; Remø et al., 2014; Waagbø et al., 2010). In these studies, histidine supplementation has been shown to minimize, but not eliminate cataract development, thus highlighting the multifactorial causation and possibly interactions between sub-optimal dietary levels of other nutrients as well as rearing temperature. Overall, the lowest cataract scores were seen in fish transferred to 8 °C, increased at 12 °C and again at 16 °C. These findings are supported by previous studies showing a higher risk of cataract development at higher temperatures (Sambraus et al., 2017; Waagbø et al., 2010). Rapid growth has also been linked to a higher risk of cataract development (Bjerkås et al., 1996; Breck and Sveier, 2001; Waagbø et al., 1996), and the fish transferred to 8 °C had a lower growth rate compared to 12 and 16 °C. Although no differences were seen in growth rate at 12 and 16 °C, higher cataract scores were seen at 16 °C compared to 12 °C, which may be linked to temperature dependent effects on carbohydrate metabolism, osmoregulation and redox homeostasis in the lens (Remø et al., 2017).

The FW diets influenced cataract development in the groups transferred to 12 and 16 °C, while no differences were seen between diets in the fish transferred to 8 °C or after 3 months in net pens where the temperature was also low. Sub-optimal dietary levels of methionine have been linked to cataract development in rainbow trout (Cowey et al., 1992). However, in the present study the diets supplemented with methionine (AA, B-vitamin and combined) had more severe cataract compared to the control diet at 16 °C, and it may be hypothesized that this is related to the lower dietary histidine content in these feeds compared to control and FA, rather than the present supplementation of methionine and other amino acids and vitamins.

The glutathione metabolism and redox potential are important for regulation of metabolism by opening and shutting redox switches in metabolic pathways (Jones and Sies, 2015). The fact that the dietary treatments did not affect glutathione metabolism and redox balance in FW in the present study is in line with previous results in Atlantic salmon post smolts (Hamre et al., 2010). That study reported very similar GSH and GSSG concentrations, and redox potential, in muscle and liver of salmon irrespective of large variations in dietary vitamin C, vitamin E, astaxanthin, lipid, Cu, Zn and Mn. The present results therefore support our hypothesis from then that glutathione metabolism is strictly regulated with minor effects of nutrition, to keep the redox potential of the cells within narrow limits. The redox status can, however, change in response to fish development and environmental conditions (Penglase et al., 2015). Accordingly, just after SW transfer in the present study there was an upregulation of GSH followed by a more reduced redox potential in fish fed the control diet compared to the combined diet. This can be interpreted as a protection of the fish fed the combined diet against the large increase in salinity, which may have caused oxidative stress. The highly supplemented combined diet may have had sufficient amounts of nutrients to avoid oxidative stress. There was no effect of temperature on fish glutathione metabolism, after either 24 h or 6 weeks of sea culture, even though fish held at 8 °C were in bad shape after 6 weeks in SW. Therefore, glutathione metabolism cannot be considered a good welfare indicator in this circumstance.

Regarding the effects of organ indices and liver lipids in the combined diet group, these all pointed in the same direction, as both hepatosomatic index, viscerosomatic index and liver triacylglycerol (TAG) were low in the combined group. Although both AA deficiencies (Espe et al., 2010) and unbalanced dietary FA composition (Ruyter et al., 2006; Sissener et al., 2017) have been shown to increase liver TAG in previous studies, neither can explain the present results as the combined diet had the same AA composition as the AA diet and the same FA composition as the FA diet. Hence, this appears to be an interaction effect of AA and FA composition. These effects were only apparent at the end of the FW period, and were not seen in the SW stage. Similar to the organ indices, both vertebrae deformities and welfare scores did not differ between the diet groups in the SW stage, showing no differences in fish robustness.

Fish in this trial were subjected to the stress of SW transfer in itself, combined with transfer at different SW temperatures without prior acclimation, and also to the varying environmental conditions in open net pens. However, in a short-term trial run in open net pens, it is a bit "random" what the fish ends up being exposed to. Since the trial was conducted in winter, there were no sea lice infestations on the fish, and they also escaped environmental problems that can occur during summer, such as high water temperatures followed by reduced oxygen and algal blooms. Furthermore, as far as we know, the fish did not get any infectious diseases during this trial. Particularly in certain disease conditions, amino acids can promote health by improving anabolism, reducing stress and modulate immunology. Further research using fish from these FW diet groups will more thoroughly investigate immunological responses and potential robustness when facing diseases, both in a cell model (Holen et al., in prep) and in a challenge trial (Remø et al., in prep) with the fish.

5. Conclusion

The current study showed no positive effects on the robustness, survival and growth of the tested dietary improvements compared to the control diet (similar to a commercial feed), indicating that the control diet was already sufficient. All groups seemed to adapt well to seawater, however, the direct transfer from 12 to 8 °C is not advisable based on the current results. Potential immunological effects of the diets will be assessed in further studies.

Author contributions

Conceptualization; N.H·S, M.E, A.J.P., K.H., K.S., E.H., S.C.R., Data curation; M.L., S.C.R., N.H·S, P.G.F., K.H., Formal analysis; M.L., S.C.R., N.H·S, P.G.F., Funding acquisition; N.H·S, M.E, A.J.P., K.H., K.S., E.H., S. C.R, P.G.F, S·S, V.V., Investigation; N.H·S, M.E, A.J.P., K.H., K.S., E.H., S. C.R, P.G.F, S·S, V.V., C.S., E.H., M.L., Methodology; N.H·S, M.E, A.J.P., K.H., K.S., E.H., S.C.R, P.G.F, C.S., E.H., M.L., B.N., Project administration; N.H·S, Writing - original draft; N.H·S, Writing - review & editing; all authors.

Declaration of Competing Interest

None.

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