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Long-term feeding of Atlantic salmon in seawater with low dietary long-chain *n*-3 fatty acids affects tissue status of the brain, retina and erythrocytes

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Abstract

In two long-term feeding trials in seawater, Atlantic salmon were fed EPA + DHA in graded levels, from 1·3 to 7·4 % of fatty acids (FA, 4–24 g/kg feed) combined with approximately 10 % 18 : 3*n*-3, at 6 and 12°C. Dietary EPA appeared to be sufficient in all diet groups, as no differences were seen in polar lipid tissue concentrations of either the brain, retina or erythrocytes. For DHA, a reduction in tissue levels was observed with low dietary supply. Effects on brain DHA at ≤1·4 % EPA + DHA of dietary FA and retina DHA at ≤2·7 % EPA + DHA of dietary FA were only observed in fish reared at 6°C, suggesting an effect of temperature, whereas tissue levels of *n*-6 FA increased as a response to increased dietary *n*-6 FA in both the brain and the retina at both temperatures. DHA levels in erythrocytes were affected by ≤2·7 % EPA + DHA at both temperatures. Therefore, DHA appears to be the limiting *n*-3 FA in diets where EPA and DHA are present in the ratios found in fishmeal and fish oil. To assess the physiological significance of FA differences in erythrocytes, the osmotic resistance was tested, but it did not vary between dietary groups. In conclusion, ≤2·7 % EPA + DHA of FA (≤9 g/kg feed) is not sufficient to maintain tissue DHA status in important tissues of Atlantic salmon throughout the seawater production cycle despite the presence of dietary 18 : 3*n*-3, and effects may be more severe at low water temperatures.

Key words: **EPA: DHA: Atlantic salmon: Requirements: Brain: Retina: Erythrocytes**

Stagnation, or even decline, in reduction fisheries[1], combined with increased aquaculture production, necessitates the use of raw materials other than fishmeal (FM) and fish oil (FO) in feed for Atlantic salmon (*Salmo salar* L.). Decreased inclusion of FM and particularly FO means decreased concentration of the long-chain *n*-3 fatty acids (FA) EPA (20 : 5*n*-3) and DHA (22 : 6*n*-3), whereas alternative lipid sources used in commercial salmon feeds generally provide 18 : 3*n*-3[2]. We have shown in two long-term trials during the seawater-stage of Atlantic salmon that low dietary EPA + DHA levels from 1·3 to 7·4 % of total FA (4–23 g/kg feed) did not affect mortality or cause severe essential FA (EFA) deficiency symptoms, but that levels >2·7 % of FA (9 g/kg feed) were required for optimal growth (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, *et al.*, unpublished results). High retention efficiency of particularly DHA (>100 %) was seen in all diet groups, with retention increasing sharply to about 200 % at the lowest dietary concentrations (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results), showing that the fish both efficiently store long-chain *n*-3 FA and also desaturate and elongate α-linolenic acid (LNA, 18 : 3*n*-3) to EPA and DHA. Such a capacity for elongation and desaturation has also been shown in salmon previously[3,4], and a study on hepatocytes showed that *n*-3 FA were incorporated in lipid fractions to a higher extent than *n*-6 FA, while EFA-deficieny increased the incorporation[5].

However, the improved growth seen with EPA + DHA both in the present trial in seawater[2] and in a previous trial on Atlantic salmon in the freshwater phase[6] may indicate that the requirements of salmon for EPA and DHA at the tissue level cannot be completely fulfilled by dietary LNA. EPA and DHA are important for ontogenesis, growth, survival, pigmentation, resistance to stress and disease as well as for the development and functionality of the brain, vision and the nervous system. Brain, retina and erythrocytes are all tissues with a high content of DHA[7]. Reduced DHA level in the brain and the retina have been seen in other animal models with severe *n*-3 FA deficiency[8,9], including marine fish larva[10,11], and has been linked to reduced neuromotor activity, learning skills and visual acuity in various animals. In Atlantic salmon, studies investigating FA composition of the brain and eye have mostly been carried out with dietary long-chain *n*-3 FA far above the expected requirement[12,13]. These studies observed that brain and retina DHA levels were highly conserved and not affected by diet, whereas tissue *n*-6 FA and to some extent EPA were more affected by diet. A recent trial with a regression on DHA

Abbreviations: ARA, arachidonic acid; EFA, essential fatty acid; FA, fatty acid; FM, fishmeal; FO, fish oil; LNA, α linolenic acid; NL, neutral lipids; PL, polar lipids.

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(0·5–7·6 % of FA) and relatively constant EPA content showed an impact on *n*-6 FA and EPA in brain polar lipids (PL), but also a significant regression of DHA[14]. However, the regression explained little of the total variation in the data set (R^2 0·30), and seemed to be caused mainly by the lowest diet group. In addition, the feeding trial only lasted 9 weeks, which is not sufficient for tissue EPA and DHA levels to stabilise relative to the diet at such low dietary levels (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results).

Changes in tissue FA composition, for instance, in the erythrocyte membranes, may affect their robustness. Although testing the fragility of erythrocytes by exposure to hypotonic solutions has most commonly been used for assessment of vitamin E/antioxidant status[15], an increased incorporation of long-chain *n*-3 FA in the cell membranes may also protect them from lysis. In rainbow trout (*Oncorhynchus mykiss*), erythrocytes of fish fed FO were less fragile than erythrocytes of fish fed linseed or safflower oil in semi-purified diets not containing any EPA or DHA[16]. In Atlantic salmon and channel catfish (*Ictalurus punctatus*), the resistance of erythrocytes to lysis has also been observed to increase with increasing dietary FO inclusion[17,18].

The lipid composition of fish cell membranes may also be affected by changes in water temperature, via a process known as homeoviscous adaptation. Membranes become more rigid at low temperatures, and this effect is offset by a higher proportion of unsaturated FA, which restore the fluidity of the membrane[19,20]. A consequence of this might be a higher dietary requirement for EPA and DHA at low temperatures. In support of this, higher activity of Δ6-desaturase in rainbow trout maintained at 5°C compared with 20°C has been reported[21], and the production of DHA from EPA was higher at 5°C than at 12°C in hepatocytes of Atlantic salmon[22]. These studies suggest that the fish may be able to adapt to lower temperatures by increasing elongation and desaturation of LNA to EPA and DHA, and therefore an increased dietary supply of EPA and DHA may not be necessary.

The aim of the present study was to assess the effects of low dietary EPA + DHA in the presence of dietary 18 : 3*n*-3 on tissue FA status of the brain, retina and erythrocytes in Atlantic salmon maintained at two different temperatures throughout the seawater production cycle.

Methods

Diets and experimental design

Two long-term feeding trials were conducted at Skretting ARC Fish Trials Station, Stavanger, Norway, and are described in detail by G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener (unpublished results). Both trials were conducted according to the guidelines of the Norwegian State Commission for Laboratory Animals, at an approved facility for research with animals and were approved by the responsible person for animal ethics at the facility.

Trial 1. The first trial was run from June 2011 to August 2012, starting with 750 fish with a mean weight of 162 (SEM 0·9) g distributed in five tanks. The fish were fed the five experimental diets for 7 months at 12°C before the initial organ sampling – when the fish reached about 1450 g in size. These 7 months were meant as a pre-feeding period to reduce body stores of EPA + DHA, so that results from the feeding trial would reflect FA available from the diets rather than from body stores. During these 7 months, no samples were collected for detailed studies of animal physiology and organ FA composition. However, whole fish and fillets were sampled at every 500 g growth and analysed for FA, in order to see when the composition stabilised after the diet change, which happened after a 7-fold increase in body weight (6 months) (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results). After the initial organ sampling, fish from each tank were split into two groups, and one tank from each diet group was further maintained at 12°C, whereas for the other five tanks the temperature was gradually decreased to 6°C. The final sampling at 12°C was conducted after another 142 d at 12°C (12 months total feeding period), whereas the final sampling at 6°C was conducted after 202 d at this temperature (14 months total feeding period), so that fish grown at each temperature would be of approximately the same size at the final sampling (approximately 3000 g). Five experimental diets were used with 10 % FM of the total recipe, whereas the remainder of the protein was supplied by plant sources. The diets had five graded levels of EPA + DHA of 1·4, 2·7, 3·4, 4·3 and 5·2 % of total FA (5–17 g/kg feed), achieved by increasing the FO content. The main lipid source in all five diets was a vegetable oil blend consisting of rapeseed oil, palm oil and linseed oil (4:2:1). The analysed composition of the final feed batch, including FA composition, can be found in Table 1, whereas further details on diet ingredients and formulations are given in the study by G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener (unpublished results).

Trial 2. Owing to the growth rate of fish maintained at 12°C in the first trial being lower than that expected for fish of this size at this temperature (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results), a second follow-up trial was run at 12°C to verify the results from the first trial. In the second trial, a common diet with 3 % EPA + DHA of total FA was used for the pre-feeding period, starting with approximately 450 g fish and lasting for 5 months. For the next 5 months, fish were fed four different diets with 1·3, 2·7, 4·4 and 7·4 % EPA + DHA of total FA (4–25 g/kg feed), in duplicate tanks (560 fish in total), and grew from approximately 1450 to 3000 g. The choice of the common pre-feeding diet was based on results from trial 1, and this diet had 3 % EPA + DHA, which would ensure optimal growth and not compromise the physiology of the fish, while still reducing tissue levels of EPA and DHA compared with before the onset of the experiment. Diets were very similar to the diets used in the first trial, except that EPA + DHA was brought close to 7·5 %, which was a common target level for commercial salmon grower diets at the time this trial was started. In the following sections, the diet groups are named according to the number of the trial (1 or 2) and the level of EPA + DHA, for example, 1: 2·7 is the group from trial 1 fed 2·7 % EPA + DHA of total FA.

Sampling

For organ sampling, feeding was stopped 12 h before sampling for each tank. The fish were anaesthetised with FINQUEL MS222 at a concentration of 7 g/l, killed by a blow to the head and individually weighed and measured. Blood was collected using vacutainers from six fish per tank at the initial organ sampling in trial 1 and from eight fish per tank at the final samplings in both trials. Whole blood was maintained on ice for testing osmotic fragility of the erythrocytes in trial 2. Erythrocytes for FA analysis were collected using a Pasteur pipette after centrifugation of blood samples (3000 ***g***, 10 min, 4°C), and washed three times with physiological saline before the samples were flash frozen. The whole brain and one eye were sampled from three fish per tank and frozen on dry ice. All tissue samples were stored at −80°C. For dissection of the retina before FA analysis, the sampled eyes were partially defrosted and cut in half; the lens and vitreous humor were removed, whereas the retina including the pigment layer was carefully removed from the underlying tissues.

Osmotic resistance of erythrocytes

Osmotic fragility of the erythrocytes was tested based on the method described previously[23]. In brief, 20 µl of heparinised blood was added to a series of nine concentrations of 5-ml saline solution (0–9 g NaCl/l) in phosphate buffer. After gentle mixing and 1 h incubation at 4°C, the suspensions were centrifuged (3000 rpm, 10 min, 5°C) and the absorbance of supernatants was measured at 540 nm. Results are given as the salinity causing 50 % lysis of the erythrocytes.

Preparation of samples for lipid class and fatty acid analyses

Lipids from the brain, retina and erythrocytes were extracted in a mixture of chloroform–methanol 2:1 (Merck) with 1 % 2,6-di-tert-butyl-4-methylphenol (Sigma-Aldrich) as described by Torstensen *et al.*[24]. In brief, chloroform–methanol was added to the samples at approximately twenty times the weight of the sample, whereas for brain samples methanol was added to the tissue and shaken for 2 h before adding chloroform to improve the extraction of PL. The samples were frozen at −20°C until further analysis.

Fatty acid composition of neutral and polar lipids

For brain and retina samples, neutral lipids (NL) and PL were separated by solid-phase extraction before analysis of FA composition. Lipids were evaporated to dryness and recovered in chloroform to a concentration of 50 mg/ml lipids. An aliquot of 200 µl (10 mg lipids) was applied to a solid-phase extraction column (Isolute; Biotage). NL were eluted with 10 ml chloroform–methanol (98:2, v/v), and PL were eluted with 20 ml methanol. An internal standard (methylated 19:0) was added to both lipid fractions. FA methyl esters from each fraction were obtained from total lipids using boron trifluoride (12 % BF_3 in methanol) following saponification, as described previously[25]. To determine the FA composition of each lipid fraction, methyl esters were separated on a Thermo Finnegan Trace 2000 GC equipped with a fused silica capillary column (CP-sil 88; Chrompak Ltd), with temperature programming of 60°C for 1 min, 160°C for 28 min, 190°C for 17 min and finally 220°C for 10 min, with all intervening temperature ramps being 25°C/min. Individual methyl esters were identified by retention time in comparison with standard mixtures of methyl esters (Nu-Chek). All samples were integrated using software Chromeleon® version 6.8, connected to the GLC.

Fatty acid composition of erythrocytes

The FA composition of erythrocytes was analysed by ultra-fast GC, a quicker method than the one used above, but with the limitation that MUFA are not separated according to the position of the double bond. An internal standard (19 : 0) was added to 50 mg of the sample before samples were saponified and methylated by adding 1 ml NaOH (0·5 M) and 2 ml BF_3 (20 % in methanol) and boiling at 100°C for 1 h. Samples were purified using hexane and adjusted to a concentration of 0·2–0·3 mg/ml before injection onto a Trace GC Ultra (Thermo Corporation), equipped with an SSL injector and a flame ionisation detector (Thermo Scientific), and the column was a Wax column (P/N UFMC00001010501, 5-m long, 0·1 mm. i.d., 0·1-µm film thickness). He was used as the carrier gas, with an initial oven thermal gradient from 100 to 220°C at 50°C/min to a final temperature of 250°C at 80°C/min. Chromeleon was the integrator used (Thermo Scientific).

Statistical analysis

Statistical analysis was carried out using Statistica software (version 11; StatSoft). Owing to the fact that only one tank was available per diet group per temperature in trial 1, individual fish from the same tank could be considered pseudoreplicates, although all possible care was taken to avoid tank variation. Linear regression analysis was the main statistical analysis used and was conducted on tank means for the different parameters. However, FA profiles are very robust with low individual variation within diet groups and unlikely to be affected by any potential tank variations. Thus, FA concentrations were also analysed using data for individual fish, both by two-way ANOVA to elucidate any temperature differences and interactions between diet and temperature and by one-way ANOVA to check for differences between diet groups at each temperature separately. The latter was carried out because of marked temperature differences in the concentrations of many FA, making it less useful to investigate dietary effects simultaneously at both temperatures. In trial 2, there were duplicate tanks for each diet group; thus, nested ANOVA was applied for the FA data to account for possible tank effects. However, no tank effects were detected, and thus regular one-way ANOVA was used for this trial as well, supporting the use of ANOVA in trial 1. Tukey's honest significant difference *post hoc* test was used on data from both the trials. For all statistical tests, P values $<0{\cdot}05$ were considered to be significant. However, P values $<0{\cdot}10$ may be included and discussed as trends.

Results

Dietary composition

Proximate composition and FA profiles of the feeds have been previously published (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results), but are also included in this study (Table 1) for a better understanding of the presented results. All diets had similar proximate composition, whereas FA profiles varied. In the feeds for trial 1, EPA + DHA exhibited a nice gradient in the five feeds, from 1·4 to 5·2 % of total FA. Total PUFA were relatively constant, but as expected *n*-6 FA decreased from diet 1:1·4 to 1:5·2, while *n*-3 FA increased. EPA + DHA caused a gradient in total *n*-3 FA, but this was partially offset by higher LNA in the diets with more plant oil. Diets in trial 2 were very similar to trial 1, but with EPA + DHA levels of 1·3, 2·7, 4·4 and 7·4 % of total FA, respectively.

Fatty acid composition of the brain

The FA of the brain NL fraction at both temperatures and in both trials generally reflected dietary composition (data not shown), with clear gradients in major FA from vegetable oils, such as 18 : 2*n*-6 and 18 : 1*n*-9. There were low levels of EPA and DHA compared with the PL fraction, but with gradients according to diet both for EPA and for DHA.

For PL in the brain at 6°C in trial 1, there were no significant linear regressions for any of the presented FA (Table 2). There were tendencies both for sum *n*-3 FA, DHA and EPA + DHA (all $P = 0{\cdot}09$) to increase with increasing dietary EPA + DHA. However, these tendencies appeared to be caused by DHA in diet group 1:1·4 being lower than the other diet groups, although not significantly. EPA levels were very similar in all diet groups. Diet group 1:1·4 had significantly higher levels of *n*-6 FA than all other diet groups, including both sum *n*-6 FA, 18 : 2*n*-6 and arachidonic acid (ARA, 20 : 4*n*-6) (Table 2). However, other individual *n*-6 FA also exhibited the same pattern (Fig. 1).

For PL in the brain at 12°C in trial 1, EPA and DHA concentrations were very stable across all diet groups. Total *n*-6 FA decreased and the *n*-3:*n*-6 ratio increased with increasing dietary EPA + DHA (Table 2), an effect that appeared to be caused by decreasing 18 : 2*n*-6 and 20 : 3*n*-6 and similar trends both for ARA and for 20 : 2*n*-6. In trial 2, only brain samples from the two most 'extreme' diet groups in the design (2:1·3 and 2:7·4) were analysed, as no differences in EPA or DHA were expected based on the results from trial 1. This turned out to be the case, and results were consistent with observations from trial 1 at 12°C (online Supplementary Table S1).

Fatty acid composition of the retina

The FA profile of the NL fraction at both 6 and 12°C generally reflected dietary composition (data not shown) similar to what was seen in brain NL.

In the retina PL fraction of fish reared at 6°C, total *n*-3 FA increased from diet group 1:1·4 to 1:5·2 (Table 3). DHA was significantly lower in diet group 1:1·4 and 1:2·7, compared with the three groups fed higher EPA + DHA. An opposite gradient

Table 1. Analysed diet composition in trial 1 and 2, including analysed proximate composition, selected fatty acids (FA) and classes of FA of the batches of 8-mm feeds

Diets*	1:1·4	1:2·7	1:3·4	1:4·3	1:5·2	2:1·3	2:2·7	2:4·4	2:7·4
Proximate composition (g/kg)									
Protein	395	385	388	388	389	382	383	386	382
Fat	355	359	356	348	352	337	343	347	331
Moisture	60	60	62	64	68	74	67	68	69
FA composition (% of total FA)									
18 : 1*n*-9/7	42·8	42·4	39·1	37·7	35·6	42·7	40·7	37·8	31·8
LA (18 : 2*n*-6)	16·6	15·6	14·8	14·3	13·5	16·5	15·6	14·3	12·6
LNA (18 : 3*n*-3)	13·2	12·0	11·5	10·7	10·1	11·7	11·1	10·1	9·0
ARA (20 : 4*n*-6)	0·0	0·1	0·1	0·1	0·2	0·0	0·1	0·2	0·3
EPA (20 : 5*n*-3)	0·7	1·4	1·8	2·3	2·8	0·6	1·4	2·4	4·1
DHA (22 : 6*n*-3)	0·7	1·3	1·6	2·0	2·4	0·7	1·3	2·0	3·3
Sum SFA	18·3	19·0	19·3	19·9	20·4	19·1	19·1	20·2	21·4
Sum MUFA	45·9	45·3	45·0	44·7	43·7	46·4	45·5	44·5	42·2
Sum *n*-6 FA	16·7	16·0	15·2	14·8	14·2	16·6	15·9	14·8	13·5
Sum *n*-3 FA	14·8	15·4	15·7	16·1	16·7	13·2	14·5	15·5	18·4
EPA + DHA	1·4	2·7	3·4	4·3	5·2	1·3	2·7	4·4	7·4
EPA + DHA (g/kg feed)	5	9	11	14	17	4	9	14	23

LNA, *α*-linolenic acid; ARA, arachidonic acid; LA, linoleic acid.
* The diet groups are named according to the number of the trial (1 or 2) and the level of EPA + DHA, so that, for example, 1: 2·7 is the group from trial 1 fed 2·7 % EPA + DHA of total FA.

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Table 2. Selected results from brain fatty acid (FA) composition in polar lipids at the final samplings in trial 1 of salmon maintained at 6 and 12°C† (Percentages of total fatty acids with their pooled standard errors)

Diets‡	1:1·4	1:2·7	1:3·4	1:4·3	1:5·2	SEM	Regression	ANOVA
Brain, polar lipid fraction (6°C)								
Sum *n*-3	29·3	30·4	31·3	30·3	31·7	0·3	NS, $P=0·09$	NS
Sum *n*-6	9·5^{a}	3·9^{b}	4·6^{b}	3·7^{b}	4·4^{b}	0·3	NS	**
n-3:*n*-6	3·4^{a}	7·8^{b}	7·2^{b}	8·2^{b}	7·3^{b}	0·2	NS	**
LA (18 : 2*n*-6)	3·9^{a}	1·2^{b}	1·6^{b}	1·2^{b}	1·3^{b}	0·2	NS	**
20 : 3*n*-6	1·7^{a}	0·6^{b}	0·5^{b}	0·4^{b}	0·3^{b}	0·0	NS	***
ARA (20 : 4*n*-6)	3·0^{a}	1·7^{b}	1·9^{a,b}	1·7^{b}	1·8^{b}	0·1	NS	**
LNA (18 : 3*n*-3)	1·3	0·4	0·6	0·4	0·6	0·1	NS	NS, $P=0·05$
20 : 4*n*-3	0·9^{a}	0·4^{b}	0·4^{b}	0·4^{b}	0·4^{b}	0·0	NS	*
EPA (20 : 5*n*-3)	6·4	6·2	6·3	6·2	6·2	0·0	NS	NS
DHA (22 : 6*n*-3)	17·7	20·5	20·9	20·5	21·5	0·3	NS, $P=0·09$	NS
EPA + DHA	24·1	26·7	27·2	26·7	27·7	0·3	NS, $P=0·09$	NS
Brain, polar lipid fraction (12°C)								
Sum *n*-3	31·2	30·0	30·9	32·2	31·1	0·1	NS	NS
Sum *n*-6	3·8^{a}	3·2^{b}	3·0^{b,c}	2·8^{b,c}	2·6^{c}	0·0	R^2 0·85, *	****
n-3:*n*-6	8·2^{a}	9·5^{b}	10·4^{b,c}	11·3^{c,d}	11·9^{d}	0·1	R^2 0·98, ***	****
LA (18 : 2*n*-6)	0·9^{a}	0·7^{b}	0·7^{b}	0·7^{b}	0·5^{c}	0·0	R^2 0·81, *	****
20 : 3*n*-6	0·7^{a}	0·5^{b}	0·4^{b,c}	0·3^{c}	0·3^{c}	0·0	R^2 0·91, **	****
ARA (20 : 4*n*-6)	1·6	1·5	1·4	1·4	1·4	0·0	NS	NS
LNA (18 : 3*n*-3)	0·3^{a}	0·2^{b}	0·2^{b}	0·2^{a,b}	0·2^{b}	0·0	NS	**
20 : 4*n*-3	0·4^{a}	0·4^{a,b}	0·3^{a,b}	0·3^{a,b}	0·3^{b}	0·0	R^2 0·90, **	*
EPA (20 : 5*n*-3)	5·8	5·5	5·4	5·9	5·6	0·0	NS	NS
DHA (22 : 6*n*-3)	21·7	20·9	22·2	23·0	22·1	0·2	NS	NS
EPA + DHA	27·5	26·4	27·6	28·8	27·7	0·2	NS	NS

LA, linoleic acid; ARA, arachidonic acid; LNA, *α*-linolenic acid.

^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) by ANOVA.

Although only *P* values <0·05 are considered significant, all *P* values <0·10 are given in the table. *P* values ≤0·05 are indicated by *, *P* values ≤0·01 by **, *P* values ≤0·001 by *** and *P* values ≤0·0001 by ****.

† Samples from three individual fish are analysed for each diet group at each temperature. For significant linear regressions, the adjusted R^2 is also given.

‡ The diet groups are named according to the number of the trial (1 or 2) and the level of EPA + DHA, so that, for example, 1: 2·7 is the group from trial 1 fed 2·7 % EPA + DHA of total FA.

was seen for 20 : 4*n*-3. A clear gradient was seen in the total *n*-6 FA content, decreasing from diet group 1:1·4 to 1:5·2, and also in the *n*-3:*n*-6 ratio and for 18 : 2*n*-6, 20 : 3*n*-6 and ARA.

For the retina PL from fish reared at 12°C in trial 1, EPA, DHA and ARA were stable across all diet groups, whereas total *n*-6 FA, 18 : 2*n*-6, 18 : 3*n*-3, 20 : 3*n*-6 and 20 : 3*n*-3 all decreased from diet group 1:1·4 to 1:5·2. Results from trial 2 were similar to the 12°C results from trial 1 (online Supplementary Table S2).

Fatty acid profile and osmotic resistance of erythrocytes

Trial 1. The FA composition of erythrocytes in trial 1 was also analysed at the initial organ sampling in January (approximately 1450 g fish, 12°C), as this tissue was assumed to respond more quickly than the brain and retina to the dietary FA (Table 4). Dietary effects were seen both at the initial sampling and the two temperature samplings for total *n*-3 FA, which decreased with decreasing dietary EPA + DHA. An opposite pattern was seen for total *n*-6 FA, and consequently the *n*-3:*n*-6 ratio decreased as dietary EPA + DHA levels decreased. Linear regressions for EPA were non-significant at all three samplings, whereas significant regressions were seen for DHA at two of the samplings, with a similar trend at the third sampling (final sampling 12°C). ANOVA also showed significant effects on DHA at all samplings, with reduced levels in group 1:1·4 at the initial organ sampling, whereas levels were further reduced in group 1:1·4 and also reduced in group 1:2·7 at the final samplings. At 6°C, the intermediates in the pathway of elongation from LNA to EPA and DHA were elevated in diet groups with low dietary EPA + DHA; 18 : 4*n*-3 was only detected in erythrocytes from fish in diet groups 1:1·4 and 1:2·7, whereas 20 : 4*n*-3 and 22 : 5*n*-3 both decreased linearly with increasing dietary EPA + DHA. A similar pattern was not seen for the two sampling points at 12°C.

ARA was elevated in diet group 1:1·4 at all three sampling points. For the final sampling at 6°C, the values from individual fish found in diet group 1:1·4 ranged from 4·2 to 6·2 % of total FA, and were significantly higher than the other diet groups where the values ranged from 3·1 to 4·1 %. For the two samplings at 12°C, diet group 1:1·4 was significantly higher in ARA than most of the other diet groups, but this pattern was less pronounced than at 6°C. MUFA was also significantly elevated in erythrocytes of fish fed diet 1:1·4 compared with the other diet groups at the final samplings at both temperatures. This appeared mainly because of 18 : 1*n*-9 – a FA that increases in the diet with increasing vegetable oil inclusion. This effect was most pronounced at 6°C, where diet group 1:2·7 was also significantly higher in MUFA than diet group 1:5·2.

Trial 2. Similar results were observed as in trial 1 (online Supplementary Table S3), with significant differences in DHA, with values of 29·6, 32·6, 35·2 and 36·6 for diet groups 2:1·3 to 2:7·4, respectively. Only the two latter groups were not significantly different from each other. No differences were seen in EPA. No differences were observed in the fragility of

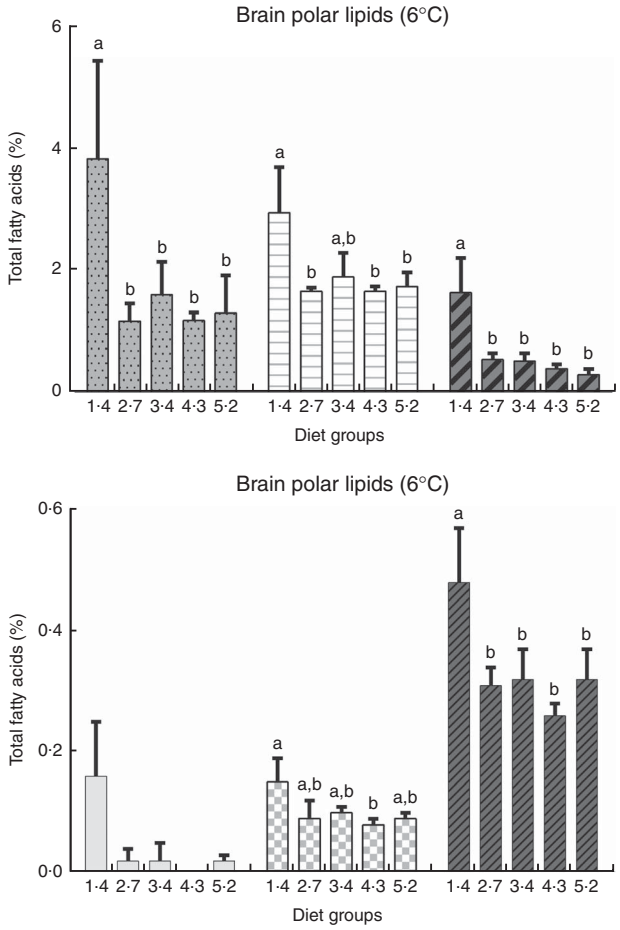

Fig. 1. *n*-6 Fatty acids in brain polar lipids of Atlantic salmon reared at 6°C in trial 1, fed dietary EPA + DHA from 1·4 to 5·2 % of total fatty acids. Values are means, and standard deviations represented by vertical bars for three individually analysed fish per diet group. [a,b] Mean values with unlike letters were significantly different ($P < 0·05$) by ANOVA followed by Tukey's honest significant difference *post hoc* test. No statistical analysis was conducted on 18 : 3*n*-6 because of several samples being below the limit of quantification. ▩, 18 : 2*n*-6; □, 22 : 4*n*-6; ▨, 20 : 3*n*-6; □, 18 : 3*n*-6; ▩, 22 : 4*n*-6; ▨, 20 : 2*n*-6.

erythrocytes; IC50 values (the concentration where hemolysis occurs in 50 % of the blood cells) were 6·77, 6·65, 6·97 and 6·81 for diet groups 2:1·3 to 2:7·4, respectively, with overlapping CI and no significant differences.

Temperature effects on tissue fatty acid composition

The two-way ANOVA revealed several effects of temperature on the FA composition of brain and retina PL and erythrocytes (Table 5). In all three tissues, SFA was significantly higher at 12°C, whereas PUFA was significantly lower. MUFA was higher at 12°C in erythrocytes, but unchanged in the two other tissues. In erythrocytes, the difference in PUFA between the two temperatures was caused by decreases in both total *n*-3 FA and total *n*-6 FA, whereas in the brain and retina there was only a difference in *n*-6 FA. The *n*-3:*n*-6 ratio was increased at 12°C in the brain and retina, whereas no temperature difference was seen in erythrocytes. Among individual FA, EPA was significantly lower at 12 compared with 6°C in all three tissues. DHA, on the other hand, was significantly higher at 12 compared with 6°C in the brain, with a similar tendency being apparent in the retina as well. ARA was significantly higher at 6 compared with 12°C in all three tissues.

In brain PL, there were a few significant interactions between diet and temperature; however, this occurred for ARA and total *n*-6 FA (Table 5). Retina PL and erythrocytes differed from the brain in this regard, and were more similar to each other, as significant interactions between diet and temperature were seen for SFA, total *n*-3 FA and EPA + DHA in both tissues. There was a significant interaction for ARA in the retina, with a similar trend in erythrocytes. An interaction effect on total PUFA was seen in the retina only, whereas there was a significant interaction effect on total *n*-6 FA in erythrocytes.

Discussion

Despite the inclusion of dietary LNA at approximately 10 % of FA, as well as graded levels of EPA + DHA from 1·3 to 7·4 % of FA, we observed reduction of DHA levels in the brain, retina and erythrocytes, demonstrating that dietary LNA alone is not sufficient to maintain DHA levels in these tissues and that dietary EPA + DHA > 2·7 % of FA is required for this. This is the same level that we concluded was required for optimal growth (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results). The aim of these feeding trials was not to induce severe deficiency symptoms by excluding all EFA from the diets, but rather to investigate how much EPA + DHA levels can be reduced in practical, high-energy diets and still achieve maximum performance in fish.

The tendency of reduced DHA in the brain of fish fed 1·4 % EPA + DHA and significantly reduced DHA in the retina of fish fed 1·4 and 2·7 % EPA + DHA was only seen at 6°C, whereas at 12°C an effect was only observed in erythrocytes in groups fed ≤2·7 % EPA + DHA of total FA in both trials. There seems to be a hierarchy within these three tissues, with DHA being most conserved in the brain, followed by the retina and then erythrocytes, consistent with reports that DHA is conserved in the brain and the retina when animals are fed diets deficient in *n*-3 FA[26]. Although not intended in the trial design, the 6°C fish grew to a slightly bigger final size, because of the 12°C group growing slightly slower than that expected in trial 1. This may have played a role in the DHA levels being affected in the brain and retina only at 6°C, as growing to a larger final size increased the dilution effect of pre-existing body stores of DHA. Alternatively, the production, turnover or transport of DHA differs with temperature, but as retention of DHA was similar at the two temperatures (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results) this seems unlikely.

An important question is 'why, despite a net production of DHA and considerable amounts of DHA found in muscle lipids of the groups fed ≤2·7 % EPA + DHA (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results), are fish not able to maintain tissue levels in the brain, retina and erythrocytes?'. A possible explanation could be limitations in transport to these tissues/mobilisation from muscle tissue. In salmon par fed a semi-purified diet with no *n*-3 or *n*-6 FA, there

Table 3. Selected results from fatty acid composition in polar lipids in the retina of salmon in trial 1 kept at 6 and 12°C† (Percentages of total fatty acids with their pooled standard errors)

Diets‡	1:1·4	1:2·7	1:3·4	1:4·3	1:5·2	SEM	Regression	ANOVA
Retina, polar lipid fraction (6°C)								
Sum *n*-3	37·8^{a}	40·9^{a,b}	45·6^{b}	45·5^{b}	42·8^{a,b}	0·4	R^2 0·29, *	**
Sum *n*-6	12·4^{a}	9·6^{a,b}	9·8^{a,b}	8·7^{b}	6·8^{b}	0·2	R^2 0·68, ****	**
n-3:*n*-6	3·1^{a}	4·4^{a,b}	4·7^{b}	5·2^{b,c}	6·3^{c}	0·1	R^2 0·80, ****	***
LA (18 : 2*n*-6)	4·6^{a}	4·0^{a,b}	4·1^{a,b}	3·4^{a,b}	2·5^{b}	0·1	R^2 0·54, ***	*
20 : 3*n*-6	2·2^{a}	1·4^{b}	1·2^{b}	1·0^{b,c}	0·6^{c}	0·0	R^2 0·85, ****	****
ARA (20 : 4*n*-6)	4·5^{a}	3·6^{a,b}	3·7^{a,b}	3·8^{a,b}	2·9^{b}	0·1	R^2 0·38, **	*
LNA (18 : 3*n*-3)	1·1	1·3	1·3	1·0	0·7	0·1	NS	NS
20 : 4*n*-3	0·8	0·8	0·8	0·7	0·5	0·0	R^2 0·34, **	NS, $P=0·06$
EPA	4·6	8·3	7·7	7·9	5·3	0·3	NS	NS, $P=0·05$
DHA	29·1^{a}	28·1^{a}	33·8^{b}	34·1^{b}	34·2^{b}	0·5	R^2 0·34, **	*
EPA + DHA	33·6^{a}	36·5^{a,b}	41·5^{b}	42·0^{b}	39·5^{a,b}	0·4	R^2 0·39, **	**
Retina, polar lipid fraction (12°C)								
Sum *n*-3	44·0	42·5	40·0	41·2	41·0	0·4	NS, $P=0·09$	NS
Sum *n*-6	9·7^{a}	8·2^{a,b}	8·3^{a,b}	6·9^{b}	7·0^{b}	0·1	R^2 0·64, ***	**
n-3:*n*-6	4·6^{a}	5·2^{a,b}	4·8^{a,b}	6·0^{b}	5·9^{a,b}	0·1	R^2 0·42, **	*
LA (18 : 2*n*-6)	4·1^{a}	2·9^{b}	3·2^{a,b}	2·5^{b}	2·4^{b}	0·1	R^2 0·57, ***	**
20 : 3*n*-6	1·8^{a}	1·4^{a,b}	1·3^{b}	0·8^{c}	0·8^{c}	0·0	R^2 0·87, ****	****
ARA (20 : 4*n*-6)	3·1	3·0	2·8	2·8	3·0	0·0	NS	NS
ALA (18 : 3*n*-3)	1·3^{a}	0·7^{b}	0·8^{b}	0·7^{b}	0·7^{b}	0·0	R^2 0·37, **	**
20 : 4*n*-3	0·8^{a}	0·6^{a,b}	0·6^{a,b}	0·4^{b}	0·4^{b}	0·0	R^2 0·61, ***	**
EPA	5·3	4·0	4·2	4·5	5·1	0·1	NS	NS
DHA	34·8	35·5	32·9	34·0	33·1	0·5	NS	NS
EPA + DHA	40·1	39·5	37·1	38·5	38·2	0·4	NS	NS

LA, linoleic acid; ARA, arachidonic acid; LNA, *α*-linolenic acid.
^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) by ANOVA.
P values ≤0·05 are indicated by *, *P* values ≤0·01 by **, *P* values ≤0·001 by *** and *P* values ≤0·0001 by ****.
† Samples from three individual fish are analysed for each diet group at each temperature.
‡ The diet groups are named according to the number of the trial (1 or 2) and the level of EPA + DHA, so that, for example, 1: 2·7 is the group from trial 1 fed 2·7 % EPA + DHA of total FA.

was no change in DHA as a percentage of total carcass lipid during the experimental period, whereas DHA decreased significantly in the blood and liver[27]. A low-fat diet and poor growth probably meant that there was very limited lipid deposition and the fat content in the fish was reduced by half during the study, but still the results indicate that there is no mobilisation of DHA from the muscle tissue. However, this differs from the situation in our study where DHA was continuously supplied by the diet and presumably also produced by desaturation and elongation in the liver, but still seems to be transported to and incorporated in muscle tissue in relatively high amounts (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results).

A study in juvenile Atlantic salmon showed that DHA is strictly regulated in the brain and retina irrespective of dietary concentrations, whereas EPA is not regulated to the same extent[12]. In trial 1 at 6°C, reduced levels of DHA were seen despite this strict regulation, suggesting deficiency. In the retina, DHA provides the flexible environment required for the pigment rhodopsin to undergo the structural transitions needed to enable vision in low-light conditions[28]. Reduced feeding behaviour and presumably reduced visual acuity at low light intensity has been observed in herring (*Clupea harenegus*) larvae deficient in DHA[11], and DHA deficiency also reduced schooling behaviour in larval yellowtail (*Seriola quinqueradiata*)[29]. However, the reduction in retinal DHA levels in the study by Bell *et al.*[11] was much larger than in our study. No difference was seen in feed intake in our study, but good light conditions for feeding were used during the experiment.

In herring, decreased DHA in the retina seemed to be compensated for by increased EPA and 22 : 5*n*-3[11], whereas no compensatory increase in these FA were seen in our study. The gradients seen in *n*-6 FA in this study may to some extent be compensatory changes for reduced levels of *n*-3 FA. However, the increase in *n*-6 FA was generally larger than the decrease in *n*-3 FA, both in the brain and in the retina, suggesting that this is not the complete explanation. Increased dietary *n*-6 FA seem to cause increased tissue levels in our fish regardless of whether or not there is a deficiency in *n*-3 FA, and it has been suggested that the FA composition of fish brain and retina may be more sensitive to dietary input than these tissues are in mammals[30]. However, the brain at 6°C did not exhibit a gradient for *n*-6 FA like the other tissues and brain sampled at 12°C, but rather showed highly elevated levels in diet group 1:1·4. Furthermore, LNA and 20 : 4*n*-3 were both elevated to a much higher extent in diet group 1:1:4 at 6°C, and the *n*-3:*n*-6 ratio was less than half of any other diet group including the groups fed equally low EPA + DHA at 12°C. We interpret this as a response to a DHA deficiency in the brain at 6°C. Such a response in other FA in the brain was not observed in a salmon trial where only DHA varied between the diets[14], but the reduction in brain DHA was less than that in our trial, despite only 0·9 % EPA + DHA of dietary FA (approximately 1·5 g/kg feed) being used, probably due to the short duration of the study. In a long-term study with rainbow trout, fish that were fed an *n*-3-deficient diet had approximately 50 % reduction in brain DHA, but the increase in the content of long-chain *n*-6 FA was still 2-fold the decrease in DHA[31]. The latter study

Table 4. Selected results from fatty acid composition in erythrocytes†
(Percentages of total fatty acids with their standard errors)

Diets‡	1:1·4	1:2·7	1:3·4	1:4·3	1:5·2	SEM	Regression	ANOVA
Initial sampling (12°C)								
Total SFA	29·4	30·3	29·2	29·3	29·4	0·1	NS	NS
Total MUFA	13·0	11·3	11·7	12·7	11·2	0·2	NS	NS
Total PUFA	57·3^{a}	58·1^{a,b}	59·0^{b}	57·8^{a,b}	59·2^{b}	0·1	NS	**
Sum *n*-3	44·1^{a}	47·1^{b}	49·0^{b,c}	48·6^{b,c}	50·7^{c}	0·2	R^2 0·83, *	****
Sum *n*-6	13·2^{a}	11·1^{b}	10·0^{b,c}	9·3^{b,c}	8·5^{c}	0·1	R^2 0·92, **	****
n-3:*n*-6	3·3^{a}	4·3^{b}	4·9^{b,c}	5·2^{c}	6·0^{d}	0·1	R^2 0·97, ***	****
LA (18 : 2*n*-6)	5·4^{a}	4·6^{a,b}	4·4^{a,b}	4·3^{a,b}	3·4^{b}	0·1	R^2 0·86, **	**
20 : 3*n*-6	2·6^{a}	2·0^{b}	1·6^{c}	1·1^{d}	0·8^{e}	0·0	R^2 0·98, ***	****
ARA (20 : 4*n*-6)	4·1^{a}	3·5^{a,b}	3·1^{b}	2·8^{b}	3·4^{a,b}	0·1	NS	**
LNA (18 : 3*n*-3)	1·4	1·3	1·3	1·3	1·1	0·0	R^2 0·76, *	NS
20 : 4*n*-3	1·6^{a,b}	1·5^{a,b}	1·8^{a}	1·4^{b,c}	1·1^{c}	0·0	NS	**
EPA	5·7^{a}	6·6^{a,b}	8·0^{b}	7·2^{a,b}	7·6^{a,b}	0·1	NS	*
DHA	32·4^{a}	34·3^{a,b}	34·2^{a,b}	35·2^{a,b}	38·0^{b}	0·3	R^2 0·83, *	*
EPA + DHA	38·0^{a}	41·0^{a,b}	42·2^{b,c}	42·4^{b,c}	45·6^{c}	0·2	R^2 0·89, **	***
Final sampling (12°C)								
Total SFA	28·2^{a}	30·3^{b}	30·4^{b}	30·2^{b}	30·2^{b}	0·0	NS	****
Total MUFA	13·7^{a}	10·3^{b}	10·3^{b}	10·3^{b}	10·2^{b}	0·0	NS	****
Total PUFA	57·7^{a}	58·9^{a,b}	58·8^{a,b}	59·1^{a,b}	59·2^{b}	0·0	NS, $P=0·07$	***
Sum *n*-3	45·0^{a}	48·2^{b}	49·6^{b,c}	50·2^{c}	51·1^{d}	0·1	R^2 0·85, *	****
Sum *n*-6	12·8^{a}	10·7^{b}	9·2^{c}	8·9^{c}	8·1^{d}	0·2	R^2 0·87, **	****
n-3:*n*-6	3·5^{a}	4·5^{b}	5·4^{c}	5·7^{c}	6·3^{d}	0·0	R^2 0·95, **	****
LA (18 : 2*n*-6)	5·8^{a}	4·6^{b}	3·9^{c}	3·8^{c}	3·2^{d}	0·0	R^2 0·86, **	****
20 : 3*n*-6	2·4^{a}	1·6^{b}	1·2^{b}	0·9^{b}	0·8^{b}	0·0	R^2 0·88, **	****
ARA (20 : 4*n*-6)	3·5^{a,b}	3·8^{a}	3·4^{b}	3·4^{b}	3·5^{a,b}	0·0	NS	*
LNA (18 : 3*n*-3)	1·9^{a}	1·3^{b}	1·2^{b,c}	1·2^{b,c}	1·0^{c}	0·0	NS	****
20 : 4*n*-3	2·1^{a}	1·6^{b}	1·4^{b,c}	1·3^{b,c}	1·3^{c}	0·0	R^2 0·81, *	****
EPA	6·8^{a,b}	6·7^{a}	6·4^{a}	7·3^{a,b}	7·5^{b}	0·0	NS	*
DHA	30·0^{a}	34·7^{b}	37·0^{b,c}	36·6^{b,c}	37·5^{c}	0·1	NS, $P=0·06$	****
EPA + DHA	36·8^{a}	41·4^{b}	43·4^{c}	43·9^{c,d}	45·0^{d}	0·1	R^2 0·80, *	****
Final sampling (6°C)								
Total SFA	26·9^{a}	28·2^{b}	27·9^{a,b}	27·3^{a,b}	28·6^{b}	0·1	NS	**
Total MUFA	12·2^{a}	10·1^{b}	8·9^{b,c}	8·9^{b,c}	8·8^{c}	0·1	NS	****
Total PUFA	60·6^{a}	61·7^{a,b}	63·1^{b,c}	63·8^{c}	62·6^{a,b,c}	0·1	NS	**
Sum *n*-3	46·1^{a}	50·6^{b}	53·2^{c}	54·4^{c}	54·0^{c}	0·1	R^2 0·76, *	****
Sum *n*-6	14·5^{a}	11·1^{b}	9·9^{c}	9·4^{c}	8·6^{d}	0·0	R^2 0·82, *	****
n-3:*n*-6	3·2^{a}	4·6^{b}	5·4^{c}	5·8^{c}	6·3^{d}	0·0	R^2 0·92, **	****
LA (18 : 2*n*-6)	5·6^{a}	4·7^{b}	3·9^{c}	3·8^{c}	3·4^{c}	0·0	R^2 0·91, **	****
20 : 3*n*-6	2·8^{a}	1·9^{b}	1·4^{c}	1·1^{c}	0·8^{d}	0·0	R^2 0·90, **	****
ARA (20 : 4*n*-6)	5·0^{a}	3·7^{b}	3·8^{b}	3·6^{b}	3·6^{b}	0·0	NS	****
LNA (18 : 3*n*-3)	1·7^{a}	1·4^{b}	1·2^{b,c}	1·2^{b,c}	1·1^{c}	0·0	R^2 0·80, *	****
20 : 4*n*-3	2·4^{a}	2·3^{a}	1·8^{b}	1·7^{b}	1·5^{b}	0·0	R^2 0·91, **	****
EPA	7·9^{a}	9·4^{b}	8·7^{a,b}	9·1^{a,b}	9·4^{b}	0·1	NS	**
DHA	29·3^{a}	32·7^{b}	37·4^{c}	38·5^{c}	38·1^{c}	0·1	R^2 0·78, *	****
EPA + DHA	37·1^{a}	42·0^{b}	46·1^{c}	47·6^{c}	47·5^{c}	0·1	R^2 0·81, *	****

LA, linoleic acid; ARA, arachidonic acid; LNA, *α*-linolenic acid.
^{a,b,c,d,e} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$) by ANOVA.
P values ≤0·05 are indicated by *, *P* values ≤0·01 by **, *P* values ≤0·001 by *** and *P* values ≤0·0001 by ****.
† From the initial sampling, three samples per dietary group are analysed, whereas eight samples per group are analysed at the final samplings at each temperature. The regression analyses are conducted on tank means.
‡ The diet groups are named according to the number of the trial (1 or 2) and the level of EPA + DHA, so that, for example, 1: 2·7 is the group from trial 1 fed 2·7 % EPA + DHA of total FA.

corresponded well with our results and indicates that a large increase in *n*-6 FA is part of the picture of a DHA deficiency in the salmonid brain, although the reasons or mechanisms for this are unknown. The role of *n*-6 FA is also reflected in the significant interaction between diet and temperature on ARA and total *n*-6 FA in brain PL in our study.

In the erythrocytes, in contrast to the brain and retina, total *n*-6 FA were only increased to compensate for the reduction in *n*-3 FA, probably to maintain membrane fluidity[32], so that the total PUFA remained constant. A further difference from the brain and retina is that MUFA, which was not affected by diet in the brain and retina, generally increased in the erythrocytes with decreasing dietary EPA + DHA. However, this was mainly 18 : 1*n*-9, whereas mead acid (20 : 3*n*-9), which has been used as a marker for EFA deficiency[31], was not detected in the erythrocytes, nor in any other tissues of our fish. In salmon par fed a semi-purified diet with no *n*-3 or *n*-6 FA, both 18 : 1*n*-9 and 20 : 3*n*-9 were significantly increased in blood PL in the 3rd and 4th month of the study[27]. Erythrocyte data from the final sampling at both temperatures in trial 1 and in trial 2 indicate that fish fed ≤2·7 % EPA + DHA of total FA are deficient in DHA as they are unable to maintain tissue concentrations of this important FA. However, from the initial organ sampling in trial 1, after 6 months of feeding the experimental diets, a natural

Table 5. Temperature differences in the fatty acid composition of the brain and retina polar lipid fraction and erythrocytes, all diet groups combined† (Mean values with their standard errors)

	6°C	12°C	SEM	ANOVA (temperature)	ANOVA (diet)	Interaction
Brain, polar lipid fraction						
Total SFA	21·8	22·9	0·1	***	NS	NS
Total MUFA	34·9	34·6	0·2	NS	NS	NS
Total PUFA	35·8	34·2	0·3	**	*	NS
Sum *n*-3	30·6	31·1	0·2	NS	NS	NS
Sum *n*-6	5·2	3·9	0·2	****	***	**
n-3:*n*-6	6·8	10·3	0·1	****	****	NS
EPA (20 : 5*n*-3)	6·3	5·6	0·0	****	NS	NS
DHA (22 : 6*n*-3)	20·2	22·0	0·2	**	NS, $P=0·10$	NS
EPA + DHA	26·5	27·6	0·2	*	NS	NS
ARA (20 : 4*n*-6)	2·0	1·5	0·0	****	***	*
Retina, polar lipid fraction						
Total SFA	23·1	24·9	0·1	****	*	***
Total MUFA	20·6	20·5	0·3	NS	NS	NS
Total PUFA	52·0	49·8	0·4	NS	NS	**
Sum *n*-3	42·5	41·7	0·4	NS	NS	**
Sum *n*-6	9·5	8·0	0·1	***	****	NS
n-3:*n*-6	4·7	5·3	0·1	**	****	NS
EPA (20 : 5*n*-3)	6·8	4·6	0·2	***	NS	**
DHA (22 : 6*n*-3)	31·8	34·1	0·5	NS	NS	*
EPA + DHA	38·6	38·7	0·4	NS	NS	**
ARA (20 : 4*n*-6)	3·7	2·9	0·0	****	*	NS, $P=0·052$
Erythrocytes						
Total SFA	27·8	29·9	0·0	****	****	*
Total MUFA	9·8	10·9	0·0	****	****	NS
Total PUFA	62·3	58·8	0·0	****	****	NS
Sum *n*-3	51·6	48·9	0·1	****	****	*
Sum *n*-6	10·7	9·9	0·1	****	****	***
n-3:*n*-6	5·1	5·1	0·0	NS	****	NS
EPA (20 : 5*n*-3)	8·9	6·9	0·0	****	**	NS, $P=0·08$
DHA (22 : 6*n*-3)	35·2	35·2	0·1	NS	****	NS, $P=0·07$
EPA + DHA	44·1	42·1	0·1	****	****	**
ARA (20 : 4*n*-6)	3·9	3·5	0·0	****	****	****

ARA, arachidonic acid. *P* values ≤0·05 are indicated by *, *P* values ≤0·01 by **, *P* values ≤0·001 by *** and *P* values ≤0·0001 by ****.
† A two-way ANOVA was run with diet and temperature as the two factors.

conclusion would have been that only diet 1:1·4 was deficient in DHA, underlining the importance of long-term trials when investigating EFA requirements. There were no differences in osmotic fragility, suggesting that fragile blood cells may be a symptom of a more severe deficiency, thus not achieved in our trial. Diets deficient in *n*-3 FA have caused increased erythrocytes fragility in rainbow trout, but in both cases with diets completely devoid of EPA and DHA[16,33]. Only one of these studies reported the FA composition of erythrocytes, and as diets devoid also of shorter-chain *n*-3 FA were used in this case DHA in erythrocytes was reduced 4-fold or more[33], which is a dramatic reduction compared with our data. Feeding high EPA + DHA reduced osmotic fragility in salmon affected by Hitra disease, but this effect was only apparent at 6°C and not at 11°C[34]. As this test was conducted only in trial 2 in our study, we do not know whether there would have been an effect of low dietary EPA + DHA at 6°C; however, it is also plausible that either severe EFA deficiency or a disease known to affect membrane fragility, such as the Hitra disease, are needed to induce a response in blood cell fragility.

Despite FO being the only source of detectable levels of ARA in the diets, ARA in erythrocytes as well as the brain and retina was the same or higher in fish fed diets without FO inclusion (diet 1:1·4 and 2:1·3) compared with other diet groups. This clearly shows that the fish efficiently elongated and desaturated linoleic acid (LA, 18 : 2*n*-6) to ARA. The apparent increased activity in this pathway with decreasing dietary FO inclusion could either be a consequence of increased availability of substrate (LA) or to compensate for the reduction in EPA and DHA, as ARA can have a similar structural role in membranes, although its major role may be as the precursor for eicosanoid production in erythrocytes. Both at 6°C in trial 1 and in trial 2, diet groups 1:1·4 and 2:1·3 had significantly and clearly elevated ARA levels in erythrocytes, whereas the other groups had very similar levels; thus, this difference is unlikely to be a result of substrate availability alone. These were also the two samplings where DHA reduction in the erythrocytes of these dietary groups was the most pronounced, suggesting a direct balance between DHA and ARA in this tissue. The increase in twenty carbon *n*-3 intermediate FA such as 20 : 3*n*-3 and 20 : 4*n*-3 with decreasing dietary EPA + DHA in the brain, retina and erythrocytes indicates that there is elongation and desaturation activity going on along the *n*-3 pathway, but not sufficient to maintain tissue DHA levels.

If the requirements for EPA and DHA were temperature dependent, we would expect to see higher tissue levels at low temperatures, at least in groups with sufficient dietary supply.

There seems to be an increased need for both EPA and ARA in the membranes at 6°C, as these are consistently higher in all tissues at low temperatures. ARA was also the only FA with a significant or close to significant interaction effect between diet and temperature in all three tissues tested. No effect of temperature was seen on DHA in erythrocytes, and it has been observed previously that DHA did not seem to participate in the adaptation to temperature in the liver PL of marine and freshwater fish[35]. The 'temperature' effects seen in brain DHA in our study and the similar tendency in the retina were probably mainly caused by the effects of low dietary EPA + DHA at 6°C. It has been shown that functional and structural integrity of membranes upon changing temperature is primarily preserved by other means than changing the FA composition of the brain PL in fish[36], and that DHA is a molecule of extreme flexibility with rapid transitions between a large number of conformers, allowing it to function well in membranes over a large range of temperatures[28]. Although the ratio of SFA:unsaturated FA varied with temperature in brain membranes of fish adapted to 5 or 25°C, the content of DHA did not vary[36]. In erythrocytes, findings similar to our results have been reported in Atlantic cod (*Gadus morhua*), where SFA and MUFA decreased while PUFA increased at lower temperature[37]. Unlike DHA, ARA and EPA seemed to be involved in homeoviscous adaptation in the brain, retina and erythrocytes in our study, perhaps reflecting the less strict regulation of these FA compared with DHA in the mentioned tissues.

Compared with DHA, a few differences were seen in EPA in the brain, retina and erythrocytes, and the dietary effects seen in the retina and erythrocytes were not consistent between samplings and trials. Furthermore, DHA showed a high degree of enrichment in PL compared with NL, whereas this was not always the case for EPA. These observations indicate that dietary levels exceeded requirements in all diet groups, and are consistent with a lower retention for EPA than DHA reported in these trials (G Rosenlund, BE Torstensen, I Stubhaug, N Usman and NH Sissener, unpublished results). Although the role of EPA is different from DHA, being bioactive rather than structural, equal tissue levels indicate sufficient substrate for bioactive components such as eicosanoids in all diet groups. This indicates that DHA is the limiting factor in diets where EPA and DHA are provided in the ratios found in FM and FO. From these ingredients, EPA + DHA are both present as a 'package deal'. This may not be the case in the future if EPA and DHA become available from GM land plants, for instance, as some of these produce either EPA or DHA[38,39], and are being tested for use in salmon feeds[40]. In this scenario, it will be important to distinguish clearly between EPA and DHA and determine separate requirements for each of them.

In conclusion, ≤2·7 % EPA + DHA of total FA (≤9 g/kg feed) is not sufficient for maintaining DHA tissue status in the brain, retina and erythrocytes of Atlantic salmon during the seawater production cycle, even when combined with a dietary supply of 18 : 3*n*-3. The tissue effects seem more severe at low temperatures, but this requires further investigation. Finally, DHA seems to be the limiting factor when EPA + DHA are provided in the ratios found in FM and FO.

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G. R. designed and planned the trials; B. E. T. designed the trials; I. S. formulated diets; G. R. and N. H. S. participated in the samplings; and N. H. S. was mainly responsible for data analyses and writing of the manuscript. All the authors participated in the writing of the manuscript and approved the final version.

There are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S0007114516000945

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