Dietary Deficiency of Docosahexaenoic Acid Impairs Vision at Low Light Intensities in Juvenile Herring (*Clupea harengus* L.)

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ABSTRACT: In the retina of herring (*Clupea harengus* L.), rods are recruited from about 8 wk after hatching, and from this time there is a linear relationship between the number of rods in the photoreceptor cell population and the content of di22:6n-3 molecular species of phospholipids. Juvenile herring were reared from four weeks' post-hatching for 15 wk on either *Artemia* nauplii deficient in 22:6n-3 or on enriched *Artemia* nauplii containing 4.3% 22:6n-3. The visual performance of the fish was then determined at three light intensities (0.01, 0.1, and 1.0 lux) by observing their frequency of striking at live *Artemia* nauplii using infrared video recording. Herring reared on the diet containing no 22:6n-3 were less active predators, especially at the lowest light intensity where very few strikes were observed. The eyes of these fish contained greatly reduced levels of di22:6n-3 molecular species of total phospholipid, 2.1% vs. 12.0% in fish supplemented with 22:6n-3. The contribution of saturated and monounsaturated fatty acids in the molecular species of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) was virtually unchanged, while 20:5n-3 and 22:5n-3 largely replaced 22:6n-3. There was an almost complete disappearance of di22:6n-3 PC, while the amounts of di22:6n-3 PE and PS fell by 18.1 and 20.6% to 2.7 and 7.6%, respectively. The dipolyunsaturated molecular species di20:5n-3, 20:5n-3/22:5n-3, and di22:5n-3 made up a substantial part of the deficit. We conclude that a dietary deficiency of 22:6n-3 during the period early in rod development impairs visual performance such that the fish can no longer feed at low light intensities.

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Abbreviations: ANOVA, analysis of variance; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. Molecular species are abbreviated as follows: e.g., 16:0/22:6 PC is 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

In terrestrial vertebrates, docosahexaenoic acid (22:6n-3) is concentrated in neural tissue, especially in retina and brain (1,2) and testes (3), and this is the case also in marine vertebrates, including fish, even though fish tissues in general are richer in 22:6n-3 than mammalian tissues. Vertebrate retinae contain large amounts of di-docosahexaenoyl (22:6n-3/22:6n-3) glycerophospholipid which is concentrated in rod outer segment membranes (1,4,5). However, the concentration of 22:6n-3 in the retina of fish is particularly impressive, e.g., in cod (*Gadus morhua*) 71.8% of phosphatidylethanolamine (PE), 59.7% of phosphatidylserine (PS), and 29.3% of phosphatidylcholine (PC) occurs as 22:6n-3/22:6n-3 molecular species (6). The amounts in the retina of herring are slightly lower, equivalent to 30.6% of total glycerophospholipid in adult fish (7). Therefore, there is strong circumstantial evidence that 22:6n-3 is essential for neural function.

Docosahexaenoic acid (22:6n-3) is an essential fatty acid in carnivorous species of fish and other animals which appear to have lost the Δ5 desaturase and cannot therefore elongate and desaturate dietary precursor C_{18} polyunsaturated fatty acids (PUFA) to C_{20} PUFA (8–10). It is now believed that the final step in the formation of 22:6n-3 in mammals involves Δ6 desaturation of 24:5n-3 and subsequent chain-shortening of 24:6n-3 (11), and the same pathway probably operates in fish. Furthermore, in some species the ability to synthesize 22:6n-3 from dietary precursors at times of high demand during neural development may be inadequate, and 22:6n-3 deficiency at this time causes impairment of visual and cognitive abilities in young animals (12,13). Recent evidence suggests this is also the case in human infants, especially preterm infants, and that such deficiencies lead to visual and cognitive subperformance (14–16). This has led to the recommendation in the United Kingdom that infant formula feeds should contain 22:6n-3 (17).

Induction of 22:6n-3 deficiency in experimental animals is difficult since 22:6n-3 is retained avidly in neural tissues (12,13), and experiments to test visual and behavioral subper-

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formance are difficult to devise, especially with a view to separating effects on vision (performance of retina) and cognition (performance of brain) (18). The free-swimming larvae of marine fish offer a number of advantages over fetal mammals for studies on the effects of 22:6n-3 deficiency. Their small size and rapid growth from first-feeding mean that they have a very large demand for 22:6n-3 which must be met directly from the diet, and deficiencies of 22:6n-3 are thus easy to induce. At hatching, the larvae of many species of marine fish have almost pure cone retinae with rods appearing later in development. In herring larvae, significant numbers of rods are recruited to the photoreceptor cell population progressively from about 8 wk after hatching (length 2.5 cm) to maturity (length 25–30 cm) to give a rod/cone ratio of 15–20:1 (19). The length of the fish is a good indicator of rod development (19). It has been shown that the amount of di22:6n-3 PE, PS, and PC in herring eyes correlates with the proportion of rods in the photoreceptor population and that the pure cone retina of larvae contains about half the amount of di22:6n-3 phospholipid in adult retina (7). The present study sought to reduce the content of di22:6n-3 phospholipid in the eyes of young herring by feeding a diet lacking 22:6n-3 and to determine whether this affected the visual performance of the fish as measured by their ability to detect and strike at prey at different light intensities chosen specifically to test cone vision and rod (scotopic) vision.

TABLE 1
The Fatty Acid Composition of the Experimental Diets (wt%)[a]

Fatty acid	22:6n-3-Supplemented	22:6n-3-Deficient
18:2n-6	3.9	6.3
18:3n-3	12.2	20.6
20:5n-3	11.6	2.9
22:5n-3	0.8	0
22:6n-3	4.3	0
Total saturates	16.4	22.7
Total monounsaturates	33.8	31.1
Total n-3 PUFA	32.6	27.4
Total n-6 PUFA	4.1	6.3

[a]See Reference 20 for full details. PUFA, polyunsaturated fatty acid.

MATERIALS AND METHODS

Experimental animals. Spring spawning herring were caught in the Firth of Clyde, Scotland. The mixed progeny from artificially fertilized eggs from twelve females and six males were reared in 450-L tanks containing seawater with a salinity of 30 to 32‰. Ambient seawater temperatures were used and rose from 8.5 ± 0.4°C during the embryo stage to 13.1 ± 0.7°C during the last week of the experiment. Larvae were fed initially from eight days' post-hatching on nauplii of *Artemia* sp. (AF grade; Artemia Systems, Baasrode, Belgium). After 20 days' feeding on this diet, larvae were transferred to the experimental diets which consisted of two strains of San Francisco Bay *Artemia* nauplii with relatively high or low n-3 fatty acid contents, the former supplemented with n-3 PUFA to provide 4.3% of fatty acids as 22:6n-3 and the latter supplemented with coconut oil giving no 22:6n-3 (Table 1, see Ref. 20 for details). Two replicate tanks of larvae were fed on each of these diets for a further 102 d before sampling.

Chemicals and solvents. Phospholipase C from *Bacillus cereus* was purchased from Boehringer Corporation (London) Ltd. (Lewes, East Sussex, England). Oxalyl chloride and anthracene-9-carboxylic acid were supplied by Aldrich Chemical Co. (Gillingham, Dorset, England). All other chemicals and biochemicals were purchased from Sigma (Poole, Dorset, England), and solvents of high-performance liquid chromatography (HPLC) grade were obtained from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Measurement of visual performance. Twenty fish were sampled at random from each tank, and their feeding behavior recorded in tanks 59 cm in diameter using an infrared video recording system with strobed infrared illumination (21). Infrared illumination was provided by a tungsten halogen bulb and infrared transmitting filter with a sharp cut-off at 700 nm which is outside the reported range of spectral sensitivity for larval and adult herring (22,23) or of any other marine fish species (24). Diffuse visible light was provided by a tungsten halogen lamp through a fiber-optic light guide fitted with neutral density filters and reflected off a white background 2 m above the tanks. The light intensity was measured at the water surface with an EEL micro photometer (Evans Electroselenium Ltd., Halstead, Essex, United Kingdom). Three replicate five-minute experiments were performed with groups of 20 fish from each tank. Three visible light intensities of 0.01, 0.1, and 1 photopic lux (equivalent to 50, 500, and 5000 $\mu W \cdot m^{-2}$) were used with different randomly chosen groups of 20 fish. In order to ensure complete adaptation to the low light intensities used, the fish were first dark-adapted by keeping them in complete darkness overnight. They were not fed during this time (approximately 16 h), and their guts were empty at the start of the experiment. They were then adapted to the experimental light intensity for 30 min before introducing prey (*Artemia* sp. nauplii at a density of 1000 L^{-1}) and commencing video recording. The larvae were returned to their rearing tanks after the behavior experiments. Subsequent frame-by-frame analysis of the video recordings (20 ms intervals) revealed the total number of strikes made at prey per fish per minute. The single camera recording system allowed analysis in only two dimensions, and therefore successful and unsuccessful feeding strikes could not be distinguished reliably.

Lipid extraction and analysis. Twenty herring larvae were anaesthetized in 0.02% (wt/vol) ethyl 3-aminobenzoate methane sulphonate (MS222), their lengths measured, and their eyes removed. The eyes and carcases were immediately frozen in liquid nitrogen and subsequently stored at −70°C. Total lipids were extracted from the eyes of individual fish by sonication in 2 mL of chloroform/methanol 2:1 (vol/vol) containing 0.01% (wt/vol) butylated hydroxytoluene as antioxidant and a Folch extract prepared as previously described (25). Samples were stored at −20°C under nitrogen between

preparative procedures. The fatty acid composition of total eye lipid of five fish matched for size from each tank was determined by gas–liquid chromatography of fatty acid methyl esters using a Packard 436 gas chromatograph fitted with a fused silica capillary column (50 m x 0.25 mm) coated with FFAP phase (S.G.E., Milton Keynes, United Kingdom) using hydrogen as carrier gas (26).

Molecular species analysis. To determine the di22:6n-3 content of total phospholipid, neutral lipid was removed by thin-layer chromatography (TLC) on high-performance TLC (HPTLC) silica gel 60 plates (Merck, Darmstadt, Germany) in hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) and the phospholipid remaining on the origin scraped from the HPTLC plate. The glycerophospholipid was hydrolyzed with phospholipase C (*B. cereus*) [Boehringer Corporation (London) Ltd.] and the 9-anthroyl derivatives prepared as described previously (7,27). The eyes from ten fish matched for size from each tank were analyzed.

PC, PE, and PS were prepared from 100 μg of total lipid by HPTLC in methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aq. KCl (25:25:25:10:9, by vol) (28). The edges of the bands were visualized with iodine, and the remaining sample scraped from the plate before hydrolyzing with phospholipase C as before. The eyes from two fish matched for size (4.0 cm) from each tank were analyzed. Molecular species were separated by HPLC on an Ultrasphere C18 column (25 x 0.46 cm, 5 μm particle size) (Beckman Instruments U.K. Ltd., High Wycombe, United Kingdom) using isocratic elution with acetoni-trile/propan-2-ol, 7:3 (vol/vol) at a flow rate of 1.0 mL/min and methanol/propan-2-ol, 4:1 (vol/vol) at a flow rate of 1.0 mL/min to separate any unresolved peaks. Peaks were detected using a Waters (Watford, United Kingdom) 470 scanning fluorescence detector (excitation 360 nm, emission 460 nm) and quantified with a Shimadzu CR3A recording integrator (Tokyo, Japan). Peaks were identified with reference to standard lipids and samples of known composition (6,7,29).

Data analysis. The behavioral data and the total di22:6n-3 phospholipid content were analyzed with a SAS statistical package by repeated measures multivariate two-way analysis of variance (ANOVA). The molecular species composition of individual lipid classes was analyzed by one-way ANOVA.

RESULTS

At the end of the experimental period, there was no significant difference ($P = 0.3366$) in the size of fish fed the two diets or those used in the behavior experiments. Lengths ranged from 2.5–4.9 cm with mean sizes in the replicate tanks of 3.48 ± 0.60 cm and 3.68 ± 0.41 cm for the 22:6n-3-supplemented fish and 3.68 ± 0.37 cm and 3.87 ± 0.58 cm for the 22:6n-3-deficient fish.

The threshold light intensity for visual feeding by dark-adapted adult herring is 0.01 lux (30). At this light intensity, there was a significant difference in the behavior of the fish reared on the two dietary regimes (ANOVA, $P = 0.0044$). Fish

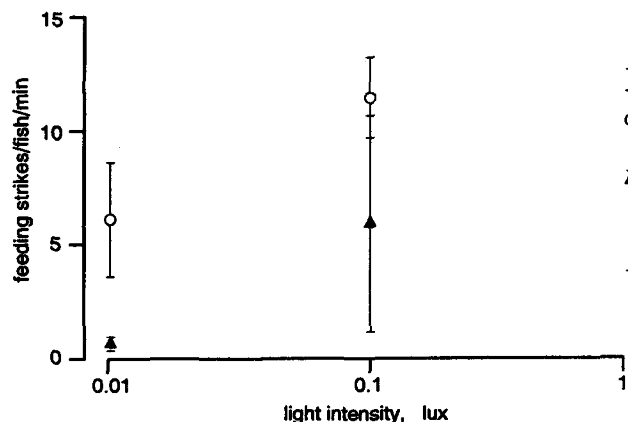

FIG. 1. The effect of light intensity on feeding behavior of herring larvae reared on diets either supplemented with 22:6n-3 (O) or lacking 22:6n-3 (▲). Symbols represent means ± 95% confidence intervals ($n = 6$) for three replicate behavior experiments for each of two rearing groups on each dietary treatment.

reared on the 22:6n-3-supplemented diet fed well, but almost none of the fish reared on the diet lacking 22:6n-3 attacked the prey (Fig. 1). At a light intensity of 0.1 lux, the threshold for larvae (23) before rods develop in the retina, the fish deficient in 22:6n-3 still fed less well, but the difference between dietary treatments was similar to that between replicate tanks and was no longer significant. There was little difference in feeding performance between fish reared on the two diets at 1 lux.

Fatty acid and molecular species analysis of the lipids from the eyes of fish from the replicate tanks showed only small differences in composition, none of which were significant ($P > 0.05$). Therefore, in the following presentation, the data for replicate tanks has been combined. Fish reared on the diet lacking 22:6n-3 showed a large decrease in the 22:6n-3 content of total lipid from eyes with concomitant increases in

TABLE 2
The Fatty Acid and di22:6n-3 Phospholipid Content of Eyes from Herring Reared on 22:6n-3-Supplemented and -Deficient Diets (mole %)[a]

	22:6n-3-Supplemented	22:6n-3-Deficient
16:0	19.3 ± 1.6	19.5 ± 0.8
16:1	3.9 ± 0.6	3.9 ± 0.6
18:0	8.4 ± 0.8	8.8 ± 0.4
18:1	15.4 ± 1.0	18.5 ± 0.7
18:2n-6	1.9 ± 0.3	2.7 ± 0.3
20:4n-6	3.8 ± 0.3	5.1 ± 0.3
20:5n-3	9.5 ± 1.0	14.6 ± 1.4
22:5n-3	3.5 ± 0.4	6.6 ± 0.3
22:6n-3	21.0 ± 3.1	6.6 ± 2.6
di22:6n-3	12.0 ± 3.3	2.0 ± 1.1

[a]Mean ± 1 SD, $P < 0.0001$ in all cases. Ten fish were analyzed for fatty acids and 20 for molecular species; 16:1 and 18:1 include both n-9 and n-7 isomers. Only fatty acids present at >2% are shown. 14:0, 15:0, 16:3n-3, 17:0, 18:3n-6, 18:3n-3, 20:1n-9 and n-7, and 20:4n-3 were also present in small amounts.

TABLE 3
The Molecular Species Composition of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), and Phosphatidylserine (PS) from the Eyes of Juvenile Herring Reared on 22:6n-3-Supplemented (control) and 22:6n-3-Deficient (experimental) Diets (mole %)[a]

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine	
Molecular species	Control	Experimental	Control	Experimental	Control	Experimental
20:5/20:5	trace	0.2 ± 0.1	0.8 ± 0.2	2.9 ± 0.4	trace	0.7 ± 0.2
20:5/22:6	0.4 ± 0.1	trace[b]	3.4 ± 0.5	3.1 ± 0.3	2.6 ± 0.4	2.8 ± 0.3
20:5/22:5	0	0.2 ± 0.1[c]	0	4.0 ± 0.6[a]	0	4.2 ± 0.8[a]
22:6/22:6	5.0 ± 0.7	0.3 ± 0.2[a]	20.8 ± 4.1	2.7 ± 0.6[a]	28.2 ± 5.4	7.6 ± 0.7[b]
22:6/22:5	0.5 ± 0.1	0.2 ± 0.1[c]	5.5 ± 0.7	6.9 ± 0.3	7.6 ± 1.3	8.5 ± 1.4
22:5/22:5	0	1.4 ± 0.2[a]	0	4.4 ± 0.5[a]	0	4.9 ± 0.9[a]
Total diPUFA	5.9 ± 0.8	2.4 ± 0.6	30.5 ± 5.4	26.0 ± 1.6	38.4 ± 6.4	28.7 ± 3.7
16:0/20:5	10.4 ± 1.8	15.7 ± 0.6[c]	6.1 ± 0.8	8.7 ± 0.6[b]	2.9 ± 0.8	5.7 ± 1.2[c]
16:0/22:6	15.0 ± 1.4	3.9 ± 0.4[a]	10.7 ± 1.6	3.2 ± 0.2[a]	5.5 ± 1.2	1.8 ± 0.2[b]
16:0/20:4	4.0 ± 0.3	5.9 ± 0.5[b]	1.7 ± 0.1	2.5 ± 0.2[b]	0.8 ± 0.3	1.7 ± 0.4
16:0/22:5	6.8 ± 1.5	9.1 ± 0.2[a]	5.3 ± 1.0	5.5 ± 0.3	4.9 ± 1.2	5.4 ± 0.7
18:0/20:5	3.5 ± 0.5	6.6 ± 0.3[a]	4.6 ± 0.8	9.5 ± 0.2[a]	2.7 ± 0.7	6.5 ± 0.8[b]
18:0/22:6	10.8 ± 0.9	4.0 ± 0.4[a]	9.6 ± 1.0	4.3 ± 0.4[a]	17.1±1.4	7.2 ± 0.8[a]
18:0/20:4	1.1 ± 0.1	2.0 ± 0.1[a]	2.2 ± 0.3	3.1 ± 0.4[c]	1.6 ± 0.3	4.0 ± 1.9
18:0/22:5	3.9 ± 0.2	4.4 ± 0.1[b]	3.0 ± 0.3	5.1 ± 0.2[a]	7.5 ± 1.4	16.1 ± 0.5[a]
Total SFA/PUFA	51.8 ± 1.6	51.6 ± 0.8	43.2 ± 5.2	41.7 ± 1.3	43.0 ± 6.5	48.8 ± 3.8
16:1/22:6	0.8 ± 0.1	0[a]	1.8 ± 0.4	0[a]	1.1 ± 0.2	0[a]
18:1/20:5	3.2 ± 0.5	5.7 ± 0.3[a]	4.3 ± 0.4	9.8 ± 0.3[a]	1.0 ± 0.0	2.4 ± 0.4[b]
18:1/22:6	1.4 ± 1.1	1.3 ± 0.1	5.9 ± 1.4	3.4 ± 0.4	5.9 ± 1.2	4.3 ± 0.9
18:1/20:4	0	trace	2.2 ± 0.4	3.1 ± 0.4	0.8 ± 0.6	1.1 ± 0.3
18:1/22:5	1.8 ± 1.5	2.3 ± 0.2	3.2 ± 0.5	6.6 ± 0.3[a]	3.8 ± 0.1	7.0 ± 0.5[a]
Total MUFA/PUFA	7.1 ± 1.2	9.3 ± 0.2	17.4 ± 1.7	22.9 ± 0.4	12.6 ± 0.6	14.8 ± 1.5
16:0/16:0	2.8 ± 0.1	2.3 ± 0.2[b]	0.4 ± 0.2	0.2 ± 0.1	0.7 ± 0.2	1.0 ± 0.4
16:0/18:1	19.7 ± 1.4	22.9 ± 0.8[c]	2.5 ± 0.3	2.6 ± 0.6	2.0 ± 0.7	2.6 ± 0.7
18:0/18:1	1.6 ± 0.1	2.0 ± 0.1[c]	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.4	0.8 ± 0.5
18:0/20:1	0.2 ± 0.0	0.2 ± 0.1	0	0	0	0
18:1/18:1	2.3 ± 0.3	2.7 ± 0.2	2.0 ± 0.2	2.5 ± 0.4	1.0 ± 0.3	1.2 ± 0.3
18:1/24:1	0.3 ± 0.1	0.4 ± 0.1	0	0	0	0
Total SFA + MUFA	27.0 ± 1.7	30.5 ± 1.0	5.2 ± 0.6	6.0 ± 0.6	4.4 ± 1.4	5.7 ± 1.9

[a]Significantly different from the 22:6n-3-supplemented fish at [a]$P < 0.0001$, [b]$P < 0.001$, [c]$P < 0.01$. Data are means ± 1 SD of eyes from four fish (two from each replicate tank) matched for size; trace = <0.1%. The order of fatty acids does not imply any stereospecificity on the glycerol. The n-9 and n-7 isomers of monounsaturated fatty acid (MUFA) were not resolved. 20:4 Fatty acid was the n-6 isomer and 22:5 the n-3 isomer. Molecular species containing 18:2n-6 could not be resolved from those containing 22:5n-3. However, 22:5n-3 was two to three times more abundant than 18:2n-6 in both dietary groups (4.0 ± 0.4% vs. 1.8 ± 0.3% and 7.6 ± 0.4% vs. 2.6 ± 0.3% in the 22:6n-3-supplemented and -deficient fish, respectively). PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

20:5n-3 and 22:5n-3 ($P < 0.0001$, all cases) (Table 2). The total glycerophospholipids from eyes of fish on the diet lacking 22:6n-3 had only 17.6% of the di22:6n-3 content of control fish ($P < 0.0001$) (Table 2). The amounts of 18:1, 18:2n-6 and 20:4n-6 were slightly but significantly ($P < 0.0001$) elevated in the eyes of the 22:6n-3-deficient fish (Table 2).

The changes in the n-3 PUFA composition of the lipid from eyes were reflected in large changes in the molecular species of PC, PE, and PS (Table 3). In the diPUFA class of molecular species, di22:6 PC fell from 5.0 to 0.3%, di22:6 PE from 20.8 to 2.7%, and di22:6 PS from 28.2 to 7.6% (Table 3). There was little change in the amounts of 20:5/22:6 and 22:5/22:6 in all three lipid classes, while increases in 20:5/20:5, 20:5/22:5, and 22:5/22:5, particularly in PE and PS, redressed part of the balance. However, the total diPUFA species were decreased in all three lipids classes (Table 3). These changes are summarized in Figure 2.

The other PUFA-containing molecular species, i.e., saturated fatty acid (SFA)/PUFA and monounsaturated fatty acid (MUFA)/PUFA species, followed the same patterns as above with 16:0/20:5, 18:1/20:5, and 18:0/20:5 being elevated in each phospholipid class from the 22:6n-3-deficient fish and 16:0/22:6, 18:1/22:6, and 18:0/22:6 being decreased (Table 3). Although all molecular species containing 22:5n-3 were also elevated, the differences were less marked, and only the changes in 16:0/22:5 and 18:0/22:5 PC and in 18:1/22:5 and 18:0/22:5 PE and PS were significant (Table 3). All arachidonyl-containing molecular species were also elevated in all three phospholipid classes from the 22:6n-3-deficient fish (Table 3), but most changes were small and not significant. In contrast to the marked changes in PUFA-containing molecular species, there was virtually no change in those molecular species containing only SFA and MUFA (Table 3).

The selectivity of particular lipid classes for particular molecular species followed well-established trends found in other studies (6,7,29). Thus, in PC, 16:0/PUFA species were

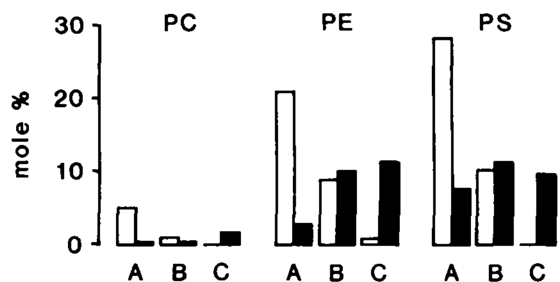

FIG. 2. The dipolyunsaturated fatty acid molecular species composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) from the eyes of juvenile herring fed on diets supplemented with (open bar) or deficient in (filled bars) 22:6n-3. A = 22:6/22:6, B = 20:5/22:6 + 22:5/22:6, C = 20:5/20:5 + 20:5/22:5 + 22:5/22:5.

abundant with low amounts of diPUFA species and a large amount of 16:0/18:1 (Table 3). In PE, 16:0/PUFA species were less prominent, while 18:1/PUFA species were 10–14% more abundant than in PC and diPUFA species contributed 26.0–30.5% (Table 3). PS contained the largest percentage of diPUFA species (28.7–38.4%) in both dietary groups, and in this phospholipid the decrease of 18:0/22:6 was balanced almost completely by an increase in 18:0/22:5 in the fish fed the 22:6n-3-deficient diet.

DISCUSSION

Adult herring have good scotopic vision and feed visually at any time whenever both suitable prey and adequate illumination are available. The present study emphasizes the essential role of 22:6n-3 in the visual process by showing that it is possible to induce a dietary deficiency of 22:6n-3 in predatory, visual feeding fish, so that their ability to feed at low (natural) light intensities is impaired. Though not tested here, predator evasion behavior could also be affected. Late larval and juvenile stages of herring are susceptible to predation by other fish and develop the ability to respond to visual looming stimuli typical of such attacks at the same time as rods appear in the retina (31). A reduction in the ability to detect attacks by predators would markedly decrease the likelihood of survival. The size of the eyes precluded routine preparation of retinas. In adult herring, about 70% of the total phospholipid from eyes was found in retina, and about half the remainder was in the iris muscle which contained half the amount of 22:6n-3 of retina (Bell, M.V., and J.R. Dick, unpublished observations). Retinal phospholipids are therefore the major pool of 22:6n-3 in whole eyes, and even assuming total depletion of 22:6n-3 in all other ocular tissues, the deficit of 22:6n-3 induced in retina in this study would have been substantial.

Herring (22,23) and most other species of marine fish (24) are unable to detect ultraviolet or infrared light; their spectral sensitivity is similar to humans. Herring are totally dependent on photopic vision at first feeding and can only use vision to attack prey at light intensities above 0.1 lux (23). Rods do not occur in significant numbers in herring retinae until eight weeks after hatching when larvae are about 2.5 cm in length and the retinomotor responses of adult fish only develop after this time (19). Adult herring are completely light adapted at 1 lux but are dark adapted, and therefore dependent on scotopic vision, at 0.01 lux (19), the threshold for visual feeding by adults (30). Since rods are recruited to the photoreceptor population from about eight weeks' post-hatching whereas cones are present at hatching, a deficiency of 22:6n-3 during development would be expected to produce a larger effect on scotopic vision than on cone vision. The result found here is consistent with that interpretation. It was not possible to determine in this study whether the decreased proportion of 22:6n-3 in ocular phospholipids was due to decreased amounts in rod outer segment membranes, to a reduced length of the outer segment leading to smaller amounts of outer segment membrane, or to a reduced number of rod cells. The latter two possibilities would also be consistent with a lower sensory threshold. The fact that a substantial part of the deficit of di22:6n-3 molecular species was compensated by increases in molecular species containing 20:5n-3 and 22:5n-3, especially in PE and PS, suggests that the amounts of outer segment membrane were similar in the eyes of both groups of fish. Preliminary microscopic examination of retinas showed rod cells and retinomotor responses present in fish fed on both dietary regimes (Batty, R., and R. Harvey, personal observations). A much more detailed study of retinas in a further experiment would be necessary to eliminate the possibility that a deficiency of 22:6n-3 has an effect on rod development *per se*.

Docosahexaenoic acid is present in a variety of other cell membranes in fish and especially in excitable membranes, including synapses. It is probable therefore that 22:6n-3 was depleted in all other tissues with possible deleterious effects on their functions. This experiment measured the ability of fish to perform a task (feed) at three different light intensities, the lower two chosen to select for vision using cones or rods. Subperformance in consummatory behavior can be due either to a reduced ability to perform the task or to changes in motivational status (or an interaction of the two). A change in motivational status under the more demanding sensory conditions of low light intensity would necessitate light, affecting another neural function directly. Herring larvae become more active in the presence of prey at light intensities below the feeding threshold even though they cannot feed. They are motivated by chemical and mechanical stimulation, but light (vision) is necessary for successful encounter and capture (32). We find it difficult to accept that fish with functioning vision at all light intensities, deprived of food and therefore hungry, might be poorly motivated to feed at low, but natural, light intensities. Difficulties in interpretation are inherent in studies of this nature where the primary events of vision in the retina are closely coupled with signal transduction and processing in the brain where other factors may then modulate responses. Since the 22:6n-3-deficient fish were able to see at higher light intensities, their cone cells and the downstream signal processing from those cells were functional. We believe the

probable lesion in this study lay in the retina since light intensities were specifically chosen which represented the visual threshold for larvae (cones only) and adults (rods and cones). The effect was specific for the lowest light intensity, suggesting impaired scotopic vision either by interrupting the primary events of photon capture by the photoreceptor membranes in rod cells or by the subsequent neural processing of the signal from the rod cells.

Herring, in common with marine fish in general, are unable to synthesise 22:6n-3 from shorter chain C_{18} or C_{20} n-3 PUFA at rates sufficient to meet their requirements. The 22:6n-3-deficient diet also lacked 22:5n-3 and contained only 2.9% 20:5n-3 compared to 11.6% 20:5n-3 in the 22:6n-3-supplemented diet. Consequently, the amounts of 22:5n-3 in the phospholipids from the eyes of the fish reared on the 22:6n-3-deficient diet are notable in that they indicate that herring are able to elongate 20:5n-3 to 22:5n-3, but appear unable to complete the final desaturation step to form 22:6n-3.

The present study has implications for the husbandry of farmed fish and possibly also for natural fish populations. Docosahexaenoic acid is largely absent from marine diatoms; dinoflagellates and prymnesiophytes represent the only substantive source of this fatty acid in marine phytoplankton (33). It is unclear how the species composition and timing of phytoplankton blooms may vary from year to year to influence the amount of 22:6n-3 available (directly and indirectly) to larval marine fish, but amounts of n-3 PUFA in phytoplankton do vary spatially and temporally (34–36). The dietary deprivation of 22:6n-3 in this study was total, and it is very unlikely that this would occur in nature. However, it is unknown whether suboptimal lipid nutrition during early development could ultimately affect recruitment to adult stocks. In herring, rod cells are recruited to the photoreceptor population relatively late in development (19). More recent investigations of other species have shown that rods begin to appear much earlier in development at the first-feeding stage (37) but are very few in number. An adequate dietary supply of 22:6n-3 is probably essential for the survival of marine fish larvae at all stages of development. In mariculture, the supply of adequate amounts of 22:6n-3 to first-feeding larvae is a continuing problem.

Since the fish on the 22:6n-3-deficient diet had impaired vision, the diPUFA molecular species containing 20:5n-3 and 22:5n-3 were unable to replace di22:6n-3 phospholipids functionally in the visual process. At present, the molecular basis for the essentiality of 22:6n-3 in biological processes is not understood. Recent molecular modeling studies have suggested that 22:6n-3-containing glycerophospholipids do not perturb bilayer packing (38,39) as had previously been thought. Furthermore, the favored angle-iron conformation adopted by 22:6n-3 shows the smallest coefficient of thermal expansion of any fatty acid (40), making these lipids particularly suited to control bilayer volume. Finally, it has been proposed that 22:6n-3-containing glycerophospholipids, and especially di22:6n-3 molecular species, provide a highly structured liquid–crystalline membrane which can accommodate the rapid conformational changes in rhodopsin associated with the light-transducing process (41,42). In recombinant bovine rod outer segment membranes, an equimolar mixture of PE and PC containing 50 mole % docosahexaenoyl chains resulted in optimal photochemical function of rhodopsin (43). It was interesting in the present study to find that PS retained 22:6n-3- containing molecular species, and especially di22:6n-3, better than PE even though the % of PS (8.1%) in total phospholipid of the eye is much less than that of PE (26.5%) (44). The total diPUFA molecular species was highest in PS from both dietary groups and the amounts of 20:5/22:5, 22:5/22:6, and 22:5/22:5, as well as 22:6/22:6, were higher in PS than in PE from the eyes of the 22:6n-3-deficient fish. Few studies have been carried out on the physical–chemical properties of PS since it is a minor component of most membranes. However, Hubbell (45) suggested that the asymmetric distribution of PS in the disc membrane was caused by interaction with rhodopsin.

Irrespective of its precise mode of action, 22:6n-3 appears to have a unique role in vertebrate rod function. Clearly, any explanation of the particular role of 22:6n-3 in the visual process must encompass the unusual prominence of di22:6n-3 molecular species of both PS and PE. Experiments, such as the present one, establish that marine fish larvae offer particular advantages for dietary intervention studies on the role of this fatty acid in vertebrate vision.

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