

Nutrient Requirements

Natural Copepods Are Superior to Enriched *Artemia* Nauplii as Feed for Halibut Larvae (*Hippoglossus hippoglossus*) in Terms of Survival, Pigmentation and Retinal Morphology: Relation to Dietary Essential Fatty Acids¹

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ABSTRACT Replicate groups of halibut larvae were fed to d 71 post-first feeding (PFF) either the marine copepod, *Eurytemora velox*, or *Artemia* nauplii doubly enriched with the marine chromist or golden algae, *Schizochytrium* sp., (Algamac 2000) and a commercial oil emulsion (SuperSelco). The fatty acid compositions of eyes, brains and livers from larvae fed the two diets were measured, and indices of growth, eye migration and skin pigmentation were recorded along with histological examinations of eye and liver. The docosahexaenoic acid [22:6(n-3); DHA]/eicosapentaenoic acid [20:5(n-3); EPA] ratios in *Artemia* nauplii enriched with the SuperSelco and Algamac 2000 were 0.4 and 1.0, respectively. The *E. velox* copepods were divided into two size ranges (125–250 and 250–400 μ m) with the smaller size range containing the highest level of (n-3) highly unsaturated fatty acids (HUFA). The DHA/EPA ratios for the two size ranges of copepods were 2.0 and 0.9, respectively. The total lipids of eyes, brains and livers of larvae fed copepods had higher levels of DHA and lower levels of EPA than those of larvae fed enriched *Artemia*. The percentage of survival of the halibut larvae was significantly higher when copepods rather than enriched *Artemia* nauplii were fed, but larval specific growth rates did not differ. The indices of eye migration were high and not significantly different in larvae fed the two diets, but the percentage of larvae undergoing successful metamorphosis (complete eye migration and dorsal pigmentation) was higher in larvae fed copepods (40%) than in larvae fed enriched *Artemia* (4%). The rod/cone ratios in histological sections of the retina were 2.5 ± 0.7 in larvae fed copepods and 1.3 ± 0.6 in larvae fed enriched *Artemia* ($P < 0.01$). Histological examination of the livers and intestines of the larvae were consistent with better assimilation of lipid from copepods than lipid from *Artemia* nauplii up to 46 d post-first feeding. Thus, marine copepods are superior to enriched *Artemia* as food for halibut larvae in terms of survival, eye development and pigmentation, and this superiority can be related to the level of DHA in the feed. J. Nutr. 129: 1186–1194, 1999.

KEY WORDS: • halibut • fish larvae • polyunsaturated fatty acids • retina • pigmentation

The production of very small, rapidly developing and highly vulnerable larvae remains a bottleneck in the commercially successful culture of many marine fish species. A particular aspect of the problem is that the very high growth and development rates of the larvae place a premium on providing optimal nutrition so that larval growth and development and, therefore, survival is maximal. Lipids are particularly impor-

tant in fish nutrition not only for supplying calorific energy but also for providing the essential polyunsaturated fatty acids (PUFA)⁵ required for normal cell membrane function (Sargent et al. 1995b). In the case of marine fish, these PUFA are the highly unsaturated fatty acids (HUFA) of the (n-3) series, eicosapentaenoic acid [20:5(n-3); EPA] and docosahexaenoic acid [22:6(n-3); DHA] (Sargent et al. 1995a). The (n-3) HUFA requirement of juvenile marine fish is ~0.5–1.0% of the dry weight of their diet, but the requirement in the early developmental stages of larvae is likely to be greater because of their rapid growth and the critical early development of specialized cells and tissues. Several investigators have studied the (n-3) HUFA requirements of a number of marine fish species

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⁵ Abbreviations used: AA, arachidonic acid; DHA, docosahexaenoic acid; EFA, essential fatty acid; EG, enrichment grade; EPA, eicosapentaenoic acid; HPTLC, high performance thin layer chromatography; HUFA, highly unsaturated fatty acids; PFF, post-first feeding; PUFA, polyunsaturated fatty acids.

(Estevez and Kanazawa, 1996, Izquierdo et al. 1989, Reitan et al. 1994, Rodriguez et al. 1994, Takeuchi et al. 1994), and all suggest that DHA is more efficacious than EPA in promoting larval health and survival.

DHA and EPA are essential fatty acids (EFA) for marine fish species because they do not have or have only a very low $\Delta 5$ -desaturase activity, which is necessary for the conversion of C-18 PUFA to long-chain HUFA (Sargent et al. 1993). In addition, although these fish may be capable of converting EPA to DHA, the rate at which they do so may be too low to satisfy the high DHA requirement during early larval growth (Sargent et al. 1995a). DHA, which is abundant in all fish tissues (Sargent et al. 1989), tends to accumulate in terrestrial vertebrates in neural and reproductive tissues, suggesting a specific functional role for DHA in particular cell membranes of these tissues (Sastry 1985, Tinoco 1982). However, neural membranes of fish are also especially rich in DHA (Bell and Dick 1991, Tocher and Harvie 1988), suggesting that the same specialized function(s) exists for DHA in fish as in terrestrial vertebrates. Studies with larval sea bass (*Dicentrarchus labrax*) and herring (*Clupea harengus* L.) fed DHA-deficient diets established a deficit of didocosahexaenoyl phospholipids in the eyes of the fish, which was correlated with impaired visual performance in the latter species (Bell et al. 1995 and 1996). In addition, normal dorsal pigmentation in flatfish larvae post-metamorphosis is influenced by dietary HUFA, and it has been suggested that albinism may be linked with impaired larval neural function (Estevez and Kanazawa 1996, Reitan et al. 1994).

The small size and poorly developed digestive function of marine fish larvae (Segner et al. 1994) means that, for most species, pelleted or microencapsulated diets are unsuitable for first feeding. The only viable alternative involves live-prey species such as rotifers (*Brachionus plicatilis*) and brine shrimp nauplii (*Artemia* spp.). However, these organisms are deficient in long-chain HUFA, especially DHA (Navarro et al. 1993) and must be enriched with these fatty acids to improve their nutritional quality before being fed to marine fish larvae. Several enrichment techniques have been developed, including microalgae, oil-based emulsions and microencapsulated preparations (Barclay and Zeller 1996, Leger et al. 1986, McEvoy et al. 1996, Southgate and Lou 1995). However, most commercially available enrichment preparations are unable to provide a DHA/EPA ratio >1 in the enriched *Artemia* (McEvoy et al. 1996, Navarro et al. 1995); thus, the efficacy of this live prey to deliver sufficient DHA to the developing larvae remains a matter of concern. Marine zooplankton offer an obvious alternative to *Artemia* nauplii as live-prey organisms for first feeding marine fish larvae, although problems exist in obtaining adequate numbers of zooplanktonic organisms to coincide seasonally with marine larval production. Marine zooplankton are the natural food organism for marine fish larvae in the wild and are naturally enriched in long-chain HUFA, generally having DHA/EPA ratios substantially >1 during their naupliar and early copepodite stages (Gronkjaer et al. 1995, Naess et al. 1995, Sargent and Henderson 1986).

The aim of this study was to determine whether enriched brine shrimp nauplii satisfy the HUFA requirements of larval Atlantic halibut to the same extent as natural marine copepods.

MATERIALS AND METHODS

Experimental design and diet preparation. The halibut feeding experiment was carried out at Seafish Aquaculture's Marine Farming Unit, Ardtoe, Scotland. All developmental stages were reared using

sea water maintained at a salinity of 33–35 g/L, filtered to 5 μm and UV-sterilized. Eggs and yolk sac larvae were reared following the method described by Gara et al. (1998). At 220 degree days post-hatch, 700 larvae were removed from the yolk sac rearing tank and stocked into each of two circular feeding tanks (150 cm diameter, 90 cm depth) containing 1300 L seawater to which marine microalgae (*Nannochloris atomus*) had been added to provide "green water" conditions. The practice of adding microalgae to the rearing water is widespread in the culture of a wide range of marine fish species. Although the mechanism of their beneficial effects is unclear, microalgae may provide a nutritional influence as well as altering the light distribution within the rearing tanks (Naas et al. 1992, Reitan et al. 1997). The water temperature was 10°C and light intensity was set at 50 lx at the water surface.

The calanoid copepods were collected from a static outdoor tank (150 m³ volume) at Ardtoe, by means of an airlift directed into a semisubmerged sieve. Copepods collected in this way were size graded by rinsing through sieves with different mesh sizes. The halibut larvae were initially fed copepods in the 125- to 250- μm size fraction before being switched to the 250- to 400- μm fraction on d 19 post-first feeding (PFF).

Enrichment grade (EG) cysts (INVE Aquaculture NV, Baasrode, Belgium) were used for the *Artemia*-fed halibut larvae. All sea water for incubating and rinsing the *Artemia* was filtered to 5 μm and UV-sterilized. Enrichments were carried out in 10-L polyethylene containers, at a density of 150,000 individuals/L, using vigorous aeration. Decapsulated cysts were incubated in sea water (34 g/L, 28°C) for 18 h, after which the nauplii were harvested and rinsed. The spray dried chromist *Schizochytrium* (Algamac 2000, Aquafarma Biomarine, Hawthorne, CA) was used for enrichment at a concentration of 0.6 g/L. The material was suspended and prehydrated by blending the appropriate quantity in 400 mL sea water for 1 min. *Artemia* nauplii were enriched at 27°C in this suspension for 18 h. SuperSelco (INVE Aquaculture NV) was also mixed by blending in sea water and then applied to the enrichment container in two aliquots (0.3 g/L per aliquot) over 18 h at 27°C. Samples of enriched *Artemia* and copepods were obtained for lipid analysis by collecting 25,000 *Artemia* per replicate on a nylon sieve of 64- μm mesh size. The *Artemia* were rinsed using distilled water, blotted on absorbent tissue, transferred to a cryovial, weighed and stored in liquid nitrogen. Four size fractions of copepods (64–125 μm , 125–250 μm , 250–400 μm and >400 μm) were collected similarly and stored.

Each tank received a single daily ration of 50,000 newly hatched EG grade *Artemia* nauplii from d 1 to 9 PFF. The water temperature was raised from 10 to 12°C over the first 2 d, and water exchange was started on d 5 PFF at an inflow rate of 0.24 L/min. On d 10 PFF, one tank was switched to a diet of calanoid copepods, *Eurytemora velox*. This tank was supplied with a single ration of 20,000 copepods per day, selected from the 125- to 250- μm size fraction. The second tank was switched from *Artemia* nauplii to enriched *Artemia* nauplii on d 10 PFF. This switch in live feed was based on the requirement for increased prey size in rapidly growing halibut larvae. Fifty thousand instar II *Artemia* nauplii separately enriched using SuperSelco and Algamac 2000 were provided to this tank in a single daily ration at a ratio of 1:1; the former was provided in the morning and the latter in the evening.

On d 16 PFF, 44 halibut larvae were counted from each of the two 1300-L tanks and stocked into cylindrical polyethylene tanks (diameter 50 cm, depth 70 cm) containing 120 L sea water, "greened" using *Nannochloris atomus*. Four tank replicates were set up for the copepod diet treatment and three replicates for the *Artemia* treatment. The lack of a fourth replicate tank in the *Artemia*-fed population. A diagrammatic illustration of the experimental design is shown in Figure 1. Comparison of the copepod and *Artemia* diets was continued in this replicated rearing system for 55 d until d 71 PFF. Each tank was illuminated from above by a single PAR 38 tungsten floodlight (Osram Concentra, 80 W, Specialist Lamp Distributors, Glasgow, Scotland) fitted with a dimmer switch. Initial illumination was set at 50 lx at the water surface and was increased to 1500 lx over the next 7 d. All tanks received a partial daily water exchange (20% of tank volume) via a surface inflow (300 mL/min for 2 h) and were

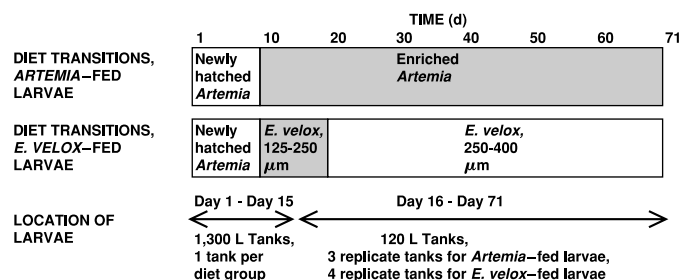


FIGURE 1 Schematic representation of diet transitions and tank transfers for Atlantic halibut larvae fed enriched *Artemia* or *Eurytemora velox*. Results are presented for the experimental period d 16–71 post-first feeding.

supplemented daily with *Nannochloris atomus*. Water temperature ranged from 12.9 to 13.5°C over the experimental period.

Halibut larvae in the *Artemia* treatment were fed four times per day with Algamac 2000- and SuperSelco- enriched *Artemia* at a ratio of 1:1. The fatty acid compositions of the two enrichments are shown in Table 1. Ration levels in the *Artemia* treatment were adjusted daily according to consumption with the aim of minimizing the levels of residual prey. Calculated individual feed rates increased from 250

Artemia per fish per day on d 16 PFF to 1500/d on d 44 PFF. By contrast, daily ration levels in the *E. velox* diet treatment were constrained by the numbers of copepods available from the outdoor collection tank. The ration levels were at all times lower than those supplied in the *Artemia* treatment and averaged 180 copepods per day throughout the experiment. The experiment was conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

Sampling. A sample of 10 larvae was collected from each of the two 1300-L tanks on d 16 PFF for calculations of mean wet and dry weight. The larvae were killed by anesthesia in 3-aminobenzoic acid ethyl ester methane sulfonate (MS222, Sigma Chemical, Poole, U.K.), then immersed in distilled water and blotted on absorbent tissue for 30 s before measuring wet weight. The larvae were then frozen (–20°C) before being freeze-dried for 24 h and reweighed to obtain dry weight.

At the end of the experiment (d 71 PFF), all surviving fry were counted to calculate survival rates and each fish was examined. The fish were killed by anesthetization and wet weight was recorded. Eye migration was classified for each individual using a 4-point scale (Gara et al. 1998). The distribution of pigment on the “ocular” (top) and “blind” (bottom) surfaces of the body was classified according to the method of Gara et al. (1998). The frequency of each pigment category was converted to a percentage of the whole population for individual replicates.

TABLE 1

Fatty acid compositions of commercial enrichment products SuperSelco and Algamac 2000 and total lipid from Artemia enriched with either SuperSelco or Algamac 2000 and two size ranges of Eurytemora velox copepods

Fatty acid	Algamac 2000	SuperSelco	<i>Artemia</i> + Algamac 2000	<i>Artemia</i> + SuperSelco	<i>E. velox</i> 125–250 µm ¹	<i>E. velox</i> 250–400 µm ²
g/100 g total fatty acids						
14:0	17.5	0.3	1.2	0.5	3.0	3.5
16:0	37.6	2.4	12.8	8.2	23.3	28.2
18:0	1.4	2.6	5.1	4.0	2.1	1.4
Total saturates ³	57.1	6.5	19.4	13.2	28.9	33.7
16:1 (n-7)	6.2	0.7	5.5	4.2	15.9	31.0
18:1 (n-9)	1.1	8.4	19.9	17.1	8.8	14.5
18:1 (n-7)	3.5	3.1	8.7	5.5	2.8	1.9
20:1 (n-9)	0.1	2.9	0.9	1.4	0.5	0.8
24:1	0.1	t	t	t	1.1	0.4
Total monoenes ⁴	11.0	18.8	35.2	29.0	29.1	49.3
18:2 (n-6)	0.4	4.6	4.0	4.6	2.0	1.7
20:2 (n-6)	t	0.2	0.2	0.2	0.3	0.1
20:4 (n-6)	0.5	1.4	1.5	1.2	1.8	1.0
22:5 (n-6)	8.6	0.6	2.5	0.3	0.9	0.2
Total (n-6)	9.8	7.1	8.3	6.4	5.3	3.2
18:3 (n-3)	1.2	0.9	19.1	20.8	1.3	1.3
18:4 (n-3)	0.2	1.5	2.1	2.2	0.6	0.6
20:3 (n-3)	0.1	t	0.3	0.4	t	t
20:4 (n-3)	0.5	1.3	0.4	0.6	0.3	0.2
20:5 (n-3)	0.6	23.3	5.3	15.3	10.8	5.4
22:5 (n-3)	0.1	4.9	t	1.7	0.2	t
22:6 (n-3)	18.1	29.2	5.1	6.1	21.8	4.6
Total (n-3)	20.8	61.1	32.3	47.1	35.0	12.1
Total PUFA ⁵	30.6	68.2	40.6	53.5	40.3	15.3
DHA ⁶ /EPA ⁷	34.7	1.3	1.0	0.4	2.0	0.9

¹ A mixture of nauplii and copepodites.

² A mixture of copepodites and immature adults.

³ Includes 15:0, 20:0 and 22:0.

⁴ Includes 20:1 (n-11), 20:1 (n-7), 22:1 (n-9) and 22:1 (n-11); t, trace value < 0.05 g/100 g.

⁵ PUFA, polyunsaturated fatty acids.

⁶ DHA, 22:6 (n-3); docosahexaenoic acid.

⁷ EPA, 20:5 (n-3); eicosapentaenoic acid.

Livers, eyes and brains were dissected from an additional six fish per treatment on d 65 PFF. The organs were pooled into three 1-mL polypropylene vials (nalgene cryovial, Nalge UK) providing three replicates from two fish for each dietary treatment. The pooled samples were weighed and stored in liquid nitrogen in preparation for lipid analysis.

Lipid extraction and analysis. Total lipids were extracted from frozen samples of *Artemia*, copepods and larval tissue samples by homogenizing in 10 volumes of chloroform/methanol (2:1, v/v) using a glass/teflon homogenizer. Total lipid was prepared and measured gravimetrically according to the method of Folch et al. (1957). Fatty acid methyl esters were prepared by acid-catalyzed transesterification of total lipids according to Christie (1982). Extraction and purification of fatty acid methyl esters was performed as described by Ghioni et al. (1996). Fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 6000, Thermo Quest, Manchester, England) using a 30 m × 0.32 mm capillary column (CP Wax 52 CB, Chrompak, London, U.K.). Hydrogen was used as carrier gas and temperature programming was from 50 to 150°C at 40°C/min and then to 230°C at 2.0°C/min. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980).

Histology. Two larvae per tank were fixed for histology (saline buffered formalin, or Bouin Picro formol) on d 32, 46 and 65 PFF. The samples were dehydrated and embedded in paraffin wax for sectioning. Sagittal sections of 3–5 µm were stained with hematoxylin-eosin, periodic acid Schiff or Masson's trichrome stain. The rod/cone ratio was measured in histological sections of the retina. In the ventrotemporal region of the retina, the numbers of nuclei in the outer nuclear layer were counted in a zone corresponding to thirty cones. The numbers of nuclei exceeding the number of cones were used as an estimate of rods (O'Connell 1963). Counts were performed on six larvae per treatment, and each value was the mean of three counts from the ventrotemporal region of the retina from one larva.

Statistical analysis. Statistics were applied to the data collected over the period d 16–71 PFF. Diet-related differences in rearing parameters, pigmentation characteristics and lipid compositions were analyzed using one-way ANOVA. Differences between means were compared using Tukey's test. Percentage data were arc-sin transformed before analysis. Chi-squared analysis was applied to the nominal eye migration data (Zar 1984). All statistical analyses were carried out using the Minitab (State College, PA) statistical package. A significance level of 95% ($P < 0.05$) was used throughout.

TABLE 2

Survival, growth and metamorphosis parameters of halibut larvae fed enriched *Artemia* or *Eurytemora velox* copepods from d 16 to 71 post-first feeding (PFF)¹

Parameter/Diet	Enriched <i>Artemia</i>	<i>E. velox</i>
Wet weight d 16 PFF, mg	39.5 ± 2.4*	24.7 ± 2.3
Dry weight d 16 PFF, mg	3.0 ± 0.1*	2.0 ± 0.2
Wet weight d 71 PFF, mg	363.8 ± 25.7*	249.6 ± 8.3
Dry weight d 71 PFF, mg	73.8 ± 10.8*	45.8 ± 3.7
Survival d 16–71 PFF, %	44.7 ± 9.5*	66.4 ± 2.3
Specific growth rate, ² d 16–71 PFF	5.91 ± 0.15	6.03 ± 0.27
Eye migration index ³	2.1 ± 0.1	2.3 ± 0.1
"Perfect" ⁴ metamorphosis, %	3.5 ± 3.1*	39.7 ± 1.9

¹ Values are means ± SD, $n = 3$ (enriched *Artemia*-fed larvae) or $n = 4$ (*E. velox*-fed larvae). Values assigned an asterisk are significantly different from *E. velox*-fed larvae ($P < 0.05$).

² Specific growth rate calculated as $\{\ln(\text{dry weight T1}) - \ln(\text{dry weight T0})\}/T \times 100$, where T0 = d 16 PFF, T1 = d 71 PFF and T = length of experiment in days.

³ Eye migration index of eye migration covers range 0 (no migration) to 3 (full migration).

⁴ "Perfect" metamorphosis refers to fish having complete eye migration and correct ocular and blind pigmentation.

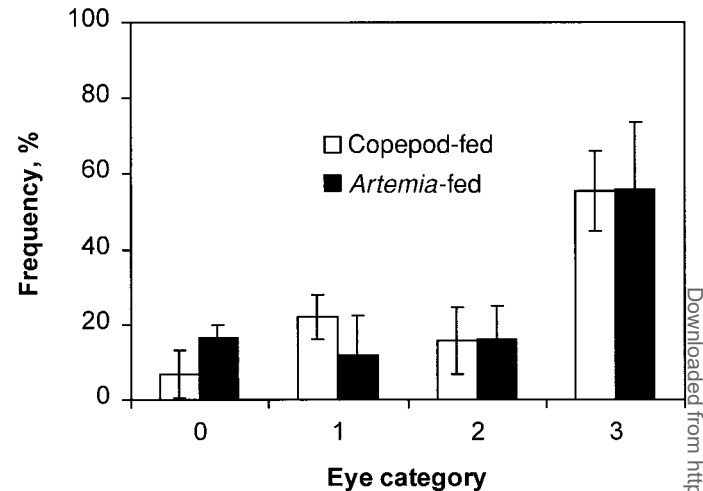


FIGURE 2 Percentage of frequency distribution of halibut eye categories (at d 71 post-first feeding) after receiving enriched *Artemia* or *Eurytemora velox* diets for 61 d. Values are means ± SD, from $n = 3$ tank replicates for *Artemia*-fed larvae and $n = 4$ tank replicates for *E. velox*-fed larvae. Eye categories as described in Gara et al. (1998).

Materials. TLC and high performance TLC (HPTLC) plates, coated with silica gel 60, were obtained from Merck, Darmstadt, Germany. All solvents were of HPLC grade and were obtained from Fisher Scientific (Loughborough, U.K.).

RESULTS

Halibut larvae were fed *Artemia* nauplii, enriched with either SuperSelco (fed morning) or Algamac 2000 (fed evening), or *Eurytemora velox* copepods, from d 16 to 71 post-first feeding (PFF). The fatty acid compositions of total lipid of the enriched *Artemia* and various size ranges of *E. velox* copepods are shown in Table 1. The Algamac 2000-enriched *Artemia* contained similar amounts of DHA and EPA, resulting in a DHA/EPA ratio of 1; in addition, it contained 2.5% of 22:5(n-6). The SuperSelco enrichment produced *Artemia* containing slightly more DHA compared with the Algamac enrichment but appreciably more EPA, resulting in a DHA/EPA ratio of 0.4. The smaller size range of *E. velox* copepods, which comprised largely nauplii and copepodites, contained high levels of DHA and EPA and had a DHA/EPA ratio of 2.0. The larger size class of copepods, which comprised largely immature and mature adults, contained much lower levels of DHA and EPA and had a DHA/EPA ratio of 0.9. The percentage decrease in (n-3) HUFA in the larger size copepods was due to increased accumulation of saturated and particularly monounsaturated fatty acids.

The survival and growth parameters of the two groups of halibut over the period d 16–71 PFF are summarized in Table 2. No significant differences between replicate tanks, fed the same experimental diets, were observed. Halibut larvae fed copepods exhibited a significantly greater mean survival rate ($66.4 \pm 2.3\%$) compared with those fed enriched *Artemia* ($44.7 \pm 9.5\%$). The halibut larvae fed *Artemia* attained significantly higher final weights than those fed copepods. However, differences in mean specific growth rates were not significant because of the different start weights. The indices of eye migration on d 71 PFF were not significantly different between dietary treatments with mean values >2 in both cases (Table 2). The eye data are summarized in the frequency distributions in Figure 2 and establish that ~55% of the

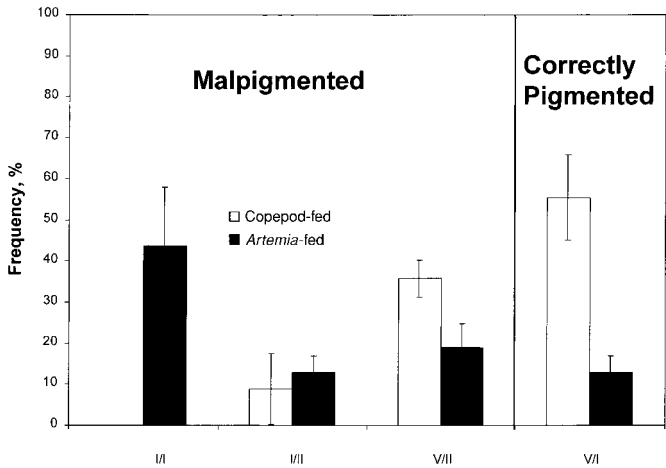


FIGURE 3 Percentage of frequency distribution of halibut pigment categories (at d 71 post-first feeding) after receiving enriched *Artemia* or *Eurytemora velox* diets for 61 d. Values are means \pm SD, from $n = 3$ tank replicates for *Artemia*-fed larvae and $n = 4$ tank replicates for *E. velox*-fed larvae. Pigment categories as described in Gara et al. (1998). Category I/I = no pigmentation on ocular and blind side; I/II = no pigmentation of ocular side/partial pigmentation of blind side; V/II = correct pigmentation of ocular side/partial pigmentation of blind side; V/I = complete pigmentation of ocular side/no pigmentation of blind side.

halibut fry in both diet treatments exhibited complete eye migration (index of 3).

The mean proportion of “perfectly” metamorphosed halibut fry, i.e., those exhibiting correct pigment distribution and complete eye migration differed significantly according to diet (Table 2). Almost 40% of halibut larvae fed copepods exhibited perfect metamorphosis attributes, whereas only 3.5% of the fish fed *Artemia* fell into this category. The large difference in metamorphosis attributes is accounted for by differences in pigment distribution between the two groups of halibut fry. By reference to frequency distributions of the pigment categories (Fig. 3), it can be seen that ~45% of the halibut fed *Artemia* were “albino” (pigment category I/I), whereas this category was not recorded at all in fish fed copepods. Conversely, only 13% of the halibut fed *Artemia* exhibited correct pigmentation of the ocular and blind surfaces (category V/I) compared with

55% of the larvae fed copepods. Fish receiving both diet treatments displayed an abnormal trait on the blind body surface of partial pigmentation of the skin (Fig. 3, pigment categories I/II and V/II).

The measurement of the rod/cone ratio in retinas of the halibut at d 65 PFF is shown in Table 3. The rod/cone ratio was significantly higher in the copepod-fed group. Light microscope sections of liver were analyzed at d 46 and 65 PFF. At d 46 PFF, lipid-containing vacuoles filled most of the hepatocytes in the copepod-fed larvae, whereas these vacuoles were much smaller in the *Artemia*-fed larvae (Fig. 4). However, at d 65 PFF, lipid-containing vacuoles were equally abundant in the livers of both groups of larvae (data not shown).

The fatty acid compositions of total lipids from halibut eyes at d 65 PFF are shown in Table 4. The eyes from fish fed copepods contained significantly greater amounts of 16:0, 16:1(n-7), 24:1 and 22:6(n-3) and significantly lower amounts of 18:1(n-9), 18:1(n-7), 18:2(n-6), 20:4(n-6), total (n-6) PUFA, 18:3(n-3), 20:3(n-3), 20:5(n-3) and 22:5(n-3), compared with those fed enriched *Artemia*. The larvae fed copepods had a significantly higher DHA/EPA ratio compared with larvae fed enriched *Artemia*, but the EPA/arachidonic acid (AA) ratio was not different. The long-chain HUFA [22:6(n-3), 20:5(n-3) and 20:4(n-6)] compositions of brains and livers from halibut larvae fed either enriched *Artemia* or copepods are shown in Figures 5 and 6. The percentages of these three essential HUFA in brain and liver were affected similarly by dietary treatment to the changes described for halibut eyes. In brain, DHA was significantly greater, whereas EPA and AA were significantly lower, in fish fed the copepods compared with those fed enriched *Artemia*. The EPA/AA ratios were significantly higher in brains of *Artemia*-fed fish compared with those fed copepods (3.4 ± 0.1 and 3.1 ± 0.1 , respectively). In liver, DHA was significantly greater and EPA significantly lower in the copepod-fed halibut compared with those fed enriched *Artemia*. In both tissues, the DHA/EPA ratio was increased in fish fed copepods compared with those fed enriched *Artemia*. The EPA/AA ratios in livers were significantly higher in fish fed the enriched *Artemia* compared with those fed copepods (3.6 ± 0.4 and 2.9 ± 0.1 , respectively).

TABLE 3

Rod/cone ratio in retinas of larval halibut fed either enriched Artemia or Eurytemora velox copepods¹

<i>Artemia</i> -fed larvae			<i>E. velox</i> -fed larvae		
Cones	Rods	Rod/Cone	Cones	Rods	Rod/Cone
30	59	1.98	30	82	2.74
30	55	1.84	30	62	2.07
30	42	1.39	30	49	1.63
30	35	1.18	30	80	2.68
30	12	0.39	30	70	2.34
30	34	1.12	30	111	3.70
Mean \pm SD	40 \pm 17	1.32 \pm 0.57		76 \pm 21	2.53 \pm 0.71*

¹ Values for the rod/cone ratio were measured in histological sections of the retina. In the ventrotemporal region of the retina, the numbers of nuclei in the outer nuclear layer were counted in a zone corresponding to 30 cones. The numbers of nuclei exceeding the number of cones were used as an estimate of rods (O’Connell 1963). Counts were performed on six larvae per treatment and each value was the mean of three counts from one larva. *The rod/cone ratio in *E. velox*-fed larvae was significantly higher than in enriched *Artemia*-fed larvae ($P < 0.01$).

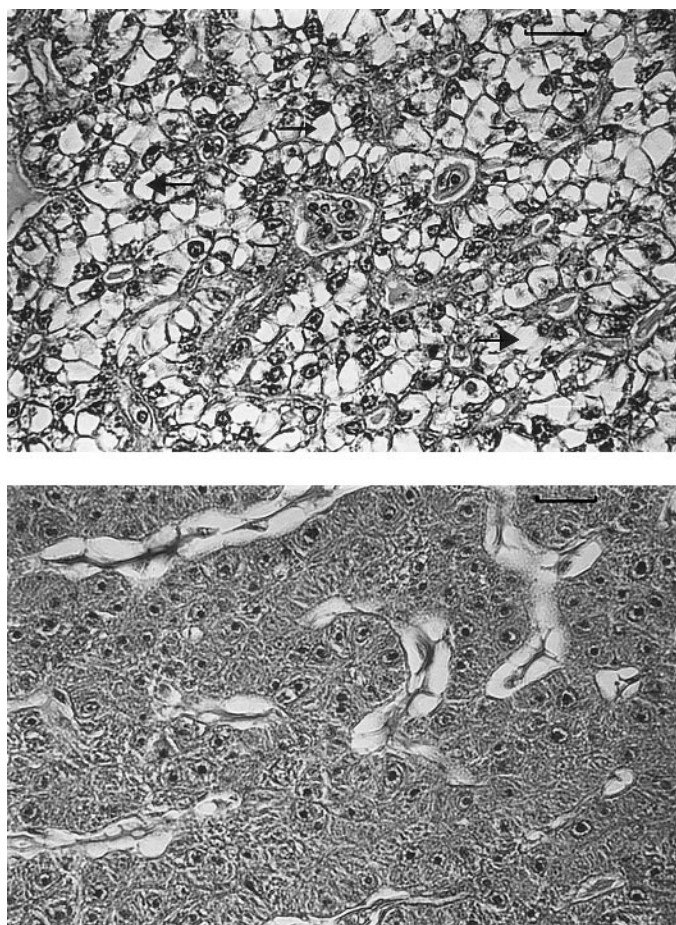


FIGURE 4 Light micrographs of liver sections from halibut larvae at 46 d post-first feeding (PFF). The presence of many lipid-containing vacuoles can be seen (arrowed) in livers from larvae fed *Eurytemora velox* copepods (top), whereas these are largely absent in livers from larvae fed enriched *Artemia* (bottom). Scale bar = 20 μ m.

DISCUSSION

In this study, halibut larvae fed *Eurytemora velox* copepods or enriched brine shrimp, *Artemia*, did not differ significantly in their specific growth rates and eye migration indices. However, copepod-fed halibut exhibited a significantly better mean survival rate and better pigmentation characteristics than those receiving *Artemia*. More than 40% of the halibut fed enriched *Artemia* were classified as "albinic," i.e., without any pigment expression on either the ocular or blind side. By contrast, none of the copepod-fed halibut were albinic and 55% exhibited correct pigmentation of both body surfaces. Naess et al. (1995) similarly reported better pigmentation characteristics for zooplankton-fed halibut larvae compared with those fed SuperSelco-enriched *Artemia*.

In experiments with larval turbot (*Scophthalmus maximus*), increased pigmentation success was positively correlated with an increased dietary and tissue ratio of DHA/EPA (Reitan et al. 1994). In this study, the ratio of DHA/EPA in copepod nauplii and copepodites was significantly greater than in enriched *Artemia*; consequently, the DHA/EPA ratio in eyes, brains and livers of halibut larvae fed copepods was always greater than in those fed *Artemia*. We conclude, therefore, that the superior performance of copepods compared with *Artemia* nauplii reflects, at least in part, a relative deficiency of DHA in the supplemented *Artemia*. Moreover, because there

was little or no difference in the EPA/AA ratio in larvae fed copepods and those fed supplemented *Artemia*, there is no reason to believe that the levels of EPA or AA are in any way unsuitable in the supplemented *Artemia*, most notably in relation to possible influences on eicosanoid metabolism in the larvae.

In addition to the benefits of maintaining a high DHA/EPA ratio in live-prey and larval tissues, there exists the potential importance of dietary phospholipid and vitamin A in preventing pigmentation abnormalities in larval flatfish (Kanazawa 1991 and 1993). The naupliar and copepodite stages of the *E. velox* copepods used in this study contained an excess of phospholipids over triacylglycerols, whereas the opposite composition was always evident in enriched *Artemia* (results not shown). Evidence suggests that phospholipids are more easily digested by larval fish compared with triacylglycerols, and their presence may enhance digestion of other lipids in the rudimentary digestive tract of larval fish (Kanazawa et al. 1983).

TABLE 4

Fatty acid compositions of total lipid from eyes of halibut larvae fed either Artemia enriched with SuperSelco and Algamac 2000 or Eurytemora velox copepods¹

Fatty acid/diet	Enriched <i>Artemia</i>	<i>E. velox</i>
g/100 g total fatty acids		
14:0	0.9 \pm 0.1	0.8 \pm 0.1
16:0	16.2 \pm 1.0*	18.0 \pm 0.3
18:0	10.5 \pm 0.4	9.8 \pm 0.5
Total saturates ²	28.0 \pm 1.2	28.9 \pm 0.1
16:1 (n-7)	3.9 \pm 0.3*	8.2 \pm 1.1
18:1 (n-9)	12.5 \pm 1.3*	9.8 \pm 0.6
18:1 (n-7)	7.2 \pm 0.5*	4.2 \pm 0.5
20:1 (n-9)	0.6 \pm 0.1	0.4 \pm 0.0
24:1	0.5 \pm 0.1*	1.0 \pm 0.1
Total monoenes ³	24.9 \pm 1.9	23.7 \pm 1.8
18:2 (n-6)	2.1 \pm 0.1*	1.0 \pm 0.2
20:2 (n-6)	0.2 \pm 0.0	0.1 \pm 0.0
20:3 (n-6)	0.2 \pm 0.1	0.2 \pm 0.0
20:4 (n-6)	3.1 \pm 0.1*	2.7 \pm 0.1
22:5 (n-6)	2.5 \pm 0.3*	0.5 \pm 0.1
Total (n-6)	8.1 \pm 0.2*	4.3 \pm 0.1
18:3 (n-3)	4.5 \pm 0.1*	0.4 \pm 0.1
18:4 (n-3)	0.3 \pm 0.1	0.2 \pm 0.1
20:3 (n-3)	1.1 \pm 0.1*	0.1 \pm 0.0
20:4 (n-3)	0.3 \pm 0.0	0.2 \pm 0.1
20:5 (n-3)	9.0 \pm 0.6*	7.0 \pm 0.4
22:5 (n-3)	2.9 \pm 0.2*	1.5 \pm 0.1
22:6 (n-3)	18.0 \pm 3.5*	30.9 \pm 1.9
Total (n-3)	35.9 \pm 4.2	40.1 \pm 1.6
Total PUFA ⁴	44.0 \pm 4.5	44.4 \pm 1.4
DHA ⁵ /EPA ⁶	2.0 \pm 0.2*	4.4 \pm 0.4
EPA/AA ⁷	2.9 \pm 0.1	2.7 \pm 0.2

¹ Values are means \pm SD, $n = 3$. Values assigned an asterisk are significantly different from *E. velox*-fed larvae ($P < 0.05$). SD < 0.05 is tabulated as 0.0.

² Includes 15:0, 20:0 and 22:0.

³ Includes 20:1 (n-11), 20:1 (n-7), 22:1 (n-9) and 22:1 (n-11).

⁴ PUFA, polyunsaturated fatty acids.

⁵ DHA, 22:6 (n-3); docosahexaenoic acid.

⁶ EPA, 20:5 (n-3); eicosapentaenoic acid.

⁷ AA, 20:4 (n-6); arachidonic acid.

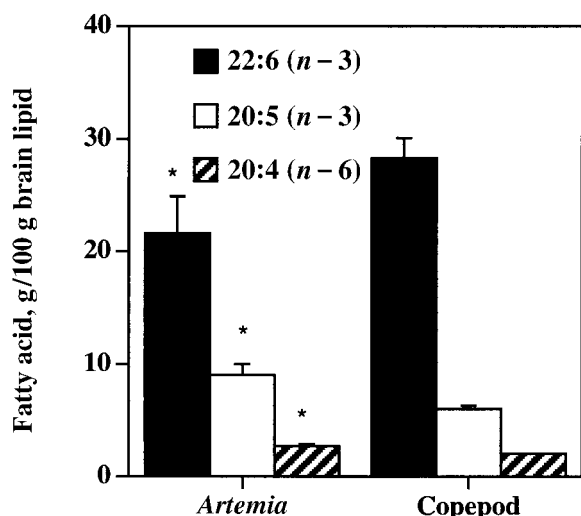


FIGURE 5 Levels of 22:6(n-3), 20:5(n-3) and 20:4(n-6) in brain total lipid from halibut larvae fed either enriched *Artemia* or *Eurytemora velox* copepods. Values are means \pm SD, $n = 3$. *Significantly different from copepod-fed larvae ($P < 0.05$).

Koven et al. 1993). Further studies have identified the superior growth and development in a number of larval fish species, including red sea bream, ayu, striped jack, carp, sea bass and turbot, when diets contained lecithin (phosphatidylcholine) supplements, suggesting an absolute requirement for lecithin in developing larvae (Geurden et al. 1995 and 1997, Kanazawa 1985, Takeuchi et al. 1992). The benefits of dietary lecithin cannot be explained by their HUFA composition alone because both soy and tuna lecithin were equally effective in promoting growth and development in some of these studies. It is possible, however, as suggested by Geurden et al. (1995 and 1997) that fish larvae have only a limited ability to biosynthesize phospholipids de novo such that intact phospholipids in the diet are essential for maximal growth. It may be that *Artemia* nauplii are limiting in this respect, particularly given the high level of triacylglycerols in their total lipid.

Histological examination (data not shown) of the developing larval gut has indicated that in the period following first feeding (up to d 46 PFF), lipid absorption was occurring largely in the hind gut; only later, when the stomach was more fully developed, did absorption of lipid occur in the fore gut (Luizi et al. 1998). During early development, lipid droplets were visible in the mucosal epithelium of the hind gut of copepod-fed larvae, but largely absent in the hind gut of larvae fed *Artemia*. In addition, during the early part of the experiment, *Artemia* appeared to pass through the gut largely undigested, whereas copepods were more completely assimilated. The observations of Luiz et al. (1998) support the histological analysis of the liver in larvae at d 46 PFF (Fig. 3), which showed a high degree of lipid vacuolation in larvae fed copepods; this is consistent with an active assimilation of dietary lipid and was not present in the livers of larvae fed *Artemia*. However, sections of liver analyzed at d 65 PFF, at which point gut development would more easily allow assimilation of ingested *Artemia*, showed lipid vacuoles present in larvae fed both copepods and enriched *Artemia*.

Studies performed by Japanese researchers have suggested that, in addition to the provision of essential (n-3) HUFA, supplementation with adequate vitamin A is vital for successful skin pigmentation (Kanazawa 1993, Miki et al. 1990). Pigmentation may require signal transmission via the visual system to the brain, which allows increased melanocyte-stim-

ulating hormone production and consequent synthesis of melanin (Estevez and Kanazawa, 1996, Kanazawa 1993). A deficiency in dietary vitamin A, which is a precursor of rhodopsin, will disrupt transmission between the eye and the brain. Marine copepods are rich in carotenoid pigments (8.2–43.6 μmol astaxanthin/g lipid for the four *E. velox* size ranges, highest value in 125- to 250- μm size fraction), including mono- and diesters of astaxanthin as well as unesterified astaxanthin, whereas *Artemia* contain lower quantities of the related carotenoid canthaxanthin (4.5–5.9 μmol canthaxanthin/g lipid for the enriched *Artemia* used in this study), which is present only in the unesterified form (Krinsky 1965, J. McEvoy, Institute of Aquaculture, University of Stirling). Although both canthaxanthin and astaxanthin can be converted to vitamin A in fish (Olson 1989), the higher quantity of total carotenoid in *E. velox*, coupled with the apparent increased digestibility of copepods in early developing larvae, may allow more efficient uptake and metabolism of these vitamin A precursors in halibut fed copepods compared with those fed *Artemia*.

Marine fish are naturally enriched with 22:6(n-3) and their neural tissues are especially rich in this HUFA (Bell and Dickerson 1991, Tocher and Harvie 1988). Membranes that are highly enriched in DHA such as those of the rod outer segment membrane are highly "stressed" due to the packing properties of di-22:6(n-3)-containing phospholipids; this can facilitate rapid conformational changes of the membrane structure as seen during the *cis* to *trans* transition that occurs on light activation of rhodopsin (Brown 1994). Thus, DHA may have a quite specific role in visual cell membranes. In addition, DHA-rich membranes are cycled between the retinal epithelium membrane and the photoreceptor membrane, and dietary DHA deficiency can interfere with this process (Bazan et al. 1992). In young developing mammals, dietary (n-3) PUFA deficiency is known to impair visual acuity (Hrboticky et al. 1991, Neuringer et al. 1984). A similar loss in visual function has been observed in larval herring (*Clupea harengus* L.); DHA deprivation caused impaired visual performance, particularly at low light intensities when rod cells are active (Bell et al. 1995). A linear relationship has been shown between the recruitment of rods in the herring retina and its content of di-DHA molecular species of phospholipids (Bell and Dickerson 1991).

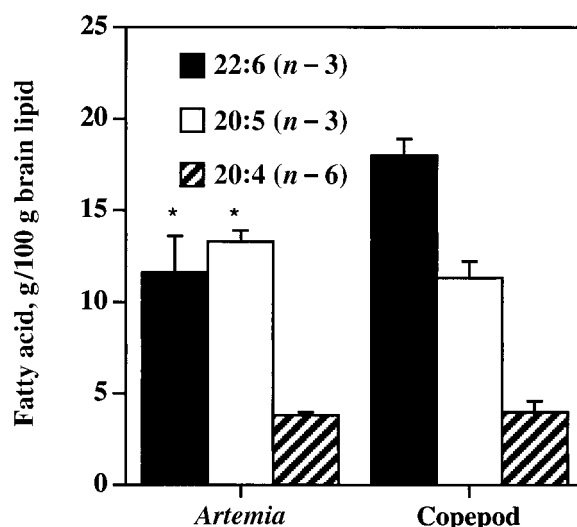


FIGURE 6 Levels of 22:6(n-3), 20:5(n-3) and 20:4(n-6) in liver total lipid from halibut larvae fed either enriched *Artemia* or *Eurytemora velox* copepods. Values are means \pm SD, $n = 3$. *Significantly different from copepod-fed larvae ($P < 0.05$).

1993). On the basis of these findings, it can be inferred that the significantly lower number of rod cells present in the retinas of *Artemia*-fed larvae may reflect a relative lack of dietary DHA and, consequently, retinal membrane DHA, in comparison to copepod-fed larvae.

Although problems in obtaining a sufficient supply of copepods to satisfy demand are likely to prevent their exclusive or even extensive use in marine fish culture, copepods clearly represent a more nutritionally optimized live prey organism than rotifers or *Artemia*. Naess et al. (1995) demonstrated that halibut pigmentation characteristics benefit even from partial supplementation of an *Artemia*-based diet with copepods, and this combined feeding strategy may offer a practical means of overcoming the developmental abnormalities associated with the use of enriched *Artemia*. Whether the superior qualities of marine copepods can be assigned to their high (n-3) HUFA content alone or to a combination of factors including carotenoid and phospholipid content remains the subject of continuing research.

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LITERATURE CITED

- Ackman, R. G. (1980) Fish lipids, part 1. In: *Advances in Fish Science and Technology* (Connell, J. J., ed.), pp. 86–103. Fishing News Books Ltd., Farnham, U.K.
- Barclay, W. & Zeller, S. (1996) Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. *J. World Aquacult. Soc.* 27: 314–322.
- Bazan, N. G., Gordon, W. C. & Rodriguez de Turco, E. B. (1992) Docosahexaenoic acid uptake and metabolism in photoreceptors: retinal conservation by an efficient retinal pigment epithelial cell-mediated recycling process. *Adv. Exp. Med. Biol.* 318: 295–306.
- Bell, M. V., Batty, R. S., Dick, J. R., Fretwell, K., Navarro, J. C. & Sargent, J. R. (1995) Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids* 30: 443–449.
- Bell, M. V. & Dick, J. R. (1991) Molecular species composition of the major diacyl glycerophospholipids from muscle, liver, retina and brain of cod (*Gadus morhua*). *Lipids* 26: 565–573.
- Bell, M. V. & Dick, J. R. (1993) The appearance of rods in the eyes of herring and increased di-dicosahexaenoyl molecular species of phospholipids. *J. Mar. Biol. Assoc. U. K.* 73: 679–688.
- Bell, M. V., McEvoy, L. A. & Navarro, J. C. (1996) Deficit of docosahexaenoyl phospholipid in the eyes of larval sea bass fed an essential fatty acid deficient diet. *J. Fish Biol.* 49: 941–952.
- Brown, M. F. (1994) Modulation of rhodopsin function by properties of the membrane bilayer. *Chem. Phys. Lipids* 73: 159–180.
- Christie, W. W. (1982) *Lipid Analyses*, 2nd ed., pp. 52–56. Pergamon Press, Oxford, U.K.
- Estevez, A. & Kanazawa, A. (1996) Fatty acid composition of neural tissues of normally pigmented juveniles of Japanese flounder using rotifer and *Artemia* enriched in n-3 HUFA. *Fish. Sci.* 62: 88–93.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497–509.
- Gara, B., Shields, R. J. & McEvoy, L. A. (1998) Feeding strategies to achieve correct metamorphosis of Atlantic halibut, *Hippoglossus hippoglossus* L., using enriched *Artemia*. *Aquac. Res.* 29: 935–948.
- Geurden, I., Coutteau, P. & Sorgeloos, P. (1997) Effect of a dietary phospholipid supplementation on growth and fatty acid composition of European sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) juveniles from weaning onwards. *Fish Physiol. Biochem.* 16: 259–272.
- Geurden, I., Radunz-Neto, J. & Bergot, P. (1995) Essentiality of dietary phospholipids for carp (*Cyprinus carpio* L.) larvae. *Aquaculture* 131: 303–314.
- Ghioni, C., Bell, J. G. & Sargent, J. R. (1996) Polyunsaturated fatty acids in neutral lipids and phospholipids of some freshwater insects. *Comp. Biochem. Physiol.* 114B: 161–170.
- Gronkjaer, P., Jorgensen, S. B., Fredricksen, M., St. John, M., Clemmensen, C. & Stotttrup, J. G. (1995) The influence of essential fatty acid composition on growth of larval cod (*Gadus morhua* L.). Preliminary observations. Vol. 19 pp. 14. Baltic Fish Committee. ICES J.
- Hrboticky, N., MacKinnon, M. J. & Innis, S. M. (1991) Retina fatty acid composition of piglets fed from birth with linoleic acid-rich vegetable oil formula for infants. *Am. J. Clin. Nutr.* 53: 483–490.
- Izquierdo, M. S., Watanabe, T., Takeuchi, T., Arakawa, T. & Kitajima, C. (1989) Optimal EPA levels in *Artemia* to meet the EPA requirements of red seabream (*Pagrus major*). *Proc. 3rd Int. Symp. Feeding and Nutr. in Fish. Toba (Japan)*, pp. 221–232.
- Kanazawa, A. (1985) Essential fatty acid and lipid requirement of fish. In: *Nutrition and Feeding in Fish* (Cowey, C. B. Mackie, A. M. & Bell, J. G., eds.), pp. 281–298. Academic Press, London, UK.
- Kanazawa, A. (1991) Nutritional mechanisms causing abnormal pigmentation in cultured marble sole larvae, *Limanda yokohamae* (Heterosomata). In: *Larvi '91- Fish and Crustacean Larviculture Symposium* (Sorgeloos, P., Lavens, P., Jaspers, E. & Ollevier, F., eds.), European Aquaculture Society Spec. Publ. 15, pp. 20–22.
- Kanazawa, A. (1993) Nutritional mechanisms involved in the occurrence of abnormal pigmentation in hatchery-reared flatfish. *J. World Aquacult. Soc.* 24: 162–166.
- Kanazawa, A., Teshima, S., Inamori, S. & Matsubara, H. (1983) Effects of dietary phospholipids on growth of the larval red sea bream and knife jaw. *Mem. Fac. Fish Kagoshima Univ.* 32: 109–114.
- Koven, W. M., Kolkovski, A., Tandler, A., Kissil, G. W. & Sklan, D. (1993) The effect of dietary lecithin and lipase, as a function of age, on n-9 fatty acid incorporation in the tissue lipids of *Sparus aurata* larvae. *Fish Physiol. Biochem.* 10: 357–364.
- Krinsky, N. I. (1965) The carotenoids of the brine shrimp, *Artemia salina*. *Comp. Biochem. Physiol.* 16: 181–187.
- Leger, P., Bengtson, D. A., Simpson, K. L. & Sorgeloos, P. (1986) The use and nutritional value of *Artemia* as a food source. *Oceanogr. Mar. Biol. Annu. Rev.* 24: 521–623.
- Luizi, F. S., Gara, B., Shields, R. J. & Bromage, N. R. (1999) Further description of the development of digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia* prey. *Aquaculture*, in press.
- McEvoy, L. A., Navarro, J. C., Hontoria, F., Amat, F. & Sargent, J. R. (1996) Two novel *Artemia* enrichment diets containing polar lipid. *Aquaculture* 144: 339–352.
- Miki, N., Taniguchi, T., Hamakawa, H., Yamada, Y. & Sakurai, N. (1990) Reduction of albinism in hatchery reared flounder "hirame," *Paralichthys olivaceus*, by feeding rotifer enriched with vitamin A. *Suisanzoshoku* 38: 147–155.
- Naas, K. E., Naess, T. & Harboe, T. (1992) Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.) in green water. *Aquaculture* 105: 143–156.
- Naess, T., Germain-Henry, M. & Naas, K. E. (1995) First feeding of Atlantic halibut (*Hippoglossus hippoglossus*) using different combinations of *Artemia* and wild zooplankton. *Aquaculture* 130: 235–250.
- Navarro, J. C., Batty, R. S., Bell, M. V. & Sargent, J. R. (1993) Effects of two *Artemia* diets with different contents of polyunsaturated fatty acids on the lipid composition of larvae of Atlantic herring (*Clupea harengus*). *J. Fish Biol.* 43: 503–515.
- Navarro, J. C., McEvoy, L. A., Amat, F. & Sargent, J. R. (1995) Effects of diet on fatty acid composition of body zones in larvae of the sea bass *Dicentrarchus labrax*: a chemometric study. *Mar. Biol.* 124: 177–183.
- Neuringer, M., Connor, W. E., van Petten, C. & Barstad, L. (1984) Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. *J. Clin. Invest.* 73: 272–276.
- O'Connell, C. P. (1963) The structure of the eye of *Sardinops caerulea*, *Engraulis mordax*, and four other pelagic marine teleosts. *J. Morphol.* 113: 287–329.
- Olson, J. A. (1989) Provitamin A function of carotenoids: the conversion of β -carotene into vitamin A. *J. Nutr.* 119: 105–108.
- Reitan, K. I., Rainuzzo, J. R., Oie, G. & Olsen, Y. (1997) A review of the nutritional effects of algae in marine fish larvae. *Aquaculture* 155: 207–221.
- Reitan, K. I., Rainuzzo, J. R. & Olsen, Y. (1994) Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. *Aquac. Int.* 2: 33–48.
- Rodriguez, C., Perez, J. A., Lorenzo, A., Izquierdo, M. S. & Cejas, J. R. (1994) n-3 HUFA requirement of larval gilthead sea bream *Sparus aurata* when using high levels of eicosapentaenoic acid. *Comp. Biochem. Physiol.* 107A: 693–698.
- Sargent, J. R., Bell, J. G., Bell, M. V., Henderson, R. J. & Tocher, D. R. (1993) The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: *Aquaculture: Fundamental and Applied Research*. Coastal and Estuarine Studies (Lahlou, B. & Vitiello, P. eds.), vol. 43, pp. 103–124. American Geophysical Union, Washington, DC.
- Sargent, J. R., Bell, J. G., Bell, M. V., Henderson, R. J. & Tocher, D. R. (1995a) Requirement criteria for essential fatty acids. *J. Appl. Ichthyol.* 11: 183–198.
- Sargent, J. R., Bell, M. V., Bell, J. G., Henderson, R. J. & Tocher, D. R. (1995b) Evolution and roles of (n-3) polyunsaturated fatty acids in marine organisms. In: *Phospholipids: Characterization, Metabolism and Novel Biological Applications*, pp. 248–259. AOC Press, Champaign, IL.
- Sargent, J. R. & Henderson, R. J. (1986) Lipids. In: *The Biological Chemistry of Marine Copepods* (Corner, E.D.S. & O'Hara, S.C.M., eds.), pp. 59–108. Oxford University Press, New York, NY.
- Sargent, J. R., Henderson, R. J. & Tocher, D. R. (1989) The lipids. In: *Fish Nutrition* (Halver, J. E., ed.), 2nd ed., pp. 153–218. Academic Press, San Diego, CA.

- Sastry, P. S. (1985) Lipids of nervous tissue: composition and metabolism. *Prog. Lipid Res.* 24: 69–176.
- Segner, H., Storch, V., Reinecke, M., Kloas, W. & Hanke, W. (1994) The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus*. *Mar. Biol.* 119: 471–486.
- Southgate, P. C. & Lou, D. C. (1995) Improving the n-3 HUFA composition of *Artemia* using microcapsules containing marine oils. *Aquaculture* 134: 91–99.
- Takeuchi, T., Arakawa, T., Satoh, S. & Watanabe, T. (1992) Supplemental effect of phospholipids and requirement of eicosapentaenoic and docosa-hexaenoic acids of juvenile striped jack. *Nippon Suisan Gakkaishi* 58: 707–713.
- Takeuchi, T., Feng, Z., Yoseda, K., Hirokawa, J. & Watanabe, T. (1994) Nutritive value of DHA-enriched rotifer for larval cod. *Nippon Suisan Gakkaishi* 60: 641–652.
- Tinoco, J. (1982) Dietary requirements and functions of α -linolenic acid in animals. *Prog. Lipid Res.* 21: 1–45.
- Tocher, D. R. & Harvie, D. G. (1988) Fatty acid compositions of the major phosphoglycerides from fish neural tissues: (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. *Fish Physiol. Biochem.* 5: 229–239.
- Zar, J. H. (1984) *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, NJ.