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Cofeeding of phospholipids to turbot *Scophthalmus maximus* L. larvae as a tool to reduce live food consumption

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Abstract

The possible benefits of feeding a formulated diet, together with *Artemia*, to first-feeding turbot larvae were investigated. Five dietary regimes were used: control (*Artemia* only), cofeeding with a diet rich in phospholipid (PL) from bovine brain, cofeeding with a diet rich in free fatty acids (FFA) from the same source, cofeeding with a commercial diet (Lansy A2), and a formulated feed only (PL-rich diet). All treatments with artificial diets and the control treatment that received *Artemia* exclusively gave a similar survival rate of the fish at day 29. Cofeeding with artificial diets resulted in a lower growth rate than the control treatment. Cofeeding the larvae with PL-rich or FFA-rich diets did not affect the pigmentation or resistance to salt stress of the larvae. Complete substitution of *Artemia* by the phosholipid diets from day 20, however, resulted in severe stress and pigmentation problems. Cofeeding might have interesting features for application since it may reduce the amount and cost of live food and also offer to the fish larvae essential nutrients that are not contained in sufficient amounts in the (enriched) live food.

KEY WORDS: *Artemia*, cofeeding, HUFA, phospholipids, turbot

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Introduction

It is generally accepted that for all marine fish species with small larvae, live feed is essential to ensure high survival and growth rates. This feeding strategy is expensive, requiring manpower and equipment. Moreover, nutritional and sanitary measures for optimal prey conditioning are hard to control. It could be economically advantageous to substitute inert diets directly at first feeding, or at least minimize the duration of the live-feed period (Person Le Ruyet 1989). For production of 45-day-old sea bass, live prey feeding represents up to 70% of the production costs. By progressively replacing the live feed with manufactured diets at earlier larval developmental stages, the amounts of live feed required for juvenile production will not only be reduced, but the weaning of the larvae can be completed at an earlier age (Person Le Ruyet *et al.* 1993). However, this feeding strategy should have minimal impact on quantitative as well as qualitative outputs of the hatchery operation. Since the mid-1970s, it has been repeatedly emphasized that *Artemia* cysts shortages combined with the expansion of aquaculture will result in a higher-priced and possibly lower-quality product (Sorgeloos 1979).

The possibility of replacing live feed with manufactured diets from the onset of exogenous feeding has been investigated in several studies. Inferior larval performance was commonly reported when artificial diets were used solely, suggesting that one of the main factors was the incomplete functioning digestive system of larvae (Holt 1993; Kolkovski *et al.* 1993; Walford & Lam 1993; Zambonino-Infante & Cahu 1994). Although digestive enzymes are found in turbot larvae at the onset of exogenous feeding, exogenous enzymes provided by the live feed may increase digestive capacity (Munilla-Moran *et al.* 1990; Holt 1993; Munilla-Moran 1994). Positive results have been reported when cofeeding fish larvae, suggesting that this strategy increases the supply of more suitable nutrients, which in turn stabilizes and improves the nutritional status of the larvae (Kanazawa *et al.* 1989; Holt 1993; Leu *et al.* 1991; Abi-Ayad & Kestemont 1994; Rosenlund & Stoss 1995).

The combined feeding of live and inert diets from an early larval age, referred to as cofeeding (Rosenlund & Stoss 1995) is considered a promising feeding strategy for turbot larvae. The dietary requirement for phospholipids and *n*-3 highly

unsaturated fatty acids (HUFAs), particularly docosahexaenoic acid (DHA), and their role in larval development has been reviewed by many authors (Kanazawa *et al.* 1983a,b; Sargent *et al.* 1993; Watanabe 1993; Mourente *et al.* 1993; Bell *et al.* 1995). The live prey *Brachionus plicatilis* and particularly *Artemia* are deficient in DHA, hence, their content has to be increased prior to offering them to the larvae, essentially by prefeeding them with *n*-3 HUFA-rich products (Léger *et al.* 1987; Sorgeloos & Léger 1992). This enrichment technique in *Artemia* nauplii has its limitations, since most strains selectively catabolize DHA (Sorgeloos *et al.* 1995). Cofeeding inert diets is advantageous since catabolism by live food during enrichment is avoided. Therefore, there is a growing demand for natural lipids highly enriched in DHA as nutritional supplements during larval development (Tocher *et al.* 1997). Obvious sources of natural lipids rich in DHA are animal brains, retinal tissue and fish roe, which can be obtained from slaughterhouses or from fishery byproducts.

In the present study, phospholipid and free fatty acid (FFA)-rich lipid fractions were obtained from bovine neural tissue and incorporated in a particulate inert diet to evaluate their efficiency on growth, survival, pigmentation and stress resistance on cofed turbot larvae.

Materials and methods

The newly hatched turbot larvae were obtained from France Turbot (Ile de Noirmoutier, France). The experiment was performed in 25 rearing tanks (60 L), equipped with individual biofilters (Dhert *et al.* 1992a). The larvae were stocked at a density of 60 larvae/L^{-1} in natural seawater (salinity 35 g L^{-1}). The temperature was increased by 0.5°C day^{-1} from 14°C at stocking to 18 ± 0.5°C. The photoperiod was maintained at constant 24 h light using fluorescent light tubes. From stocking to eye pigmentation, light intensity was 120–140 lx at the water surface. Prior to feeding it was increased to 500–600 lx until the end of the experiment. An antibiotic (Linco-Spectin 100, Upjohn NV, Belgium) was administered to each tank (5 mg kg^{-1} day^{-1}) before stocking (day 1 post hatch), at mouth opening (day 3 post hatch) and at day 6 to prevent early massive mortalities caused by bacterial infections.

Rotifers were fed on baker's yeast (Bruggeman instant, Bruggeman NV, Ghent, Belgium), supplemented with 150 g kg^{-1} on a wet-weight basis of DHA Selco (INVE Aquaculture NV, Baasrode, Belgium) and offered to the turbot larvae twice per day until day 8. Newly hatched *Artemia* nauplii (DC-EG *Artemia* cysts, INVE Aquaculture NV) were fed from day 8 to day 10, followed by 24-h DHA Selco-enriched *Artemia* from day 11 onwards.

Table 1 Formulation of the extruded basal diet. The total diet consisted of 800 g kg^{-1} extruded basal diet, 144 g kg^{-1} fat, 40 g kg^{-1} carrageenan and 16 g kg^{-1} emulsifier

	g kg^{-1}
Extruded basal diet	800
Astaxanthine[1]	0.8
Attractant premix[2]	26
Butylated hydroxyanisole[3]	0.04
Butylated hydroxytoluene[3]	0.04
α-Cellulose[4]	7
Choline chloride[5]	8.6
Codfish powder[6]	216.3
Egg white albumin[7]	104
Emulgator blend[8]	3.4
Haemoglobin powder[9]	34.6
Hydrogenated coconut oil[10]	34.6
Isolated soybean protein[11]	95.2
Mineral premix[12]	17.3
Native corn starch[13]	112.4
Vitamin C[14]	1.3
Vitamin premix[15]	17.3
Wheat gluten[16]	26
Whey protein concentrate[17]	95.2

[1] Carophyl Pink, Roche, Belgium.
[2] According to Kanazawa *et al.* (1989).
[3] Fédera, Belgium. The extruded basal diet contains 6% of fat with less than 0.35 mg g^{-1} DW of *n*-3 HUFAs.
[4] Sigma C8002.
[5] 50% purity, INVE Aquaculture NV, Belgium.
[6] Code 0271, Rieber œ Son A/S, Norway.
[7] type HG LW^{-1}, Orffa Belgium NV, Belgium.
[8] Glycerol mono-oleate/sorbitan monostearate (1:1).
[9] Vepro 95 PHF, Veos NV, Belgium.
[10] Cocos 32/34, Vandemoortele NV, Belgium.
[11] Supro 500E, Protein Technologies International, Belgium.
[12] According to Coves *et al.* (1991).
[13] Snowflake 03401, Orffa Belgium NV, Belgium.
[14] Mg-L-ascorbyl-2-monophosphate, Phosphitan C, Showa Denko KK, Japan.
[15] According to Coves *et al.* (1991) Roche, Belgium.
[16] Biogluten, Amylum NV, Belgium.
[17] Lacprodan-80, Orffa Belgium NV, Belgium.

Five dietary regimes were used in five replicates starting from day 11 onwards: control (*Artemia* only), cofeeding with a diet rich in phospholipid (PL) from bovine brain, cofeeding with a diet rich in FFAs from the same source, cofeeding with a commercial diet, and formulated feed only (PL-rich diet). The basal experimental diet formulation was based on Coutteau *et al.* (1996) with dietary protein provided by a mixture of various protein sources (Table 1). The lipid component consisted of a phospholipid-rich or a free fatty acid-rich lipid fraction extracted from bovine brain. Bovine brains were obtained from the pilot slaughterhouse of the University of Gent (Department Animal Production),

Table 2 Total lipids (g kg^{-1} DW), lipid composition (g $kg^{-1}$of total lipid) and fatty acid composition of the extracted fats and experimental diets ($n = 3$). For a description of the diets see Figure 1

	Extracted fats		Experimental diets		Control diet	
Lipid composition[1]	PL-rich bovine brain	FFA-rich bovine brain	Lansy A2	PL bovine brain	FFA bovine brain	*Artemia* DHA-enriched
Total lipid	1000	1000	181.4	192	192	296.5
Cholesterol esters	0.0	9.9	6.3	3.7	16.6	4.8
Ethyl esters	0.0	0.0	194.7	0.0	9.2	0.0
Triglycerols	0.0	14.4	450.1	229.9	231.2	712.9
Free fatty acids	0.0	448.6	25.7	7.3	345.1	74.2
Cholesterol + DAG	151.7	162.9	118.2	141.0	162.3	44.3
MAG	388.9	276.0	54.5	312.5	172.5	24.5
Total neutral lipids	540.6	911.8	849.5	603	936.9	860.6
PE[2]	69.1	0.0	19.7	35.9	4.3	42.2
PS + PI	17.5	5.0	2.2	10.6	3.3	1.6
PC	207.1	0.0	120.9	171.8	9.5	89.9
Sphingomyelin	136.5	83.3	2.7	80.6	42.3	2.0
Lyso P. choline	29.2	0.0	5.2	6.5	3.7	3.6
Total polar lipids	459.4	88.2	150.5	397.0	63.1	139.3
Fatty acid composition						
DHA (area percentage)	7.1	7.4	21.7	7.1	7.4	7.2
DHA (mg g^{-1} DW)	4.6	6.2	25.3	4.6	6.2	17.2
EPA (area percentage)	0.8	0.7	13.2	0.8	0.7	7.4
EPA (mg g^{-1} DW)	0.5	0.6	15.3	0.5	0.6	17.8
DHA/EPA	9.2	10.3	1.7	9.2	10.3	1.0
(*n*-3) HUFA (area percentage)	9.1	9.2	38.0	9.1	9.2	16.7
(*n*-3) HUFA (mg g^{-1} DW)	5.8	7.7	44.2	5.8	7.7	40.1
Total mg FAME g^{-1} DW	63.7	83.7	116.4	99.10	119.10	239.3

[1]DAG, di-acyl glycerols; DW, dry weight; FAME, fatty acid methyl ester; MAG, mono-acyl glycerides; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; PC, phosphatidyl choline; Lyso P. choline, lysophosphatidyl choline.
[2] PE can contain minor amounts of PA (phosphatidyl amine).

Belgium. Lipids were extracted using the procedure of Bligh & Dyer (1959). For each kg of brains, 4 L of solvent ($CHCl_3$–MeOH, 1:1) was used to which 1.8 L KCl (8.8 g L^{-1}) minus the water content of the tissue was added to get a final solvent ratio ($CHCl_3$–MeOH–H_2O) of 10:10:9. From this mixture, the lower phase containing the lipids was recovered and dehydrated with Na_2SO_4. After evaporation of the solvents (rotavapor, Van Der Heyden, Brussels, Belgium) the fat was redissolved in a minimal amount of $CHCl_3$ to which a four times higher amount (v/v) of acetone was added to precipitate the phospholipids. The acetone fraction containing the neutral lipids was removed. The remaining phospholipid-rich oil was collected, flushed with N_2 and kept at −18°C. A subsample diluted in $CHCl_3$–MeOH (2:1) and kept at −30°C was taken for lipid class analysis. Part of this oil was used to prepare a free fatty acid fraction. To achieve this, 100 g fat was saponified with 2 L of a 0.5 M mixture of NaOH–MeOH at 70°C under continuous stirring. 1 M HCl was added to adjust the pH (pH = 3). Free fatty acids were extracted using 5 L of hexane. After hexane evaporation the free fatty acid-rich fraction from the polar lipid of the brain was obtained. Prior to the mixture of the ingredients (800 g kg^{-1} extruded basal diet, 144 g kg^{-1} fat, 40 g kg^{-1} carrageenan, 16 g kg^{-1} emulsifier and 200 mL of hot water at 90°C), the fat was mixed with the emulgators and water (40°C). A syringe was used to extrude the pellets, which were air-dried to a water content of less than 80 g kg^{-1}, crumbled and sieved into particles with ranges of 100–200 μm, 200–300 μm and 300–500 μm. The total lipid, lipid classes and fatty acid composition of the diets obtained are presented in Table 2.

In order to evaluate and compare the effectiveness of the experimental PL (phospholipid) diet and FFA (free fatty acid) diet, a commercial diet (Lansy A2, INVE Aquaculture NV) was also cofed. The cofeeding treatments were randomly assigned to the tanks and initiated on day 11. Half of the *Artemia* ration was replaced by artificial diet so that a 1:2 ratio of *Artemia*–artificial diet (DW) was given to the fish, based on the assumption that part of the artificial diet would sink or would not be available (Fig. 1). In one treatment, PL w/A (phospholipids without *Artemia*), the *Artemia* nauplii were completely substituted by the diet supplemented with bovine brain phospholipids from day 20 onwards.

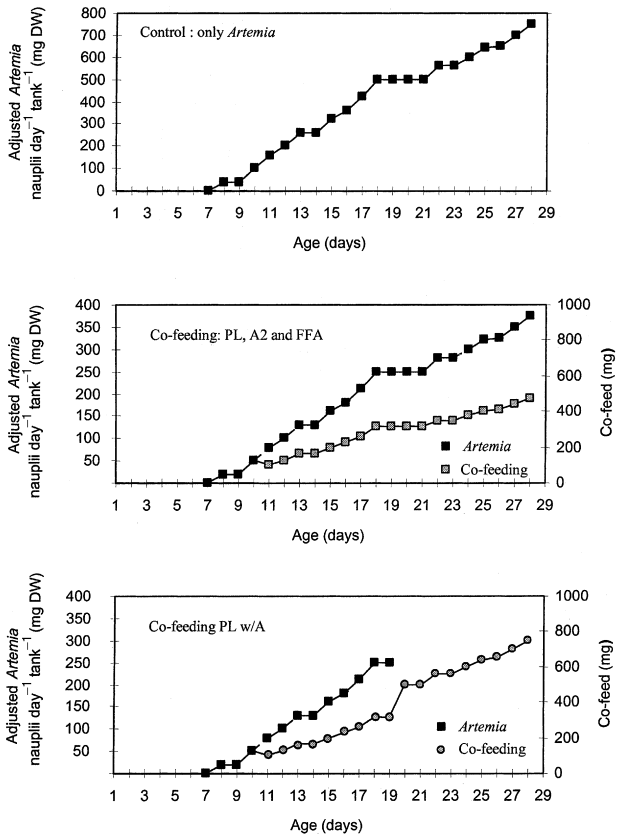

Figure 1 Feeding regime per turbot culture tank (60 L) with *Artemia* and cofeed. Control: only *Artemia* (non-enriched days 8–10; 24-h DHA Selco from day 11 onwards). PL: artificial diet coated with polar fraction of bovine brain extract. FFA: artificial diet coated with free fatty acid fraction of bovine brain extract. A2: commercial diet Lansy A. See text for details.

Total lipids were determined gravimetrically and extracted according to the method of Folch *et al.* (1957), modified by Ways & Hanahan (1964). Lipid classes were determined by Iatroscan thin-layer chromatography-flame ionization detection system (Iatroscan MK-5, TLC-FID, Iatron Laboratories Inc., Tokyo, Japan). Fatty acid analysis was performed by gas chromatography, with a Chrompack CP9001 (Varian, Brussels, Belgium) equipped with an autosampler. Fatty acid methyl esters (FAMEs) were prepared via a modified direct transesterification protocol of Lepage & Roy (1984). An internal standard (11–14 eicosadienoic acid) was added prior to the reaction in order to obtain quantitative results expressed in mg g^{-1} dry weight (DW). Fatty acid composition was also calculated as percentage of the total fatty acids (from 14:0 onwards).

The effect of the different treatments on the turbot larvae was evaluated by length and dry weight measurements taken at regular time intervals and by a salinity stress test performed on days 18 and 28, according to the method described by Dhert *et al.* (1992b). Salinities of 60 g L^{-1} and 70 g L^{-1} were used during the stress test on day 18 and day 28, respectively. All salinity stress tests were performed with five replicates per treatment.

The pigmentation percentages were obtained by individually counting pigmented and black larvae from all tanks at the end of the experiment (day 29). Survival (in percentage) of turbot larvae in all tanks was recorded at restocking (day 19) and at day 29.

The data were subjected to ANOVA and further comparison between means was subjected to Duncan's multiple range test. Results are given as mean ± SD and differences were considered significant at $P < 0.05$.

Results

Although no significant differences ($P < 0.05$) were found among treatments (Fig. 2), there was a greater tendency towards lower survival on the FFA-rich diet than in the other groups. On days 19 and 29 the highest survival rate was obtained in the treatment given the commercial diet Lansy A2 (61% and 45%, respectively) and the PL-rich diet from bovine brain (55% and 48%, respectively) as cofeed.

The mean standard length was significantly larger in the control group and smaller in the group fed formulated feed only, than in the cofed group. The PL-rich diet without *Artemia* nauplii gave the smallest animals (8.37 mm on day 29). The dry weight of the control group was also significantly greater ($P < 0.05$) than in all the other treatments on days 19, 24 and 29. The treatment PL w/A gave significantly

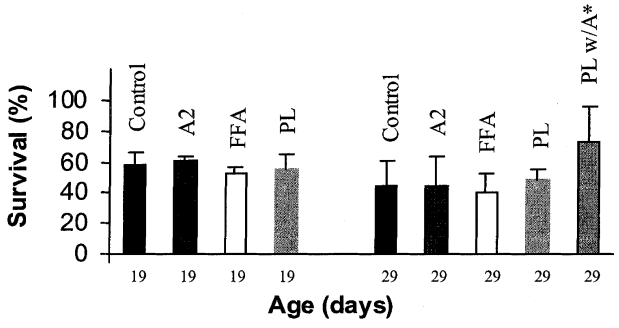

Figure 2 Survival rate (%) of turbot larvae on days 12 and 29 after hatching. For a description of the feeding regime see Figure 1 and text. Treatments on either day 19 or day 29 were not significantly different at P = 0.05. Asterisk indicates survival for the period day 19–29.

Table 3 Mean standard length (mm) and dry weight (mg) of larvae fed the experimental diets from days 19, 24 and 29 after hatching. For a description of the diets see Figure 1

	Day 19	Day 24	Day 29
Standard length (n = 5)			
Control	6.58a[1] ± 0.75	8.81a ± 0.97	13.04a ± 1.76
A2	6.05b ± 0.58	7.57b ± 1.06	11.59b ± 1.89
FFA	5.99b ± 0.62	7.09c ± 0.93	11.00b ± 1.43
PL	5.98b ± 0.60	7.57b ± 1.11	11.56b ± 1.21
PL w/A		6.46d ± 1.18	8.37c ± 1.59
Dry weight (n = 5)			
Control	1.03a ± 0.31	3.46a ± 0.74	7.16a ± 1.89
A2	0.68b ± 0.10	2.00b ± 0.021	4.81b ± 0.95
FFA	0.56b ± 0.14	1.98b ± 0.27	4.19b ± 1.03
PL	0.64b ± 0.15	2.17b ± 0.57	5.00b ± 0.89
PL w/A		1.19c ± 0.17	1.71c ± 0.21

[1] Values followed by the same letter in each column are not significantly different at $P < 0.05$.

Table 4 Mean index of stress resistance (SI; cumulative mortality) and standard deviation (n = 5) per treatment of turbot larvae on days 18 and 28. For a description of the diets see Figure 1

Treatment	SI (Day 18)	SI (Day 28)
Control	269a[1] ± 34.3	97a ± 23.7
PL	284a ± 43.4	105a ± 11.4
A2	286a ± 20.4	112a ± 21.3
FFA	315a ± 63.2	120a ± 18.7
PL w/A		125a ± 28.9

[1] Values followed by the same letter in each column are not significantly different at $P < 0.05$.

smaller animals with the lowest dry weight (8.37 mm and 1.71 mg, respectively) (Table 3).

The summed cumulative mortalities or stress index (SI) for each treatment are given in Table 4. The statistical analysis showed no significant differences ($P < 0.05$) in stress resistance among treatments on days 18 and 28.

The evaluation of pigmented, partly pigmented, nonpigmented and black larvae (stressed larvae) of the various treatments (Table 5) showed a statistical difference ($P < 0.05$) only for the PL w/A treatment, which gave the lowest percentage of pigmented and the highest percentage of black larvae.

The Iatroscan analysis of the cofeeds showed that the PL-rich diet contained a large amount of polar lipids — 397 g kg^{-1} compared with 151 g kg^{-1} (27.3 mg g^{-1}) in the A2 and 63 g kg^{-1} in the FFA diets (Table 2). The most important polar lipids in the diets were phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and, in the case of the bovine brain diets, sphingomyelin. In the analysis of turbot body tissue from day 29, the PL-rich diet yielded the highest percentage of polar lipids (497 g kg^{-1}, of which 75% was PC), but only 155 g kg^{-1} of total lipids. Statistical differences ($P < 0.05$) were detected among treatments for percentages of total lipids, cholesterol esters, triglycerols and cholesterol plus diacylglycerols (Table 6).

Table 5 Mean values of pigmented and black (stressed) turbot larvae. For the description of the diets see Figure 1

Treatment	Pigmented (%)	Partly pigmented (%)	Nonpigmented (%)	Black (%)
Control	66a[1]	14a	20a	0a
PL	59a	19a	19a	3a
A2	67a	15a	17a	1a
FFA	55a	13a	27a	5a
PL w/A	20b	17a	10b	53b

[1] Values followed by the same letter in each column are not significantly different at $P < 0.05$.

The fatty acid analysis of the cofeeds showed very high DHA:eicosapentaenoic acid (EPA) ratios (10.3 and 9.2 for the FFA-rich diet and the PL-rich diet, respectively). The A2 diet had the highest n-3 HUFA value (44.2 mg g^{-1} DW), the FFA diet had the highest value for total mg FAME g^{-1} DW (119.1 mg g^{-1} DW) (Table 2). In turbot larvae (day 29) significant differences ($P < 0.05$) were detected among parameters. The group with the highest DHA:EPA ratios was PL w/A (2.9). The larvae fed on the PL w/A diet always had the lowest amounts of total mg FAME g^{-1} DW and n-3 HUFAs, while for these parameters very high figures (129.7 mg and 26.7 mg, respectively) were obtained in the control group. The A2 group also had a high percentage of n-3 HUFAs (28.5%) (Table 7).

Discussion

Cofeeding might have attractive features for application in the hatchery rearing of commercial fish species. This technique not only allows the reduction in the cost of live food, but also allows the larvae to consume specific essential nutrients, which are not contained in sufficient amounts in the (enriched) live food. However, one would expect that a partial replacement of *Artemia* nauplii by a formulated artificial diet during early larval rearing would negatively influence the growth rate of the fish, e.g. owing to decreased ingestion rates. Hence, commercial application of cofeeding can only be considered if the quality and quantity of the hatchery production meet the requirements for a good performance during the consequent nursery phase.

In the present study, we demonstrated that cofed animals reached a growth intermediate between the 100% *Artemia*-

Table 6 Total lipid (% dry weight) and lipid composition (% of total lipid) of turbot larvae sampled on day 29. For the description of the diets see Figure 1

	Control		A2		FFA		PL		PL w/A	
Percentages[1] (%)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lipid	18.41a[2]	1.66	Missing	—	13.37a	2.09	15.45a	0.78	12.86a	0.97
Cholesterol esters	0.99a	0.31	1.35a	0.42	0.89ab	0.31	0.75ab	0.02	0.37b	0.11
Ethyl esters	0.41a	0.51	0.37a	0.33	0.88a	0.31	1.04a	0.18	0.34a	0.32
Triglycerols	18.43a	7.07	10.41b	2.24	8.74b	2.34	10.92ab	1.03	2.47b	1.12
Free fatty acids	10.28a	2.21	12.74a	12.62	13.11a	7.22	8.33a	0.13	13.11a	2.36
Cholesterol[3]	22.75a	1.96	23.33ab	2.22	27.38c	0.96	26.68bc	1.41	31.01d	1.74
Unknown neutral lipids	3.34a	1.00	4.36a	1.70	3.31a	0.53	2.63a	0.30	3.86a	0.73
Total neutral lipids	53.20a	4.40	52.56a	13.36	54.32a	5.39	50.33a	0.72	51.17a	3.08
PE[4]	5.50a	1.49	9.06a	5.85	6.91a	2.65	9.41a	1.10	10.49a	0.79
PS + PI	0.22a	0.15	0.11a	0.10	0.42a	0.27	0.19a	0.09	0.40a	0.10
PC	35.83a	4.32	36.22a	8.83	35.22a	1.43	37.14a	0.42	36.92a	0.09
Sphingomyelin	1.19a	0.18	1.21a	0.07	1.27a	0.33	1.17a	0.06	1.24a	0.01
Lyso PC	1.05a	0.54	0.84a	0.29	1.86a	0.85	1.78a	0.12	1.13a	0.38
Total polar lipids	43.80a	4.40	47.44a	13.36	45.68a	5.39	49.67a	0.72	48.83a	3.08

[1] PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; PC, phosphatidyl choline; Lyso P. choline, lysophosphatidyl choline.
[2] Values followed by the same letter in each row are not significantly different at $P < 0.05$.
[3] Cholesterol contains minor amounts of DAG.
[4] PE can contain minor amounts of PA.

Table 7 Fatty acid analysis of turbot larvae sampled on day 29. For a description of the diets see Figure 1

	Control		A2		FFA		PL		PL w/A	
Percentages[1] (%)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DHA (area %)	9.0a[2]	0.85	15.53b	1.80	11.03c	0.74	11.40c	1.70	12.97c	0.93
DHA (mg g^{-1} DW)	11.68a	0.79	13.37b	0.33	9.47a	0.32	11.10a	1.56	10.10a	0.53
EPA (area %)	7.32a	0.31	8.77b	0.47	7.13a	0.15	6.85a	0.07	4.43c	0.46
EPA (mg g^{-1} DW)	9.46a	0.43	7.5b	1.56	6.10b	0.20	6.60b	0.00	3.53c	0.76
DHA/EPA	1.2a	0.11	1.8b	0.19	1.6ab	0.10	1.7ab	0.24	2.9c	0.57
(*n*-3) HUFA (area %)	20.58ab	0.77	28.47c	1.70	22.40b	0.56	21.90ab	2.26	19.73a	0.61
(*n*-3) HUFA (mg g^{-1} DW)	26.68a	1.95	24.50ab	5.67	19.17c	0.25	21.25cb	1.91	15.43c	1.31
Total mg FAME g^{-1} DW	129.7a	10.56	86.43b	21.61	85.53b	2.63	97.2b	1.27	78.47b	8.96

[1] DHA, docosahexaenoic acid; DW, dry weight; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; HUFA, highly unsaturated fatty acids.
2 Values followed by the same letter in each row are not significantly different at $P < 0.05$.

fed control group and the group fed exclusively on artificial feed. Compared with the control, cofed larvae had reached 84–88% of the length and 59–70% of the dry weight of the 29-day-old fish fed enriched *Artemia,* corresponding to a loss of approximately 2 days of culture. However, it should be emphasized that despite the reduced growth performances no difference in larval quality was found. Survival rates were high (over 50% and 35% on days 19 and 29, respectively) in both the cofed and control group. Moreover, no difference in larval quality could be detected when subjecting the larvae to a standardized salinity stress test. In this respect, the use of cofeeding diets provides a valuable feeding strategy for commercial hatcheries, as it reduces the dependency on natural resources (irregular supply and quality fluctuations of *Artemia*) and still allows the production of standard quality fry.

The uptake of the inert food by the larvae had been observed, but had not been recorded on a regular basis. However, the proper ingestion and assimilation of the artificial diets was indicated by the clear reflection of the dietary differences in the tissues of animals fed the different cofeeding diets. DHA, which was present in different concentrations in the cofeeding diets, was reflected in the turbot proportional to the dietary DHA composition. This is especially important for DHA since it is catabolized by *Artemia* and thus difficult to be vectored through live food (Dhert *et al*. 1993). The comparison of DHA levels in turbot of the cofeeding treatments with that in fish of the control

treatment showed that the fish receiving relatively low levels of DHA (bovine brain extracts PL and FFA) had similar or even higher DHA levels measured as an area percentage (percentage of fatty acids) than those of the control treatment. This demonstrates that the cofeeding technique is a powerful tool for offering specific nutrients that are normally catabolized by live food.

In contrast to data showing a positive influence of increased dietary DHA : EPA ratios on larval pigmentation (Devresse *et al*. 1992; Reitan *et al*. 1994; Dhert *et al*. 1994), no relation between the pigmentation and biochemical composition of the fish could be detected in the present study. The fact that the control group with the lower DHA : EPA ratio reached a similar pigmentation rate as the cofed larvae may be explained by the greater amount of total *n*-3 HUFA per unit dry body matter possibly having a stronger impact on pigmentation than the DHA : EPA ratio. Conversely, the PL w/A group with the highest DHA : EPA ratio had the lowest proportion of pigmented larvae and the highest proportion of black larvae, which may indicate larval stress caused by malnutrition (Dhert *et al*. 1994). Similar observations were reported during starvation of larval turbot, where DHA : EPA ratios increased because of the preferential consumption of EPA over DHA (Planas *et al*. 1990).

The three isolipidic cofeeding diets had a very distinct lipid class composition. Diet FFA contained a large amount of free fatty acids, diet PL was rich in polar lipids and the commercial diet A2 had mainly triacylglycerols and ethyl esters. The control group fed the *Artemia* had the greatest amount of body triacylglycerol (TAG), followed by the A2, PL and FFA group. The level of TAG in fish depends on their nutritional status and is not directly related to the lipid classes fed through the diet, as clearly demonstrated in post-larval turbot by Geurden *et al*. (1998). Body lipids of turbot from that study contained an even greater amount of TAG when fed a diet rich in PL than when fed a TAG-rich diet. The body lipids of the PL w/A larvae had a very low TAG content, explaining their low stress resistance, malpigmentation and poor growth. Similarly, fish of the PL w/A group, and also of the FFA group, had the lowest total lipid level, in strong contrast to fish fed the commercial diet A2. The inferior results observed in the PL w/A group are in agreement with data from Holt (1993), Kolkovski *et al*. (1993), Walford & Lam (1993) and Zambonino-Infante & Cahu (1994), observing a lower performance or lack of success when formulated diets were used as the only food source. It is suggested that an incompletely functioning digestive system combined with low feed intake are the main reasons for the poor performance of marine fish larvae deprived of live prey organisms (Kolkovski *et al*. 1993; Webster & Lovell 1990; Weinhart & Rösch 1991).

Although not significant, there was a tendency towards a slightly higher survival, growth, total body lipid and TAG content in larvae cofed with the PL diet compared with the diet containing the FFA fraction, suggesting that phospholipids are better assimilated and used for meeting the nutritional requirements than FFA. However, little is known on the specificities and activities of lipolytic enzymes during intestinal hydrolysis in fish larvae. However, it should be noted that the present study did not demonstrate the beneficial effect of supplementing the diet with PL to the same degree as Kanazawa *et al*. (1983a, 1983b) for 10-day-old seabream/ayu or as Geurden *et al*. (1995) for start-feeding common carp. It is possible that the PL requirement of the cofed turbot was already being fulfilled by the PL provided through the consumption of the *Artemia* nauplii. Consequently, the formulation of the artificial cofeeding diets should particularly focus on nutrients being deficient in *Artemia*.

Conclusions

Cofeeding regimes can be used successfully in the larviculture of turbot and may be economically advantageous owing to the reduction in the use of *Artemia*. The use of a manufactured diet as the only food source in early stages of turbot rearing resulted in poor performance. Although a higher performance for standard length and dry weight was achieved in the control group, for the rest of the larval quality parameters measured, the cofed larvae performed as well as the control larvae. Furthermore, when cofeeding a phospholipid rich diet, an important increase in survival rates may be expected.

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