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UTILIZATION OF PROTEIN, FAT AND GLYCOGEN IN COD (*GADUS MORHUA*) DURING STARVATION

by

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ABSTRACT

Three groups of cod (*Gadus morhua*) with relative liver sizes Low, Medium and High were used in this 12 weeks starvation study. During the experiment fish were sampled for measurement of weight, relative liver size and content of water, lipid and glycogen in liver and water, protein and glycogen in fillets. During fasting total weight loss was 15%, 12% and 11% for groups Low, Medium and High, respectively. In all groups a decrease in relative liver size was found during fasting, however, largest decrease in liver lipid stores were measured in the groups with the initially lowest liver index and lipid levels. There was an increase in fillet water content in those fish with initial low liver index after 4 weeks of fasting but not in the two other groups. In group Medium this increase was observed only after 8 weeks and in group High no increase in fillet water was observed. The concentration of protein was stable, leading to an increased water:protein ratio when fillet water increased. Both liver and fillet glycogen content varied according to relative liver size before fasting. Liver glycogen was utilized as energy during fasting. However, this energy store was not depleted, neither in fillet nor in liver. The reduction in glycogen was observed during the first part of the starvation period.

INTRODUCTION

One of the guidelines from Norwegian Authorities to improve fillet quality is to starve the reared fish before slaughter at least for one week. However, longer periods of starvation is common. The present study deals with the effect of starvation upon fillet quality as well as energy utilization according to pre-fasting state of cod.

Atlantic cod is a lean fish in which the major part of its lipid content

is found in the liver. In reared cod the liver lipid content is often extremely high and there is a linear positive correlation to dietary lipid. High values for liver weight/body weight have been demonstrated (Lie *et al.* 1986).

Earlier studies with cod starved for 3 to 4 weeks showed that of a total weight loss of about 17%, decreased liver weight contributed only 3% (Hemre *et al.* 1993). This indicates that the liver cells are partly inactive during fasting. The degree of inactivity may be dependent on the pre-fasting state of the fish. In reared cod the liver often contributes 12–17% of total live weight (Lie *et al.* 1986), while in wild cod liver sizes of 2–7% is common (Hemre, unpublished results). It is claimed that reared cod use primarily muscle protein and to some extent depot-fat to maintain an adequate metabolic pool of carbon substrates during fasting, while starving wild caught cod use mainly lipid energy from the liver (Takama *et al.* 1985, Hemre *et al.* 1990; 1993). The white muscle of cod contains no fatty globules, and the lipid consists mainly of phospholipids which decrease only when tissue structures are broken down (Love 1980).

In studies evaluating the effect of increasing amounts of feed carbohydrates no effect upon liver size has been observed. It has been suggested that storage of liver glycogen is limited. During short time starvation (3–4 weeks) reared cod utilized liver glycogen for energy, however, the levels used being dependent on the pre-fasting state (Hemre *et al.* 1990, 1993). In studies with isolated liver cells from starved chinook salmon (*Oncorhynchus tshawytscha*) a rapid breakdown of glycogen has been observed (Klee *et al.* 1990). Other studies have shown that glycogen was still present in liver cells from severely starved carp (*Cyprinus carpio*) (Gas 1973).

The present study evaluates whether the utilization of lipid, glycogen and protein energy during starvation of cod depends on pre-fasting nutritional state. By analyses of liver and muscle composition during a 12 week starvation period the onset of glycogen, lipid, and protein breakdown is discussed.

MATERIALS AND METHODS

Fish trial and pre-fasting state of the fish

Ninety cod (individually tagged) hatched and reared at Austevoll Aquaculture Research Station (Institute of Marine Research, Norway), was measured ultrasonagraphically using a 450 Linear Array Scanner (Pie Medical, Holland) in order to estimate relative liver size, this technique is described by Mattson (1991) for measurements of gonad size in fish, and validated to estimations of relative liver size by Karlsen (unpublished results). According to liver size the cod were divided into three groups

of 30 fish. Mean initial weights of the three fish groups were 1402 g, 1704 g and 1940 g in the groups termed Low, Medium and High, respectively. The fish used was from the same year class and initial liver indices of analysed fish were 3.1, 8.6 and 14.3, respectively, see Table 1 for further details. The fish were starved for 12 weeks, from the end of July to October 1991 at ambient temperature and natural light. At start 10 fish from each cage were randomly sampled with a net pen. After 2, 4, 8 and 12 weeks 5 fish from each cage were sampled using the same proceedure. Sampled fish were killed by a blow on the head, thereafter stored frozen at $-20°$ C until dissections of liver and fillet. Pooled samples from five fish were used for chemical determination of water, glycogen, lipid and protein.

Analytical methods

Water content in fillets was determined gravimetrically after freeze drying for 48 hrs in an Hetrosic IP 65 freeze dryer (Hetro Birkeröd Denmark). Water content in liver was determined gravimetrically after drying for 48 hrs at $80°$ C in a heating cabinet. Total lipid was determined gravimetrically after ethyl acetate extraction. Glycogen in liver and fillet was determined using an enzymatic method as described by Hemre *et al.* (1989). Nitrogen was determined in homogenized samples by a Nitrogen determinator (LECO, FP-428; system 601-700-500). Protein was calculated as $N \times 6.25$.

Table 1. Nutritional status of the fish prior to fasting. The fish was divided according to relative liver size (hepatosomatic index). The analytical results are from pooled samples of five fish.

	Low	Medium	High
Total fish weight, g	1402	1704	1940
S.E.M.* (n=30)	38	97	72
Liver index	3.1	8.6	14.3
S.E.M. (n=10)	1.0	1.3	1.3
Liver:			
Water, %	47.7	33.5	29.4
Lipid, %	47.6	57.6	63.7
Glycogen (mg/g)	2.3	9.9	10.6
Muscle:			
Water, %	79.4	79.9	78.6
Protein, %	17.0	17.3	17.5
Glycogen (mg/g)	0.8	1.8	4.0

* S.E.M. = standard error of the mean
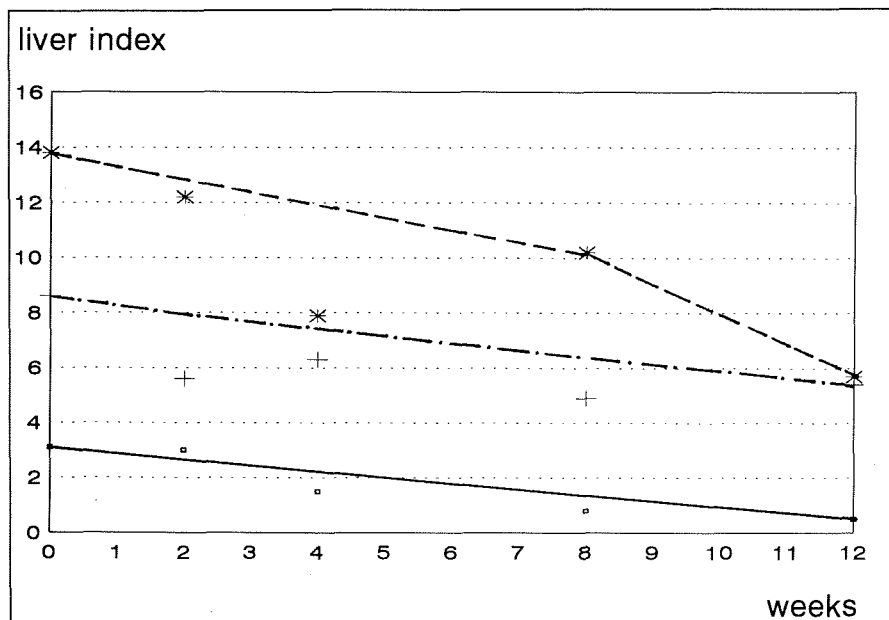[1] Liver index = (liver weight, g) $\times$ 100 / (total weight, g)

Figure 1. Liver index of sampled cod during a 12 week starvation period, initially divided into Low (unbroken line), Medium (broken line with a dot, points +) and High (broken line, points *) liver index (curve adjustment is applied). Five fish were sampled from each group after 4, 8 and 12 weeks.

Calculations and statistical evaluation of results

Correlation analyses were made by a Spearman rank order calculation. Results from each sampling were compared by a Kruskal-Wallis ranged order ANOVA test (CSS: Statistica, StatSoft, Inc. 1992).

RESULTS AND DISCUSSION

The status of the fish initially is described in Table 1. Liver lipid and glycogen were higher (p<0.001) in the heavier fish with the higher liver index. Fillet water was stable at 78–80%, while fillet glycogen increased with increasing liver index (p=0.0017, r=0.68). The chemical analyses at start confirmed that liver size may be used as a way of describing nutritional status of fish which store their lipid in the liver (Shul'man 1974).

After 12 weeks of starvation the cod in groups Low, Medium and High showed mean weight losses of 223 g (15%), 210 g (12%) and 185 g (11%), respectively, indicating a higher weight loss of fish with the initial

low liver index. Figure 1 shows that liver index decreased in all groups as the starvation period was prolonged. The most substantial loss was found in group Low where the liver index was reduced from 3.1 to 0.9 after 12 weeks of fasting. In groups Medium and High the reductions were from 8.6 to 5.6 and from 14.3 to 8.0, respectively. In group Low the initial lipid level was above 50%, after 12 weeks of fasting it was reduced to 7%. Groups Medium and High had high lipid levels in the liver initially (Table 1), in both groups decreased concentrations were found and the levels were reduced to 18% for group Medium and 29% for group High at the end of the starvation period (Figure 1). Mean total lipid contents in livers were initially 21, 64 and 154 g (p=0.0037) for groups Low, Medium and High, respectively, the values were 1, 17 and 35 g (p=0.0037) after 12 weeks of fasting (Table 2). However, the main part of liver lipids in the Medium and High groups were utilized only after 9 weeks of starvation (Figure 2). The results indicate substantial utilization of liver lipid in all groups, and the theory that too large lipid droplets in the liver cells may result in an inability to mobilize these energy reserves was not confirmed by the results in the present study.

Significantly different (p=0.014) liver glycogen values were measured initially. In group Low the glycogen level was 3 mg/g, this was reduced to 1 mg/g after 4 weeks fasting. Initial values for groups Medium and High were 11 mg/g liver, which in both groups were reduced to 1–2 mg/g liver after 4 weeks. No further reduction in liver glycogen was found in any of the groups. The results confirm that glycogen is mobilized during the first stages of fasting as also found for sea bass *(Dicentrarchus labrax)* (Stirling 1976) and yellowtail (*Seriola quinqueradiata*) (Shimeno and Hosokawa 1975) and in isolated chinook salmon liver cells (Klee *et al.* 1990). The glycogen levels were not completely depleted, in accordance with earlier results on fasting cod (Hemre *et al.* 1990, 1993).

In the present study fillet protein concentrations were stable in all groups the first 8 weeks of food deprivation, while the total amount of muscle mass (measured as total live weight and substracted weight of

Table 2. Average content of liver lipid (g) during fasting of cod. Low, Medium and High refers to amount of liver initially. The analytical results are from pooled samples of five fish.

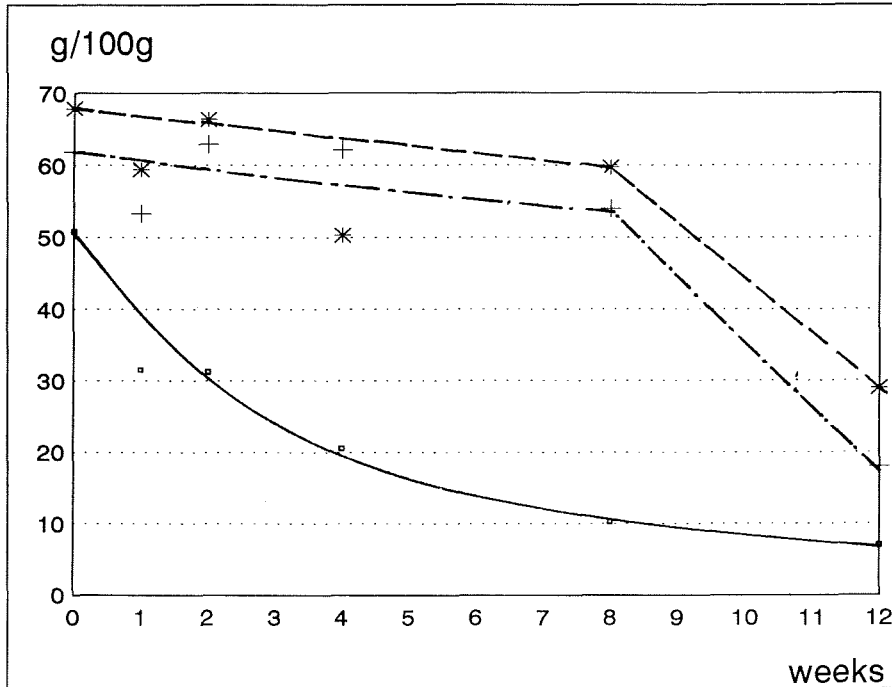|	Low	Medium	High
Initial	20.8	64.0	154
14 days	15.2	81.4	189.5
28 days	3.5	67.9	171.9
56 days	2.1	52.7	159.5
84 days	0.8	16.9	35.1

Figure 2. Lipid concentrations of sampled cod during a 12 week starvation period, initially divided into Low (unbroken line), Medium (broken line with a dot, points +) and High (broken line, points *) liver index (curve adjustment is applied). Five fish were sampled from each group after 4, 8 and 12 weeks.

head and internal organs) was significantly reduced. Fillet water content increased from 79% to 81% after 4 weeks in the fish with the initial Low liver index and further to 84% after 8 weeks and to 87% after 12 weeks (Table 3). The increase in fillet water may be a way of measuring the state of hunger (Love 1980). The fish in the present study with Medium liver index initially showed increased levels of water in the fillet only after 8 weeks of food deprivation, while in the fish with the initial High liver index nö change in fillet water was seen after 12 weeks of fasting. Fillet protein levels were stable throughout the experiment, leading to increased ratio of fillet water to protein as fillet water increased. Increased fillet water:protein ratio may lead to reduced fillet quality (Love 1980). The results from the present experiment therefore indicate that the starvation period of reared cod should not exceed 4 weeks when liver size is Low, while Medium or High liver sizes assures good fillet quality even after a longer fasting period. The weight decrease in the present experiment was from both muscle mass and liver mass indicating that fillet protein as

Table 3. Water (%) in fillet of cod starved for 84 days. Low, medium and high refer to Low, Medium and High liver index at start. The analytical results are from pooled samples of five fish.

	Low	Medium	High
Initial	79	80	78
14 days	79	80	78
28 days	81	80	78
56 days	84	81	79
84 days	87	84	78

well as liver lipid was used for maintenance of metabolic functions during starvation, in line with earlier starvation studies in cod (Hemre *et al.* 1993), saithe *(Pollachius virens)* (Bratland *et al.* 1976, Rosenlund *et al.* 1984), Atlantic salmon *(Salmo salar)* (Lie and Huse 1992) and *Micropterus salmoides* (Love 1980).

An increase in protein catabolism was long regarded as a terminal feature of starvation in mammals, marking the end of lipid and carbohydrate reserves. In species in which large parts of the energy come from protein these effects have not been found, not in the present nor in former studies (Love 1980, Hemre *et al.* 1990, 1993, Lie and Huse, 1992). Love (1980) claimed that when cod were starved in an aquarium, there was a delay of about 9 weeks during which the carbohydrate and lipid of the liver were mobilized, then the fillet water increased. The present study show that the time of this onset depends on the prefasting state of the fish. In other studies with cod (Love 1980) a clear increase in fillet water during starvation has been found simultanously with a fall in fillet glycogen. The levels of fillet glycogen in the present study were low in all groups, however, with a positive correlation between fillet glycogen and initial liver index (p=0.0017, r=0.68). Initial levels were 1 mg/g, 2 mg/g and 4 mg/g for groups Low, Medium and High respectively. In the Medium and High groups fillet glycogen levels decreased during the first three weeks of food deprivation, thereafter low and stable values of about 1 mg/g fillet were determined, the same levels as found in the liver. Carp starved for 6 months still had fillet glycogen present, as judged by histological studies (Gas 1973). Starvation of sea bass and yellowtail caused a rapid inital decrease in carbohydrate and a progressive decline in both lipid and protein (Stirling 1976, Shimeno and Hosokawa 1975). The findings in the present experiment are in accordance with those studies.

CONCLUSIONS

In the cod studied both liver and fillet glycogen were utilized during the first 4 weeks of fasting, the levels stabilizing at about 1 mg/g. Substantial use of liver lipid was seen in all groups, but the onset of lipid utilization depended on the initial liver size. Increase in fillet water was found to depend on the prefasting state of the fish. In fish with the initial Low liver index fillet water content increased, and after 4 weeks it was normal (<85%), after 12 weeks it was increased to 87%. From this it is concluded that the fish with medium to high liver index initially were able to meet food deprivation better than the fish with the initial low liver index.

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11
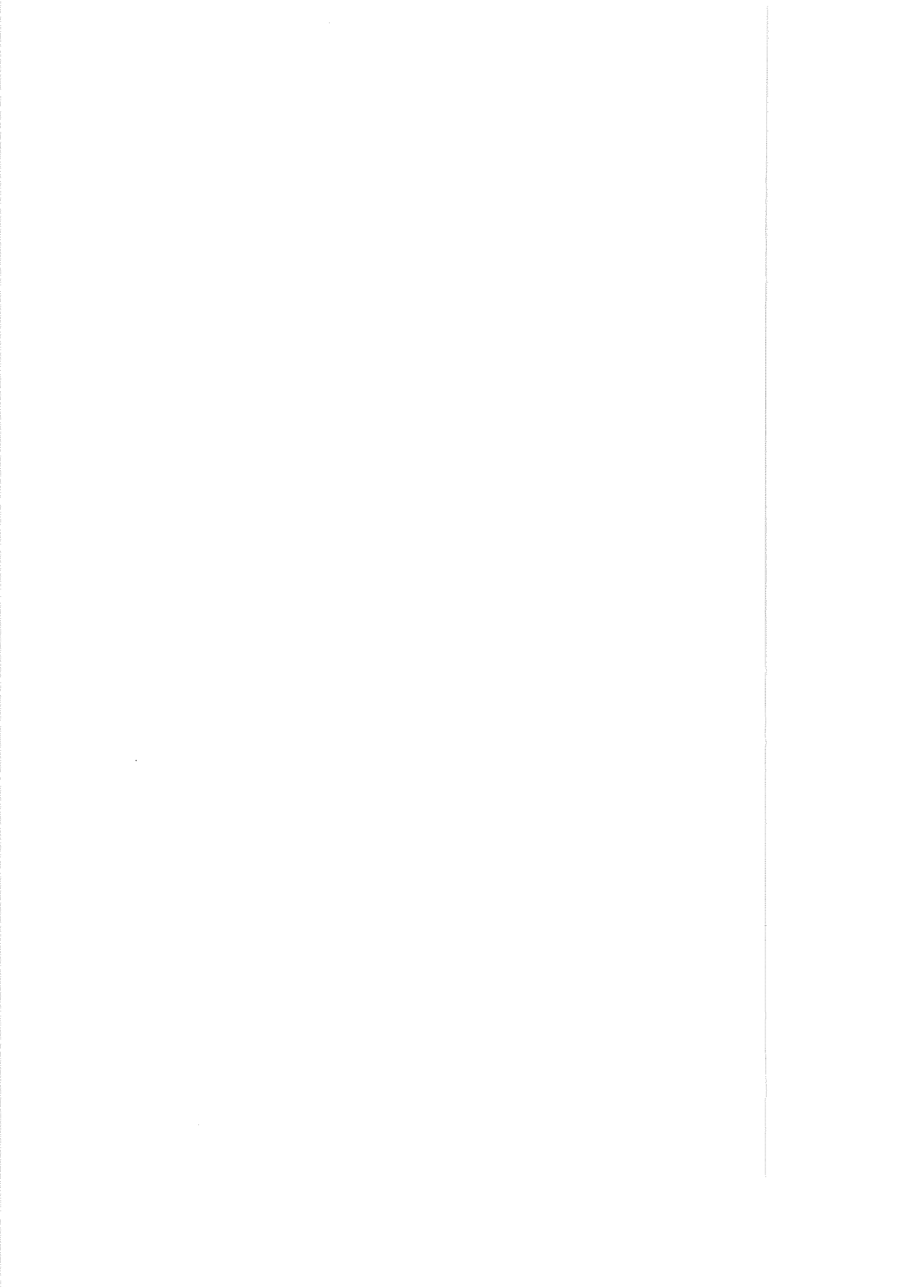
BROODSTOCK NUTRITION IN COD (GADUS MORHUA) – EFFECT OF DIETARY FATTY ACIDS

By

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\ABSTRACT

Three groups of cod (26 ± 2g), were fed diets coated with 90g/kg of either soyabean oil (A), capelin oil (B) or sardine oil (C) for 24 months to maturation, reaching a mean weight of about 2.5 Kg.

Broodfish fed on these diets differed with respect to liver fatty acid composition. Only small differences were seen in the fatty acid composition of eggs from the groups B and C, in group A, however, there were lower levels of n-3 fatty acids.

There were no differences in egg production, mean fertilization or egg size between the groups.

INTRODUCTION

Knowledge about interactions between broodstock nutrition and reproduction is scarce. Such knowledge is necessary to ensure a supply of healthy juveniles, particularly of marine species, to cover the current growth in fish farming. Some reports on the influence of maternal nutrition upon fecundity and egg viability as well as upon larval development through the yolk sac period are available (Watanabe, 1982; Sandnes *et al.*, 1984; Luquet and Watanabe, 1986; Hardy *et al.*, 1989; Mangor-Jensen *et al.*, 1993). The reproduction of red sea bream (*Chrysophrys major*) is greatly affected by the n-3 fatty acid content of the broodstock diet (Watanabe *et al.*, 1984ab) and the dietary level of 22:6n-3 influenced the brooders of carp (Ohmae *et al.*, 1979).

Several analyses of the fatty acid composititon of cod (*Gadus morhua*) egg lipids have been reported (Kaitaranta, 1981; Tocher and Sargent, 1984; Ulvsund and Grahl-Nielsen, 1988). Changes in the fatty acid composition of glycerophospholipids during embryogenesis of cod eggs

have been studied (Lie, 1993). The effect of the fatty acid composition of the parental diet on the fatty acid composition of individual phospholipids of eggs and milt was also reported (Lie, 1991). However, how these changes may influence egg quality is not known.

The present experiment was conducted to elucidate probable effects of increased level above suggested requirement for essential fatty acids in diets to fish. Three dietary oils were used, giving 15, 20 and 30% of the fatty acids as n-3. The effect on the compositon of total lipid of broodfish liver and of the eggs during embryogonesis, and on egg quality were measured.

MATERIALS AND METHODS

Broodstock

Cod, (26 ± 2g), hatched and reared at the Aquaculture Research Station Austevoll (Institute of Marine Research, Norway) were used. Three groups were fed pellets of dry feed, coated with 90g/Kg of either soyabean oil (diet A), capelin oil (diet B) or sardine oil (diet C), *ad lib*. once a day for 24 months to maturation (mean weight of 2.5kg). Further details regarding the composition of the diets are given by Lie *et al.* (1992b).

The broodfish were transferred to closed floating pens shortly prior to spawning (Mangor-Jensen *et al.*, 1993). Natural spawning took place and fertilized eggs were collected daily according to Holm and Andersen (1989).

Spawning volume, egg size and fertilization rate were measured dayly for all groups according to Mangor-Jensen *et al.* (1993).

Groups of eggs were incubated in 70L incubators supplied with a flow-through system and handled as described by Mangor-Jensen *et al.* (1993). Samples of egg were collected for chemical analysis at day 0, 4, 10, 12 14 and 16 and stored at −80° C until analyzed.

Chemical analyses

Pooled samples of 5 livers, homogenized eggs and feed were extractec and the fatty acid composition of total lipids were analysed using method described by Lie and Lambertsen (1991).

Statistics

Principal component analysis (PCA) of the fatty acid composition c feeds, livers and eggs were analysed by the SIRIUS programme (versio 3.0) (Kvalheim and Karstang, 1987).

RESULTS AND DISCUSSION

The fatty acid composition of diets A, B and C and fish livers from the same dietary groups are presented in Table 1, 2 and 3, respectively. The liver lipids from fish fed diet A (soyabean oil) contained a high level of 18:2n-6 (25.7%). Compared with the fish in groups B (capelin oil) and C (sardine oil), group A fish had the highest levels of 18:1n-9 and 18:3n-3 and lowest level of 20:5n-3 and 22:6n-3. High levels of the two long chain monoenes 20:1n-9 (10.1%) and 22:1n-11 (4.8%) were found in the lipids of group B fish, while the liver lipids of the fish from group C had the highest level of 20:5n-3 (12.2%) and 22:6n-3 (12.3%). These results are in line with previous reports regarding the influence of diet on the composition of cod liver lipids (Lie et al., 1986; Lie et al., 1992b).

The score plot (PC1 versus PC2) from the multivariate analysis of the fatty acid composition of the diets, livers and eggs from all groups showed five main classes in the data (Fig.1). Class 1 contained liver lipids from the fish fed diet A, class 2 the liver lipids of group B and class 3 the liver lipids of group C. This demonstrates three groups of broodfish different
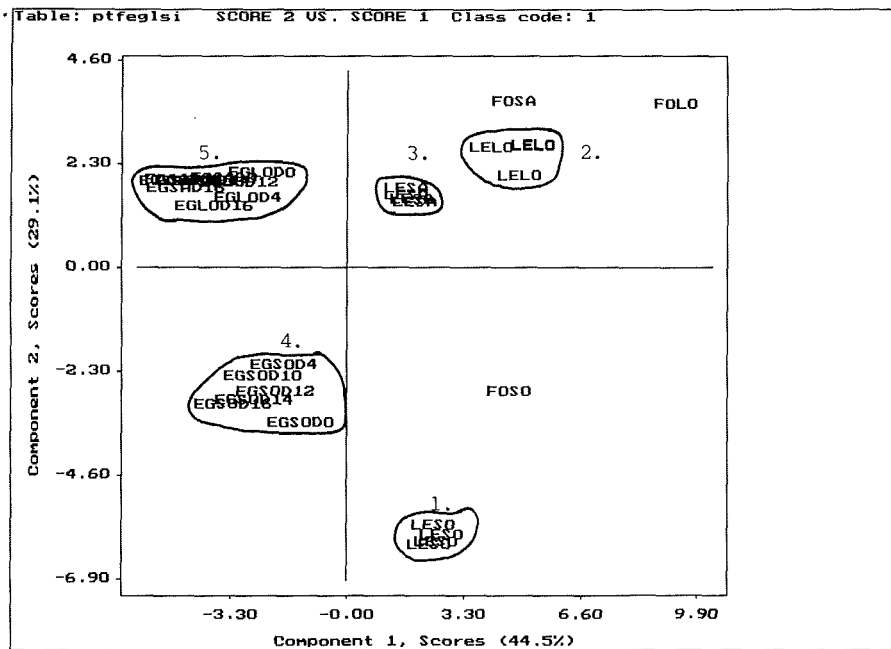

Figure 1. Score plot from the principal component analysis (PCA1 versus PCA2) of the fatty acid composition of the three different diets (FO), livers (LE) and eggs (EG) from cod. Soyabean oil (SO), Capelin oil (LO) and Sardine oil (SA).

Table 1. Fatty acid composition (% of lipid) in feed, liver and egg of cod fed diet A, with soybean oil as lipid source.

Fatty acid	Feed	Liver	Egg					
			Days after fertilisation					
			0	4	10	12	14	16
14:0	3.3	1.4	0.5	1.8	1.4	1.5	0.5	0.5
16:0	12.6	10.5	16.1	19.1	18l.6	18.9	16.5	16.1
16:1n-9	n.d.[1]	0.5	0.3	2.2	2.4	2.0	0.2	0.7
16:1n-7	4.3	1.8	1.5	1.7	1.1	1.3	2.0	0.1
18:0	2.7	3.8	4.2	4.2	5.0	5.3	5.6	5.6
18:1n-11	n.d.[1]	n.d.[1]	0.6	0.6	0.6	0.5	n.d.[1]	n.d.[1]
18:1n-9	16.2	24.8	16.6	15.5	14.6	15.0	12.2	12.6
18:1n-7	2.2	3.0	2.6	2.6	2.5	2.6	2.3	2.2
18:2n-6	28.2	36.0	24.5	22.1	21.2	21.2	20.8	19.8
18:3n-3	3.9	3.7	1.6	1.5	1.4	1.5	1.1	1.0
20:1n-11	0.3	0.5	0.1	0.1	0.1	0.1	0.1	0.1
20:1n-9	5.5	2.4	0.8	0.8	0.8	0.8	0.8	0.8
20:1n-7	0.2	0.1	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]
18:4n-3	1.4	0.5	0.1	0.2	0.2	0.2	0.1	0.1
20:2n-6	n.d.[1]	0.7	0.6	0.6	0.8	0.8	1.2	1.1
20:3n-6	n.d.[1]	n.d.[1]	n.d.[1]	0.1	0.1	n.d.[1]	0.1	0.1
20:4n-6	0.1	0.2	0.5	0.6	0.6	0.4	0.7	0.8
22:1n-11	7.1	1.6	0.3	0.2	0.1	0.1	n.d.[1]	n.d.[1]
22:1n-9	0.4	0.1	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]
20:4n-3	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3
20:5n-3	4.5	2.2	6.2	6.0	6.2	5.8	7.8	8.3
22:5n-3	0.4	0.4	1.1	1.0	1.1	0.9	1.3	1.5
22:6n-3	4.5	3.7	18.2	15.8	17.7	17.1	23.5	24.7
Sum saturated	18.7	16.4	21.5	26.0	25.8	26.3	23.3	22.8
Sum 16:1	4.4	2.3	1.8	3.8	3.5	3.3	2.2	0.7
Sum 18:1	18.3	27.8	19.8	18.6	17.7	18.1	14.5	14.8
Sum 20:1	6.0	3.0	1.0	0.9	0.9	0.9	0.9	0.9
Sum 22:1	7.5	1.7	0.3	0.2	0.1	0.1	n.d.[1]	n.d.[1]
Sum monoenes	36.7	34.9	23.2	23.8	22.5	22.6	17.7	16.6
Sum n-3	15.0	10.7	27.4	25.0	26.9	25.7	34.1	35.8
Sum n-6	29.0	36.8	25.7	23.3	22.7	22.4	22.7	21.7
Sum PUFA	43.9	47.8	53.2	48.3	49.6	48.1	56.9	57.8
n-3/n-6	0.5	0.3	1.1	1.1	1.2	1.1	1.5	1.6

[1] n.d. = not detected

15

with respect to liver composition. Liver cells are important in the process of gonadal development as a store of nutrients as well as a production site of a special lipoprotein, vitellogenin, which play a fundamental role in the process of gonadal development.

The fatty acid composition of total lipid of eggs from cod fed diet A was clearly influenced by the diet, giving high levels of 18:2n-6, 18:1n-9 and low levels of 20:5n-3 and 22:6n-3 (Table 1). In the eggs the levels of sum n-3 fatty acids increased during embryogenesis concomitant with a reduction in monoenoic and n-6 fatty acids. Minor differences in the composition of egg lipids from groups B and C were found (Table 2 and 3). Even though the levels were low, elevated levels of 20:1n-9 and 22:1n-11 were found in group B and a higher level of 20:4n-6 in group C. This is further illustrated in the score plot (PC1 versus PC2) from the multivariate analysis of the fatty acid composition (Fig. 1) were the egg lipids of cod fed diet A was included in class 4, while class 5 included egg lipids of both group B and C. However, even with a somewhat lower level of n-3 fatty acids in group A, these results suggest that the long chain n-3 polyunsatureted fatty acids are conserved in the eggs. The
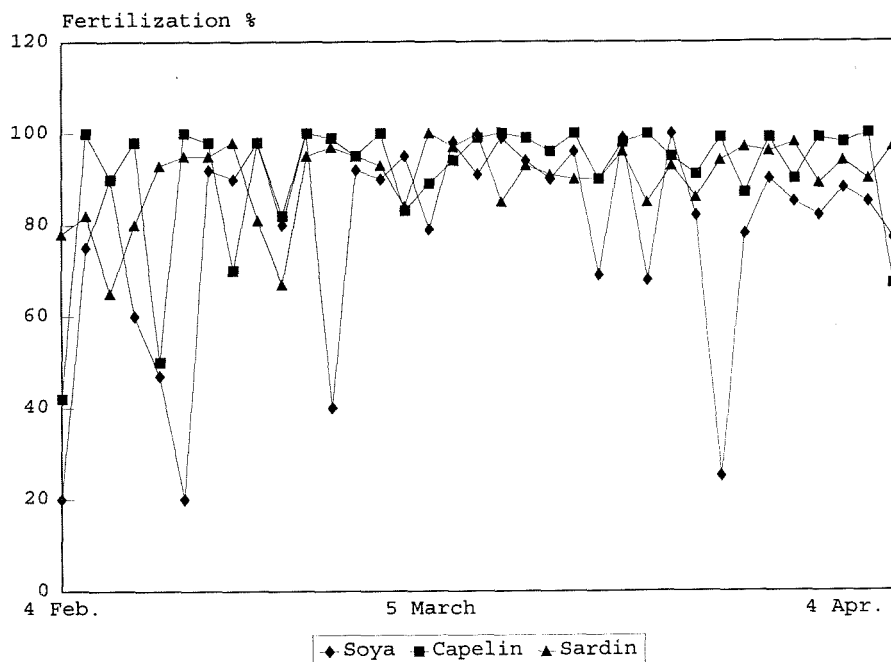

Figure 2. Fertilization of batches of egg from the three broodstock groups during the spawning season.

accumulation of 22:6n-3 in cod eggs during embryogenesis is reported by Frazer *et al.* (1988) and Lie (1993).

There were no significant differences in the egg production between the groups. Mean fertilization (%) were 79 (±22), 91 (±14) and 90 (±8) in groups A, B and C, respectively. The differences were not significant. However, larger variation and several egg batches with fertilization below 50% were found in group A (Fig. 2). The mean egg diametre declined during the spawning season, but no differences between the groups were found (Fig. 3).

No effects were found on egg quality due to increased levels of n-3 fatty acids in the broodfish diets, even if main differences in the fatty acid composition of the broodfish and eggs were found. According to Hardy
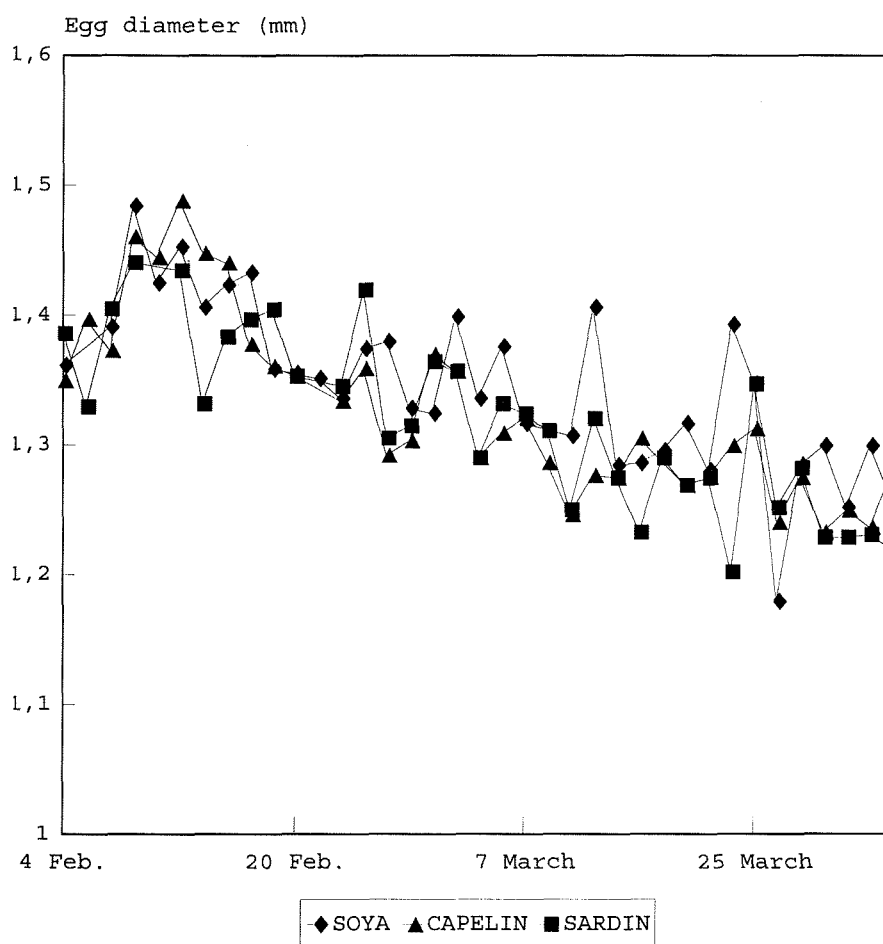

Figure 3. Egg diameter measured daily through the spawning season.

Table 3. Fatty acid composition (% of lipid) in feed, liver and egg of cod fed diet C, with sardin oil as lipid source.

Fatty acid	Feed	Liver	Egg					
			Days after fertilisation					
			0	4	10	12	14	16
14:0	6.7	3.0	2.2	1.3	1.2	0.9	0.8	0.9
16:0	14.8	13.5	22.8	19.8	19.2	19.6	19.2	17.8
16:1n-9	0.3	0.7	1.9	1.8	1.9	1.8	1.7	1.5
16:1n-7	7.6	4.9	2.5	1.9	2.0	1.6	1.6	1.6
18:0	2.1	4.4	4.3	3.6	4.9	5.6	5.3	5.8
18:1n-11	n.d.[1]	n.d.[1]	0.6	0.5	0.4	0.4	0.4	0.6
18:1n-9	9.6	19.1	11.2	10.1	9.2	8.5	8.6	10.4
18:1n-7	2.9	5.1	4.2	4.2	4.0	3.8	3.8	4.0
18:2n-6	4.1	6.2	2.9	2.8	2.5	2.2	2.1	3.2
18:3n-3	1.0	1.1	0.3	0.3	0.3	0.2	0.2	0.3
20:1n-11	0.8	1.0	0.2	0.2	0.1	0.1	0.1	0.1
20:1n-9	6.3	2.9	0.8	0.8	0.9	0.9	1.0	1.4
20:1n-7	0.3	0.1	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	0.1	n.d.[1]
18:4n-3	2.7	1.7	0.3	0.3	0.2	0.2	0.2	0.2
20:2n-6	n.d.[1]	0.3	0.2	0.2	0.2	0.2	0.3	0.4
20:3n-6	n.d.[1]	0.1	0.1	0.1	0.1	0.1	0.1	0.2
20:4n-6	0.8	0.9	1.6	2.0	1.9	2.0	1.9	2.0
22:1n-11	8.0	1.9	0.1	0.1	0.1	n.d.[1]	n.d.[1]	0.2
22:1n-9	0.5	0.2	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]	n.d.[1]
20:4n-3	0.6	0.8	0.3	0.3	0.3	0.3	0.3	0.3
20:5n-3	13.0	12.2	13.2	15.2	15.3	15.7	15.4	14.0
22:5n-3	1.6	2.1	1.6	1.9	1.8	1.8	1.8	1.7
22:6n-3	10.8	12.3	23.6	29.4	29.6	30.8	32.0	30.0
Sum saturated	24.1	21.8	30.5	25.6	26.3	26.6	26.6	25.3
Sum 16:1	8.1	5.6	4.5	3.7	3.9	3.4	3.2	3.1
Sum 18:1	12.5	24.2	15.9	14.8	13.6	12.7	12.8	14.9
Sum 20:1	7.3	4.0	1.0	1.0	1.1	1.1	1.2	1.5
Sum 22:1	8.5	2.1	0.1	0.1	0.1	n.d.[1]	n.d.[1]	0.2
Sum monoenes	37.5	36.2	21.8	19.8	18.9	17.4	17.4	19.8
Sum n-3	29.8	31.0	39.4	47.4	47.6	49.0	50.0	46.5
Sum n-6	5.0	7.5	4.8	5.0	4.6	4.5	4.3	5.9
Sum PUFA	34.7	39.2	44.4	52.6	52.5	53.6	54.5	52.6
n-3/n-6	6.0	4.2	8.2	9.4	10.3	10.9	11.6	7.9

[1] n.d. = not detected

et al. (1989) no differences in fecundity, egg viability and egg size could be observed in coho salmon (*Onchorhynchus kisutch*) as long as the dietary n-3 level was at least 1% of the diet. Similar results are found for Atlantic salmon (Waagbø *et al.*, unpublished).

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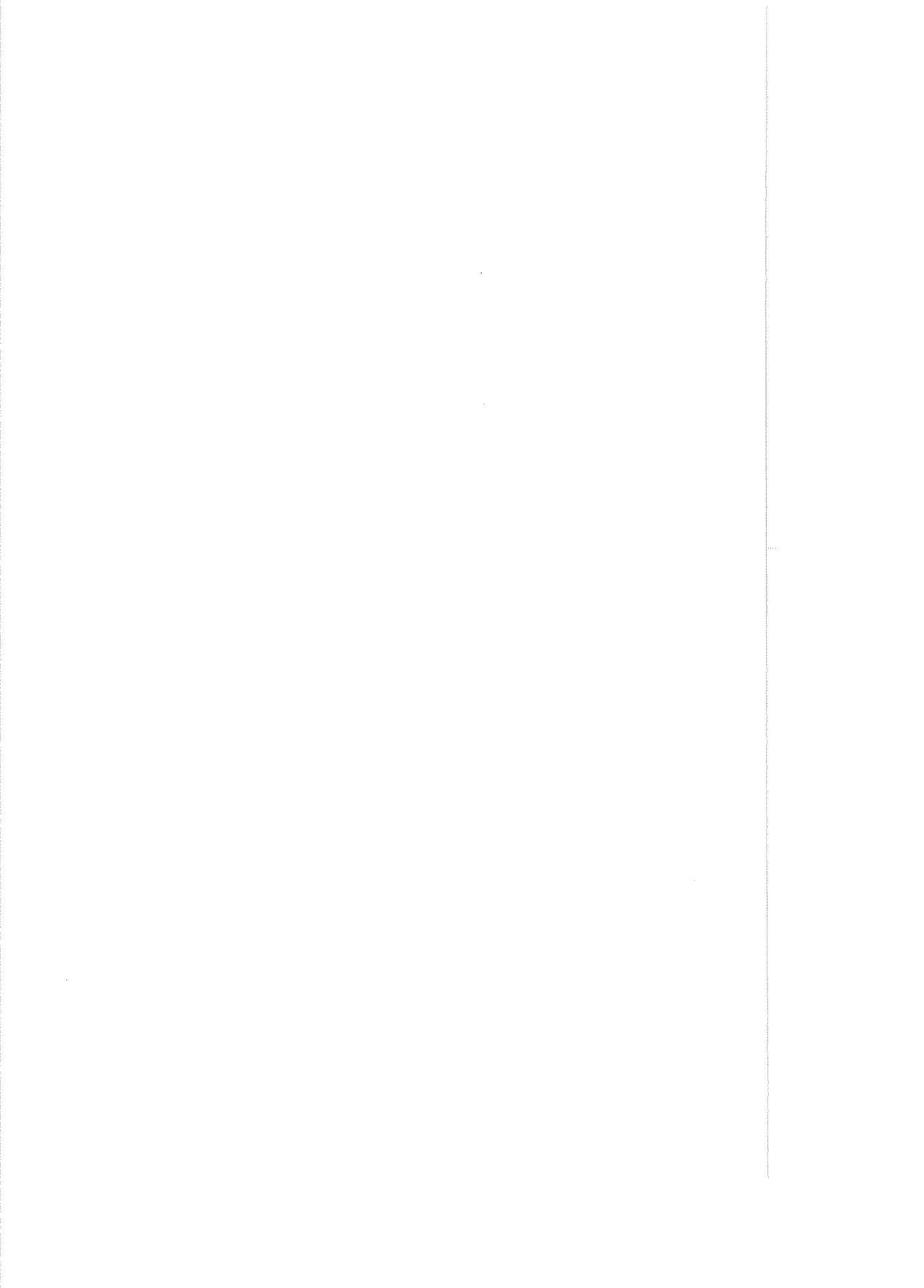
TISSUE VITAMIN B$_6$ CONCENTRATIONS AND ASPARTATE AMINOTRANSFERASE (AspT) ACTIVITY IN ATLANTIC SALMON (*SALMO SALAR*) FED GRADED DIETARY LEVELS OF VITAMIN B$_6$

By

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ABSTRACT

Atlantic salmon (*Salmo salar*) fry were start fed a dry pelleted diet supplemented with pyridoxine hydrochloride (PN.HCl) of 0, 2, 4, 6 or 8 mg/kg for 20 weeks. Growth, mortality, tissue vitamin B$_6$ concentrations, aspartate aminotransferase (AspT) activity, haemoglobin concentration, proximal composition as well as fatty acid composition in liver were recorded. A minimum vitamin B$_6$ requirement for growth of 2 - 3 mg/kg, and a minimum level of 6 - 8 mg/kg for maximizing white muscle AspT activity and whole body and muscle vitamin B$_6$ concentrations were indicated. Liver reached a saturation level in fish supplied with 4 mg PN.HCl/kg diet. It was found that more protein was synthesized the higher the content of vitamin B$_6$ in diet, concomitant with reduced lipid contents. Thus, dietary effects of higher supplementation levels related to protein and lipid metabolism should be further evaluated.

INTRODUCTION

Dietary requirement for vitamin B$_6$ have been reported in various fish species (Arai *et al.* 1972; Agrawal and Mahajan 1983b; Ikeda *et al.* 1988) and requirements for maximum growth in the range of 2 - 3 mg/kg diet have been suggested (Ogino 1965; Takeda and Yone 1971; Adron *et al.* 1978; Andrews and Murai 1979; Kissil *et al.* 1981). Studies on vitamin B$_6$ in salmonids are few. Recently, a minimum requirement for growth in young rainbow trout, *Onchorhunchus mykiss*, was reported to be 2 mg/kg diet (Woodward 1990) and 5 mg/kg diet in Atlantic salmon, *Salmo salar* (Lall and Weerakoon 1990). These estimates are far below those reported earlier based on growth response and maximum liver storage, ranging between 10 and 20 mg/kg in salmonids (Hardy *et al.* 1979; Halver 1989).

The vitamin B_6 level recommended for coldwater fish by the National Research Council is 10 mg/kg (NRC 1981).

The dietary vitamin B_6 requirement for maximum activity of the vitamin B_6 dependent enzyme aspartate aminotransferase, AspT, (EC 2.6.1.1.) in muscle of rainbow trout is three times higher (6 mg/kg) than the level which is necessary to obtain maximum growth (Woodward 1990). Estimation of dietary requirements based on AspT activity in Atlantic salmon have not been reported, but maximum activity of alanine aminotransferase (EC 2.6.1.2.) was obtained at 5 mg/kg, as for maximum growth response (Lall and Weerakoon 1990). Requirement based on tissue vitamin B_6 concentration has not been reported for Atlantic salmon.

Signs of vitamin B_6 deficiency in fish are rather unspecific and include increased mortality, reduced feed intake and nervous disorders (Halver 1989; Steffens 1989). Steffens (1989) reported rapid recovery within a day or two after administration of pyridoxine hydrochloride (PN.HCl) or of the active coenzyme pyridoxal-5-phosphate (PLP).

The present study reports on the effect of feeding graded levels of vitamin B_6 on relevant parameters for the utilization of this vitamin in Atlantic salmon. Recommendations on dietary vitamin B_6 supplementation are based on growth, concentration of vitamin B_6 in whole body, muscle and liver and on the activity of AspT in serum, muscle and liver. Serum haemoglobin levels, tissue proximal composition and liver fatty acid composition are also reported.

MATERIALS AND METHODS

Fish and diets

Atlantic salmon «swim-up» fry with an initial weight of 0.15 g were distributed into 15 fibreglass tanks (1m x 1m x 0.6m) each with approximately 2000 fish per tank. Five groups of fish (designated A, B, C, D and E) were fed a dry pelleted basal diet (Table 1) in triplicates, with the following supplements of pyridoxine hydrochloride (mg PN.HCl/kg): Group A, 0; Group B, 2; Group C, 4; Group D, 6 and group E, 8. The feeds were milled and crushed to a proper size and kept frozen at -20° C until used. The fish were fed in excess by automatic feeders for 20 weeks. The flow-trough tanks were supplied with continuously aerated water (O_2 equivalent to 7 - 8 mg/L) at a rate of about 4 L/min. Salinity were kept at 2 g/L by addition of salt well water (28 g/L) and pH was kept between 6.0 and 6.5. The mean water temperature was at the start of the experiment in March 12.3° C; in April 14.5° C; in May 15.0° C;

Table 1. Composition of the basal experimental diet

Ingredients	g/kg diet
Vitamin-free casein (Sigma)	190
Gelatin	120
Washed cod muscle (dry)	90
Herring meal	250
Dextrin	140
Vitamin premix[1]	10
Mineral premix[2]	50
Herring oil	150

[1] Vitamins supplemented per kg feed:
Vit A 0.75 mg, Vit D 0.06 mg, DL-alpha-tocopherol 30 mg, menadione sodium bisulfite 10 mg, thiamine HCl 10 mg, riboflavin 20 mg, D-calsium panthotenate 40 mg, nicotinic acid 150 mg, biotin 1 mg, folic acid 5 mg, vitamin B_{12} 0.02 mg, inositol 400 mg, ascorbic acid 400 mg, choline chloride 1500mg.

[2] Minerals supplemented per kg feed:
$Ca_2HPO_4 \cdot 2\ H_2O$ 47 g, KH_2PO_4 37,5 g, NaCl 25 g, $MgSO_4 \cdot 7\ H_2O$ 12.5 g, $FeSO_4 \cdot 7\ H_2O$ 1.25 g, $ZnSO_4 \cdot 7\ H_2O$ 1.25 g, $MnSO_4 \cdot H_2O$ 0.2 g, $CuSO_4 \cdot 5\ H_2O$ 0.05 g, KI 0.04 g, $Na_2SeO_3 \cdot x\ H_2O$ 0.001 g.

in June 13.3° C and in July 10.1° C. The supply of warm water by use of a heat exchange system was turned off in late June, resulting in a temperature drop from 15° C to 10° C overnight.

Sampling

Initial weight was measured 100 fish. Later on, 100 fish randomly collected from each tank every month were weighed individually. Mean weights of fish from each tank were used in the statistical analyses (n = 3 for each dietary regime). Sampled fish were not returned to their respective tanks. The hepatosomatic index (HSI) was calculated at the end of the experiment (n = 80 per tank).

Whole body vitamin B_6 concentrations were determined throughout the experiment in pooled samples of 10 - 20 fish per tank (n = 3). Vitamin B_6 in liver and muscle at the end of the experiment, activity of the vitamin B_6 dependent enzyme aspartate aminotransferase (AspT) in liver, muscle and serum, and total serum protein were analysed in pooled samples of 10 fish per tank (n = 3).

Dry matter, protein and fat contents were determined in the diets and in pooled samples of whole body and liver (n = 3) of fish from each dietary group, and in pooled samples of muscle (n = 3) of fish fed diets A and E. The fatty acid composition was determined in pooled liver

samples (n = 3) of fish fed diets A and E. Blood haemoglobin concentration (Hb) was analysed on individual samples at the end of the experiment (n = 10 per tank).

Analyses

Total vitamin B_6 concentration (μg/g w.w.) was determined by a microbiological assay using *Saccharomyces uvarum* (ATCC No. 9080) as test organism according to the method described by AOAC (1990), with the following modifications: 1-2 g of sample was autoclaved for 4 hrs in 100 ml H_2O, and 1M H_2SO_4 was added to give a pH of 1.7.
Pyridoxine hydrochloride was used as a standard (0.75 ng/ml) in double-distilled water. The tubes were incubated for 18 hrs. at 30° C with constant shaking. All analysed values given refer to total vitamin B_6 activity (Σ pyridoxine, pyridoxal and pyridoxamine). Two separate extracts of each sample were analysed, and mean values are given (variation between samples < 10%).

Aspartate aminotransferase activity was determined in serum, and in muscle and liver extracts prepared as described by Casillas *et al.* (1982). Stimulated AspT activity in muscle and liver homogenates was also determined by addition of the active coenzyme pyridoxal-5-phosphate (PLP) to find the vitamin B_6 level at which no further stimulation took place. Tissue homogenates (0.8 g/20 ml buffer) were centrifuged for 15 min (4° C) at 3500 rpm using a Sorvall RT6000 refrigerated centrifuge, and the supernatant was filtered through a 0.45 μm Millipore filter, type HA. AspT activity was determined within 6 hours using reagent kits from Boehringer Mannheim GmbH Diagnostica according to Sandnes *et al.* (1988). Preliminary studies showed a maximum increase in AspT activity by addition of 200 μl of a 40 μg/ml solution of PLP to 1 ml of the muscle homogenates. Control samples were diluted with physiological saline and AspT activity +/- PLP were analysed. The activation coefficient αAspT = stimulated AspT/basal AspT was calculated (Friedrich 1988). AspT activity in serum are expressed as Units per gram protein (U/g prot) and AspT activity in muscle and liver as Units per gram wet weight (U/g w.w.).

Haemoglobin (Hb) and total serum protein were analysed according to Sandnes *et al.* (1988). Total protein in feed and fish (N x 6.25) was calculated from ammonium determination in Kjeldahl digests (Crooke and Simpson 1971), and lipid was extracted using ethyl-acetate. The liver fatty acid composition was analysed following extraction by chloroform-methanol (Lie and Lambertsen 1991).

Statistics

Statistical evaluation of experimental data was carried out by ANOVA one-way analysis of variance, regression analysis and Mann-Whitney U test between two groups using Statgraphics Statistical Graphics System (Statistical Graphics corporation, Maryland, USA).

RESULTS

Feed analyses

Vitamin B_6 levels in diets A, B, C, D and E were 1.0, 2.7, 4.5, 6.2 and 8.2 mg PN.HCl/kg, respectively. The diets contained (g/kg): Protein 520, fat 150, ash 65 and dry matter 950.

Mortality

The total pooled mortality in the non-supplemented group was 58%. In groups B, C, D and E the total mortalities were 22, 21, 23 and 25%, respectively (Table 2). Mortality was uniformly distributed among triplicates throughout the study.

Growth and proximal composition

There were no significant differences in body weights between groups at any time throughout the experiment ($p > 0.05$). However, fish supplemented with the highest dietary vitamin B_6 level (8 mg/kg) showed the highest weight after 12 weeks of feeding the respective diets (Table 2). The hepatosomatic index (n = 3) calculated at the end of the experiment (Table 2) showed no significant differences between groups ($p > 0.05$).

Whole body protein content tended to increase with increasing dietary vitamin B_6. This was accompanied by reduced whole body lipid content (Table 2), the latter showing significant differences between groups ($p < 0.05$). Fat free dry matter was practically constant and ranged from 177 to 184 g/kg. The protein content in fat free dry matter (g/kg) increased significantly from 759 in the non-supplemented group to 756, 766, 785 and 795 in the supplemented groups B, C, D and E, respectively.

The proximal composition of muscle and liver did not show significant changes related to diets (data not shown). However, contrary to whole body and muscle, the liver of non-supplemented fish showed the lowest contents of dry matter and fat. Liver lipid content (g/kg w.w) increased from 49 in non-supplemented fish to 78 in the highest supplemented fish.

Table 2. Weight (Wt,g) and mortality (M,%) in Atlantic salmon fed five dietary levels of vitamin B₆ through 20 weeks. The total pooled mortality, hepatosomatic index (HSI), proximal compositon (g/Kg) and blood haemoglobin levels (Hb, g/dL) after 20 weeks are given (n=3 unless otherwise stated,SEM are given).

		Dietary vitamin B_6 (mg PN.HCl/kg)									
		0		2		4		6		8	
Week 4	Wt, g	0.5	(<0.1)	0.5	(<0.1)	0.5	(<0.1)	0.5	(<0.1)	0.5	(<0.1)
	M, %	1.3		1.2		1.5		1.3		2.1	
Week 8	Wt, g	1.0	(0.1)	1.0	(0.1)	1.0	(<0.1)	1.1	(<0.1)	1.1	(0.1)
	M, %	35.3		8.2		8.9		11.2		13.1	
Week 12	Wt, g	2.1	(0.1)	2.2	(0.2)	2.0	(0.1)	2.1	(0.1)	2.4	(0.1)
	M, %	15.0		8.1		7.3		6.2		6.3	
Week 16	Wt, g	2.9	(0.2)	2.6	(0.2)	2.7	(0.2)	2.7	(0.1)	2.9	(0.2)
	M, %	4.9		3.0		1.7		2.0		1.3	
Week 20	Wt, g	4.4	(0.3)	3.8	(0.2)	3.7	(0.3)	3.8	(0.3)	3.8	(<0.1)
	M, %	1.5		1.5		1.5		2.3		2.2	
Total pooled M,%		58.0		22.0		20.9		23.0		25.0	
HSI, g/100g		1.5	(0.1)	1.7	(0.1)	1.7	(0.1)	1.6	(0.1)	1.8	(0.1)
Dry mat., g/kg		257	(3.3)	257	(0.5)	248	(1.9)	251	(2.2)	249	(0.7)
Prot, g/kg		136	(2.5)	136	(1.8)	137	(0.6)	139	(1.6)	142	(0.7)
Fat, g/kg		78	(1.8)[1a]	77	(0.8)[a]	70	(1.3)[b]	74	(1.1)[ab]	70	(0.6)[b]
Hb, g/dL, n = 30		7.4	(0.2)	7.6	(0.1)	7.5	(0.2)	7.5	(0.1)	7.3	(0.1)

[1] Different superscript letters within a row indicate significant differences (p < 0.05).

Liver fatty acid composition and haematology

The fatty acid composition in liver was not affected by dietary vitamin B₆ (data not shown). Blood haemoglobin concentrations (Table 2) were not different between groups (p > 0.05).

Vitamin B_6 in whole body, muscle and liver

Vitamin B₆ conce. ration in the yolk sac fry one week prior to start of feeding was 2.1 μg/g (w.w.) and decreased to 1.1 μg/g at the onset of feeding. After two weeks of feeding the diets A, B, C, D and E, whole body vitamin B₆ levels were further reduced to 40, 52, 59, 66 and 75% of the initial level, respectively (Fig. 1). Fish fed diets A and B showed decreasing whole body vitamin B₆ concentrations until the sixth week of feeding, and subsequently stabilized at 0.3 μg/g and 0.5 μg/g, respectively

Vitamin B6 (ug/g)

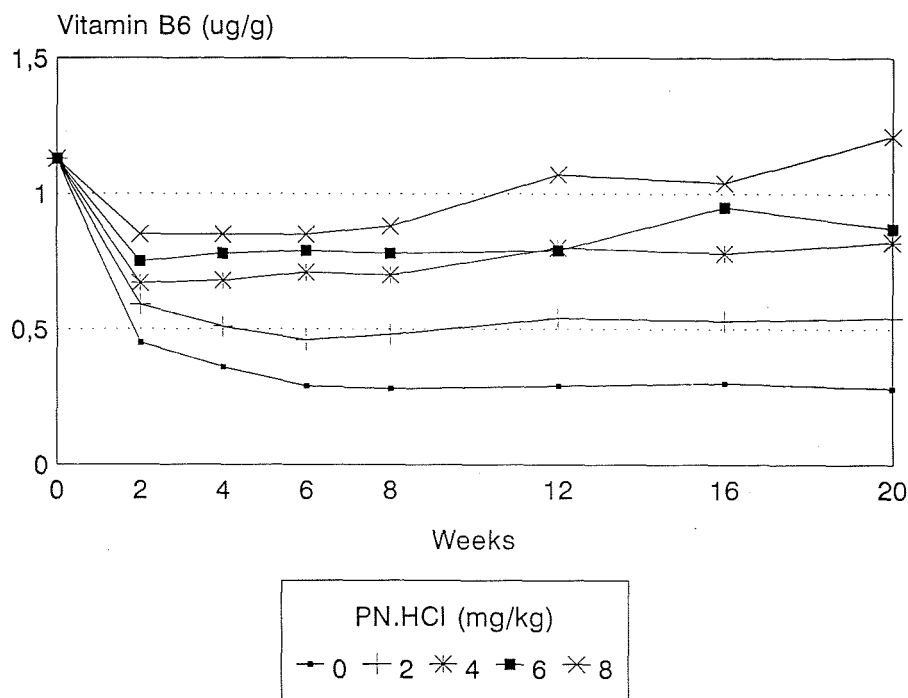Weeks

PN.HCl (mg/kg)

0   2   4   6   8

Fig. 1. Whole body vitamin B_6 concentrations ($\mu g/g$ w.w.) in Atlantic salmon fed a semisynthetic diet supplemented with 5 dietary levels of pyridoxine hydrochloride (PN.HCL) for 20 weeks.

In the other groups, the vitamin B_6 levels tended to stabilize after two weeks of feeding, and in fish fed diet E the concentration increased to a level similar to the initial level after 12 weeks of feeding. Whole body vitamin B_6 concentration attained in this group after 20 weeks of feeding was significantly higher than those found in the other groups ($p < 0.05$).

Vitamin B_6 concentration in muscle and liver, as well as in whole body at the end of the feeding experiment, were significantly correlated to the dietary vitamin B_6 levels (Table 3). However, liver concentrations were not significantly different between fish fed dietary vitamin B_6 supplementations of 4, 6 and 8 mg PN.HCl/kg ($p > 0.05$). Muscle vitamin B_6 concentrations were not significantly different between fish supplied with 6 and 8 mg PN.HCl/kg($p > 0.05$). Muscle and liver vitamin B_6 concentrations were highly correlated ($r = 0.93$).

Aspartate aminotransferase (AspT) in liver, muscle and serum

Basal AspT activity measured in white muscle, liver and serum at the end of the experiment increased with dietary vitamin B_6 levels and tended

Table 3. Vitamin B_6 concentration (μg/g w.w.) in pooled samples of whole body, muscle and liver, and aspartate aminotransferase (AspT) activity in muscle and liver (U/g w.w) and in serum (U/g protein) of Atlantic salmon fed five dietary levels of vitamin B_6 for 20 weeks (n = 3, SEM are given). Stimulated AspT activity by addition of pyridoxal-5-phosphate, PLP, are given.

		Dietary vitamin B_6 (mg PN.HCl/kg diet)				
		0	2	4	6	8
Vitamin B_6, μg/g						
Whole body		$0.3 (0.01)^{1a}$	$0.5 (0.02)^b$	$0.8 (0.02)^c$	$0.9 (0.02)^c$	$1.2 (0.02)^d$
Muscle		$0.2 (0.01)^a$	$0.7 (0.03)^b$	$1.4 (0.15)^c$	$1.7 (0.02)^d$	$1.8 (0.09)^d$
Liver		$3.4 (0.2)^a$	$4.1 (0.2)^b$	$5.1 (0.5)^c$	$5.3 (0.1)^c$	$5.5 (0.1)^c$
AspT activity, U/g						
Muscle	− PLP	$3.7 (0.6)^a$	$12.5 (1.2)^b$	$19.3 (1.8)^c$	$26.7 (2.9)^d$	$24.9 (2.2)^d$
	+ PLP	40 (2.8)	43.7 (1.7)	40.7 (1.9)	46.3 (2.3)	42.1 (1.7)
Liver	− PLP	$127 (19)^{a,c}$	$98 (11)^b$	$120 (23)^{a,c}$	$130 (13)^c$	$138 (4)^c$
	+ PLP	182 (20)	137 (11)	154 (20)	158 (3)	167 (4)
Serum	− PLP	$4.6 (0.2)^a$	$11.7 (3.3)^{2b}$	$18.2 (3.1)^c$	$21.2 (0.6)^c$	$17.9 (1.0)^c$

[1] Different superscript letters within a row indicate significant differences (p <0.05).
[2] n = 2

to approach maximum values in the higher supplemented groups for muscle and serum (Table 3). AspT activity in white muscle was significantly correlated to muscle vitamin B_6 level (p < 0.01) but showed no significant differences between fish fed the two highest dietary vitamin B_6 supplementation of 6 and 8 mg PN.HCl/kg (Table 3). Muscle and serum AspT values were positively correlated (r = 0.84).

AspT activity in liver was not correlated to liver vitamin B_6 concentration. It showed, however, a positive correlation to fish weights (p < 0.05) which was not found in muscle.

The AspT activation study showed that stimulation by addition of PLF to muscle homogenates was not different in fish fed the two highest dietary vitamin B_6 levels (6 and 8 mg PN.HCl/kg) as demonstrated by the activation coefficients αAspT = 10.8, 3.5, 2.1, 1.7 and 1.7 in muscle o fish fed diets A, B, C, D and E, respectively. The corresponding αAspT values in liver were 1.4, 1.4, 1.3, 1.2 and 1.2 in the same fish groups.

DISCUSSION

The high mortality recorded in fish fed the non-supplemented diet durin the first three months of the experiment (> 50%), may probably explai

the fact that fish weight in this group was not negatively affected by the low dietary vitamin B_6 level (1 mg/kg). After 20 weeks of feeding there was a lower frequency of small fish (< 3 g) in this group (29%) compared to the other groups (39–43%). During the second month of the experiment, an infection with costia (*Ichthyobodo necator*) and the subsequent formalin treatment and further some problems with water quality resulted in higher mortality than expected. Total mortality during this period was approximately three times as high in nonsupplemented fish (35%) as in the other groups (8–13%). This may indicate that small fish were most susceptible to the costia infection in the unsupplemented group, since all the supplemented groups showed similar mortalities.

The final weight indicated that under the experimental conditions chosen the vitamin B_6 requirement for growth was covered in the diet containing 2.7 mg PN.HCl/kg. This is fairly consistent with results reported for rainbow trout (McLaren *et al.* 1947; Woodward 1990), the latter indicated a requirement for growth of 2 mg/kg diet. In Atlantic salmon fed a purified casein based diet, the requirement for maximum growth was approximately 5 mg/kg (Lall and Weerakoon 1990). This study was later repeated, and growth results obtained at this time did not show a clear difference between fish supplied with 2.5 and 5 mg/kg (Lall, pers. comm.). The requirement for vitamin B_6 in fish may be affected by the dietary protein quality and diet processing (Fisher *et al.* 1984; Hilton 1989). The weight gains supported by use of different test diets may subsequently influence the experimental results. In the present study growth rate was not optimal for Atlantic salmon fry throughout the experiment.

A positive correlation between dietary protein level and vitamin B_6 requirement within species has been shown (Cowey 1992), and it has been suggested that the high crude protein level used in formulated diets for fish may require a high level of dietary vitamin B_6. However, growth data from the present study support the suggestion by Coburn (1992), that the vitamin B_6 requirement of carnivorous farmed fish do not appear to be higher than that of other animals.

The proximate whole body composition showed only small differences between dietary groups. The protein content in fat free dry matter increased with increasing dietary supplementations, thus indicating that slightly more protein was synthesized the higher the content of vitamin B_6 in the diets. Total fat content in the livers of unsupplemented fish were reduced, accompanied by lower HSI values, as opposed to the marked fat infiltration found in livers of vitamin B_6 deficient rainbow trout (McLaren *et al.* 1947; Jürss and Jonas 1981). Liver fatty acid composition did not show any apparent effect of dietary vitamin B_6 on the interconversion of fatty acids.

A great reduction of vitamin B_6 in the yolk sac fry was found prior to start of feeding. As there was no weight increase during this period, approximately half of the vitamin B_6 content was consumed in the course of one week. Subsequently, vitamin B_6 in whole body was correlated to the dietary levels. Fish fed diets supplemented with 0 and 2 mg PN.HCl/kg did not show an increase in whole body vitamin B_6 concentrations in the course of the experimental period, thus indicating that by this criterium the dietary requirement for Atlantic salmon at start of feeding was higher than 2.7 mg PN.HCl/kg, the level analysed in diet B.

Vitamin B_6 concentration in the liver of non-supplemented fish was at a level of approximately 60% of the concentration found in the highest supplemented fish. In muscle of non-supplemented fish approximately 11% was found. These results are in accordance with the corresponding AspT activity in each respective organ. According to Halver (1989), the activity of aspartate aminotransferase appears to be related to the oxidative capacity of the tissue.

The hepatic vitamin B_6 concentrations (3–6 μg/g) were markedly higher than those in muscle (0.2–2.0 ug/g) but due to the different sizes of these organs the total muscle vitamin B_6 content far exceeded the total liver content. The muscle was early suggested to be a storage organ for vitamin B_6 (Krebs and Fisher 1964) and later it was found that muscle was the largest single pool of vitamin B_6 in the body (Friedrich 1988). However, dietary vitamin B_6 levels $\geq$ 13 mg/kg in larger Atlantic salmon did not lead to an increase in muscle vitamin B_6 levels (Albrektsen et al. 1993), although the concentration was three times higher than in the present study. In adult wild salmon, the hepatic vitamin B_6 concentration was found to be 5–6 μg/g, while fingerlings fed 50% protein in the diet in freshwater showed values in the range of 2–3 μg/g (Halver 1989).

The criteria used to estimate the highest requirement for vitamin B_6 in the present study was for maximal whole body and muscle vitamin B_6 levels. AspT activity in white muscle gave a fairly good approximation to these criteria. The highest storage levels of vitamin B_6 in liver and the dietary level of vitamin B_6 at which no significant change was found in the hepatic AspT activity, was found in fish supplied with 4 mg PN.HCl/kg diet. Thus the present data show that muscle vitamin B_6 concentration reflects the content of vitamin B_6 in the diet better than liver. The AspT activity recorded in white muscle confirms this. Also, Jürss and Nasev (1981) found muscle AspT activity to be a sensitive indicator for the early detection of inadequate vitamin B_6 supply in rainbow trout. AspT activity was reduced after feeding a vitamin B_6 deficient diet for one week (Jürss 1978, 1981). In the present study, maximal AspT activity in white muscle was reached in fish supplied with 6 mg PN.HCl/kg, the

same level as for rainbow trout (Woodward 1990). The activation coefficient $\alpha AspT$ also showed the highest saturation of enzyme at 6 mg/kg. A tendency towards saturation of vitamin B_6 in muscle tissue was indicated, as the concentrations *versus* feed levels could be described approximately by a parabola. Only fish fed the nonsupplemented diet had a lower muscle AspT activity than that considered to be a limiting value (≤ 6 U/g) in rainbow trout (Jürss and Nasev 1981). The stimulated AspT activity did not vary between groups, thus indicating that the amount of apoenzyme present was not affected by the dietary vitamin B_6 levels.

Measurements of relevant tissue enzyme activities are frequently used to provide supplementary evidence concerning the requirement of an animal for a given vitamin. Whether maximal enzyme activity is desirable in a growing animal and a useful parameter to establish nutrient requirements has been questioned (Baker 1986). White muscle AspT activity in the present study was not influenced by fish weight and showed a positive correlation to muscle vitamin B_6 concentrations and to diet concentrations. It was therefore considered to be a useful diagnostic tool to estimate a subclinical vitamin B_6 deficiency.

Liver AspT activity reflected the dietary vitamin B_6 levels less clearly than white muscle AspT, but the stimulation study indicated differences in this organ caused by the diet. The relatively high AspT activity found in liver in the non-supplemented group was somewhat unexpected. AspT activity in liver of sampled fish was, however, affected by fish weight, which may explain the higher enzyme activity in group A fish. Hardy *et al.* (1987) found that stimulation of AspT in liver homogenates by addition of PLP was a useful indicator of vitamin B_6 deficiency, but as judged from the present data this assay is more specific using muscle as the test organ.

Anaemia has been reported in different fish species after feeding a vitamin B_6 deficient diet (Halver 1957; Smith *et al.* 1974; Agrawal and Mahajan 1983a) while it was not found in other species (Adron *et al.* 1978; Andrews and Murai 1979). In the present study it appears that the natural contents of vitamin B_6 in the feed ingredients supported normal haemoglobin concentration in the blood.

In summary, the present study indicates that feed for Atlantic salmon fry should contain a minimum vitamin B_6 level of 2–3 mg/kg to support growth, and a minimum vitamin B_6 level of 6–8 mg/kg for maximizing white muscle AspT activity and whole body and muscle vitamin B_6 concentrations. The relations found between proximal chemical composition and vitamin B_6 status warrants further investigations.

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EFFECTS OF DIETARY IRON SUPPLEMENTATION ON TISSUE IRON CONCENTRATIONS AND HAEMATOLOGY IN ATLANTIC SALMON (*SALMO SALAR*)

By

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ABSTRACT

Two experiments were conducted to study iron nutriture in Atlantic salmon, *Salmo salar*, parr. In Experiment I, parr were fed a semi-purified diet supplemented with graded levels of iron (0, 20, 40, 60, 80, 160 mg/kg diet as iron(II)sulphate) for 8 weeks. In Experiment II, fifteen fish from each group from Experiment I were given the non-supplemented diet for 6 weeks to study how fish with different iron status are affected by low dietary iron.

Results from Experiment I showed that whole fish, liver and head kidney iron concentrations responded readily to changes in dietary iron level; the other tissues analyzed did not respond. The highest iron concentrations were found in spleen, followed by the head kidney and liver. The lowest iron levels were found in the white muscle. During Experiment II the differences in hepatic iron concentration disappeared. Further the fish pre-fed the non-supplemented diet showed reduced haematological values, indicating that the group was developing anaemia. This shows that the basal diet containing 33 mg iron/kg dry feed did not meet the iron requirement of Atlantic salmon.

INTRODUCTION

Iron is an essential element involved in the physiological function of oxygen transport and cellular respiration.

Iron deficiency in fish results in microcytic anaemia as has been shown in brook trout *(Salvelinus fontinalis)* (Kawatsu 1972), red sea bream *(Chrysophrys major)* (Sakamoto and Yone 1978a), carp *(Cyprinus carpio)* (Sakamoto and Yone 1978b) and channel catfish *(Ictalurus punctatus)* (Gatlin and Wilson 1986). Iron absorption and metabolism in salmonids are poorly understood. However, in mammals the absorption of iron is affected by factors such as age, iron status, health state, conditions within the gastrointestinal tract, the amount and chemical form of the iron ingested, and

the amounts and proportions of various other components of the diet (Underwood 1977).

The minimum iron requirement in feed for Atlantic salmon was suggested to be about 60 mg/kg (Lall and Hines 1987).

Commercial Norwegian fish feeds contained from 51 to 515 mg iron/kg dry feed (Maage 1991). It was recently suggested that the iron absorption in Atlantic salmon is poorly regulated and that "normal" levels of iron in salmon feed may result in oxidative damage *in vivo* (Røed *et al.*, 1991). As a consequence, iron supplementation in Norwegian commercial fish feeds is practically abolished.

However, basal scientific knowledge of iron nutriture in Atlantic salmon is scarce. Therefore, such knowledge is required to provide the background for guidelines about dietary iron levels for the salmon industry.

The objectives of these experiments were:

1) to study the effect of feeding graded levels of iron on the iron tissue concentration and haematology in Atlantic salmon parr,
2) to study how fish with different iron status are affected by low levels of iron in the feed.

The experiments were run from October 1989 to January 1990.

MATERIALS AND METHODS

Diet formulation

An experimental diet based on cod-muscle meal as the protein source were prepared (Table 1). The diet was supplemented with 0, 20, 40, 60, 80 or 160 mg iron as iron(II)sulphate ($FeSO_4 \times 7H_2O$) per kg diet. By

Table 1. Composition the basal experimental diet

Ingredient	g/kg
Cod muscle meal	610
Capelin oil	180
Dextrin	150
Vitamin mix [a]	10
Mineral mix [b]	50

[a]) Vitamins in mg/kg of the diet: thiamine, 10; riboflavin, 10; niacin, 20; pyridoxine, 40; biotin, 25; folic acid, 20; B 12, 10; inositol, 500; ascorbic acid, 1000; Choline Chloride, 5500; Vitamin K, 50. Vitamins in IU/kg of diet: Vitamin A (retinyl palmitate), 7000; vitamin D (cholecalciferol), 3000; DL-α -tochophenyl acetate, 200.
[b]) Minerals in g/kg of the diet: $CaHPO_4·2H_2O$, 19; KH_2PO_4, 15; NaCl, 10; $MgSO_4·7H_2O$, 5; Minerals in mg/kg of the diet: $ZnSO_4·7H_2O$, 450; $MnSO_4·H_2O$, 75; $CuSO_4·5H_2O$, 20; KI, 15.

analysis the diets contained 33, 58, 75, 91, 101 and 190 mg Fe/kg dry diet, respectively. Further, the protein content was 600 g/kg and total lipids 180 g/kg.

Essential element levels in the diets were (mg/kg dry feed): P, 10700; Ca, 3500; Mg, 1790; Zn, 137; Cu, 13.4. The content of vitamin C determined by analysis at the start of the experiment was 710 mg/kg dry diet.

Fish and fish rearing

Ninety three Atlantic salmon parr (mean weight 31.3 ± 5.9 g) obtained from Matre Aquaculture Station were randomly distributed in each of 12 tanks (1m × 1m × 1m). The fish were fed the unsupplemented basal diet for two weeks prior to the experiment to allow for acclimatization and to reduce their iron status.

Duplicate groups were then fed the experimental diets for 8 weeks (Experiment I). In Experiment II, 15 fish from each of these dietary treatments (7/8 from each tank) were marked according to the previous diet and put together in one tank and fed the unsupplemented diet for 6 weeks. The fish were reared under continuous light and fed by automatic feeders every seven minutes. Sea water (1–2%) was added to the fresh water to buffer for acidity and increase the alkalinity. Iron concentration of the water was in the range of 14–34 microgram per litre. During the experiments the water temperature followed the ambient water temperature of the Matre river which declined from 9.5 to 5° C. Dead fish, if any, were removed daily and recorded.

Sampling

Prior to the samplings, fish were starved for 24 h. All the fish in each tank were anaesthetized and measured for fork length and weighed at weeks 4, 8 (Experiment I) and 14 (Experiment II). These data were used for calculation of condition factor K= $100 \cdot$weight/(fork length)^3. At the end of Experiment I ten fish were selected randomly for sampling from each tank (twenty per treatment). Ten fish from each dietary treatment were used for iron analysis of whole fish. From the remaining ten, blood samples were withdrawn by syringe from the caudal peduncle and tissues were taken for iron analysis. At the end of Experiment II only livers were dissected for iron analyses.

Chemical analyses

The samples of whole fish were ground before freeze-drying. Freeze dried samples of whole fish, liver, red muscle, white muscle, spleen, head kidney,

back-kidney, gills, intestine, vertebrae and feed were homogenized and digested in nitric:perchloric acid (9:1) according to Julshamn and Andersen (1982). Iron analyses were performed by flame atomic absorption spectroscopy (Perkin Elmer 3030) and were controlled by concomitant analyses of Oyster Tissue, Bovine Liver, Citrus Leaves and TORT standards from National Institute of Standards and Technology, USA.

Haemoglobin and haematocrit were determined as described by Sandnes *et al.* (1988).

Statistical analyses

The data were evaluated statistically by using a STATGRAPHICS software. The statistics used are described in Sokhal and Rohlf (1981). All tests for significance were made at the level $p < 0.05$, unless otherwise stated.

To assess normality of distribution of the measured parameters, a normal probability plot was used. To control whether parallel samplings could be pooled, nested ANOVA was applied. One way ANOVA analysis was performed to find differences among experimental groups. Further, correlation analyses of the parameters from individual fish were included.

RESULTS

EXPERIMENT I

Weight and condition factor
Fish from all dietary groups grew from about 31 grams to about 38 grams during 8 weeks of feeding (Table 2). Mortalities were negligible. The condition factor declined slightly from 1.21 at the start of experiment to

Table 2. Weight (g ± SD, n=80) and condition factor (± SD) of Atlantic salmon parr fed purified diets with graded levels of iron concentration for 8 weeks.

Supplemental iron (mg/kg)[1]	weight	condition factor
0	37.9 ± 8.1[ab2]	1.17 ± 0.1 [bc]
20	35.9 ± 7.3[a]	1.17 ± 0.07 [bc]
40	38.1 ± 8.1[ab]	1.19 ± 0.12[c]
60	38.6 ± 8.0 [b]	1.17 ± 0.09[bc]
80	38.3 ± 7.8[ab]	1.15 ± 0.08[ab]
160	39.0 ± 8.8[b]	1.13 ± 0.07 [a]

Initial values: body weight, 31±6 g; condition factor, 1.21±0.13.

[1] Basal diet containing 33 mg Fe/kg dry diet.
[2] Means not shearing a common superscript are significantly different (p<0.05)

about 1.16. The decline was most clear in the groups fed the highest iron supplementation.

Iron concentration in whole fish and tissues

Whole fish and tissues responded differently to changes in dietary iron level (Table 3). Correlation analyses showed that for liver, head kidney and whole fish there were significant correlations between dietary and tissue iron concentration.

The iron concentration in whole fish at the start of the experiment was 16.3 mg/kg wet weight (w.w.). After 8 weeks this value had declined and the mean values ranged between 9.5 and 12.5 mg/kg w.w. The group receiving the diet supplemented with 160 mg Fe/kg had significantly higher iron concentration than groups receiving 0 or 20 mg iron supplementation (Table 3).

The hepatic iron concentration at the start of the experiment was 117 mg/kg w.w. After 8 weeks of feeding the hepatic iron concentration varied from 65 to 118 mg/kg, and the group receiving the diet supplemented with 160 mg Fe/kg showed significantly ($p<0.05$) higher iron concentration then the groups receiving 0, 20, 40 or 60 mg supplemented Fe/kg (Table 3).

By ANOVA, the other organs showed no significant differences related to changes in the dietary iron level (Table 3). The highest iron concentration was found in the spleen (300–900 mg Fe/kg w.w.), followed by the head kidney and the liver. The lowest tissue iron concentration was found in white muscle which varied from 1.6 to 2.1 mg/kg after 8 weeks of feeding.

Blood values

The haemoglobin (Hb) and haematocrit (Hct) values showed no significant differences relative to changes in the dietary iron level (Table 3). At the start of the experiment the Hb-concentration was 12.2 g/100 ml and varied between 12.3 and 13.3 g/100 ml after 8 weeks of feeding. The Hct-level at start was 46% and after 8 weeks of feeding it varied between 47 and 51%.

EXPERIMENT II

This experiment started out with fish from Experiment I. They were fed the unsupplemented basal diet for 6 weeks.

Table 3. Iron concentration (mg/kg $\pm$ SD wet weight, n=10) in whole fish and tissues, and haemoglobin (g/100 ml$\pm$SD, n=10) and haematocrit levels (%$\pm$SD, n=10) of blood samples from Atlantic salmon parr fed semipurified diets with graded levels of iron concentration for 8 weeks.

Supplemental iron (mg/kg)[1]	0	20	40	60	80	160
Tissue iron concentration						
whole fish	9.5 ± 1.3^{a2}	9.5 ± 0.9 [a]	10.9 ± 1.4^{ab}	11.9 ± 2.4 [ab]	10.6 ± 1.7^{ab}	12.5 ± 2.9 [b]
liver	75 ± 30^{a}	65 ± 13^{a}	77 ± 16^{a}	80 ± 16 [a]	96 ± 25^{ab}	118 ± 35 [b]
red muscle	11.6 ± 2.7	12.6 ± 4.7	10.7 ± 2.7	11.2 ± 2.0	13.5 ± 2.2	12.2 ± 2.4
white muscle	1.9 ± 0.7	1.9 ± 0.4	1.8 ± 0.7	2.1 ± 1.1	1.8 ± 0.8	1.6 ± 0.9
vertebrae	17.9 ± 7.6	17.4 ± 6.5	22.7 ± 6.3	17.7 ± 6.4	19.2 ± 3.4	16.0 ± 7.1
spleen [3]	412;306	705;611	470;287	919;530	509;274	379;329
head kidney [3]	106;131	161;275	134;240	119;173	263;351	216;239
rear kidney	33.0 ± 7.0	33.2 ± 10.0	61.5 ± 20.9	46.2 ± 25.3	57.6 ± 16.3	47.5 ± 17.6
gills	21.0 ± 4.1	24.6 ± 7.8	23.9 ± 5.0	18.2 ± 3.8	21.5 ± 6.4	23.9 ± 4.8
intestine	16.3 ± 6.1	25.9 ± 22.0	14.7 ± 6.6	20.4 ± 10.6	23.4 ± 17.4	21.1 ± 22.6
Blood values						
haemoglobin	12.6 ± 2.0	12.3 ± 1.0	12.3 ± 1.3	12.8 ± 2.0	13.3 ± 1.2	12.5 ± 1.2
haematocrit (%)	50.3 ± 6.0	47.1 ± 4.0	49.9 ± 5.0	50.3 ± 7.0	50.5 ± 5.0	49.4 ± 3.5

Initial values: whole fish, 16.3$\pm$2.5; liver, 117$\pm$28; red muscle, 16.7$\pm$5.7; white muscle, 5.0$\pm$1.7; vertebrae, 18.0$\pm$7.6; spleen, 294$\pm$26, head kidney: ¡_ 160$\pm$21; rear kidney, 73$\pm$6; gills: 25.3$\pm$1.3; intestine: not analyzed, vertebrae: 18.0$\pm$7.6; haemoglobin: 12.2$\pm$1.7; haematocrit: 46$\pm$5.

[1] Basal diet containing 33 mg Fe/kg dry diet.

[2] Means not s hearing a common superscript are significantly different ($p < 0.05$)

[3] Iron concentration in two pooled samples of 5 fish each.

Weight and condition factor

Only a slight growth from a mean of 39.7 grams to 41.2 grams was seen in this experiment (Table 4). The fish previously fed the diet with the highest iron concentration had the highest final weight. The former differences in condition factor in Experiment I disappeared.

Iron concentration in whole fish and liver

Whole fish iron concentration varied between 9.3 and 11.7 mg/kg and the hepatic iron concentration varied between 72 and 86 mg/kg (Table 5). There were no significant differences between the groups.

Blood values

The haemoglobin concentration varied between 9.0 and 10.3 g/100 ml and haematocrit varied between 49 and 59% at the end of the experiment. Those fish which had been on the unsupplemented diet also 10 weeks prior to Experiment II now showed the lowest values for haematocrit and haemoglobin.

DISCUSSION

Lall (1989) stated that growth retardation is not a common feature of iron deficiency in fish. There was no differences in final weight between the group feed the highest and the lowest level of iron in the present experiments. Growth in the present experiments was rather low, especially during Experiment II. The reason was probably that the experiments

Table 4. Weight (g $\pm$ SD, n=15) and condition factor ($\pm$SD, n=15) of Atlantic salmon parr fed purified diets without iron supplement for 6 weeks.

Pretreatment [1]	weight week 6	condition factor week 6
0	39.3 $\pm$ 8.2[ab2]	1.22 $\pm$ 0.11
20	40.0 $\pm$ 6.9[ab]	1.19 $\pm$ 0.06
40	41.6 $\pm$ 6.2[ab]	1.22 $\pm$ 0.11
60	38.4 $\pm$ 8.3[a]	1.20 $\pm$ 0.13
80	39.2 $\pm$ 5.7[ab]	1.20 $\pm$ 0.08
160	48.5 $\pm$ 13.2[b]	1.22 $\pm$ 0.09

[1] Level of supplemental iron (mg/kg dry feed) in Experiment 1.
[2] Means not shearing a common superscript are significantly different (p<0.05)

Table 5. Iron concentration (mg/kg±SD wet weight, n=10) in whole fish and liver, and haemoglobin (g/100 ml±SD, n=10) and haematocrit (%±SD, n=10) in Atlantic salmon parr fed purified diets without iron supplement for 6 weeks.

Experimental group[1]	0	20	40	60	80	160
Tissue iron concentration						
Whole fish	10.6 ± 2.3	9.3 ± 3.0	9.7 ± 1.7	11.7 ± 1.3	11.2 ± 0.3	10.8 ± 2.5
Liver	75 ± 23	72 ± 18	76 ± 23	86 ± 24	74 ± 20	76 ± 15
Blood values						
haemoglobin	9.0 ± 1.2	10.0 ± 0.8	10.0 ± 1.1	10.3 ± 0.9	9.6 ± 1.4	9.9 ± 1.0
haemotocrit	49.4 ± 5.1^{a2}	53.8 ± 7.5^{ab}	58.6 ± 5.2^{b}	58.0 ± 4.2^{b}	55.1 ± 5.3^{ab}	55.1 ± 8.0^{ab}

[1] Level of supplemental iron (mg/kg dry feed) in Experiment 1.

[2] Means not shearing a common superscript are significantly different (p<0.05)

were performed during the colder months from October to January. The water temperature was depending on the ambient water temperature in the Matre River which was low and declining during the course of the experiments.

In Experiment I, the iron concentration in many tissues were analyzed. The concentrations varied from values of more than 900 mg Fe/kg w. w. in a spleen sample to only about 1.8 mg/kg in white muscle tissue. Also in rainbow trout the spleen is found to be the organ with the highest iron concentration (Walker and Fromm 1976).

Spleen and head kidney with the highest iron concentrations are the erythropoietic tissues in fish (Giersberg and Rietschel 1968). The hepatic iron concentration was relatively high and was together with head kidney and whole fish the only tissue giving significant response to the iron supplementation. Hepatic iron concentration was reported to respond readily to dietary iron in salmonids (Walker and Fromm 1976; Desjardins *et al* 1987). A major part of the difference found in whole fish iron concentration can be ascribed to difference in hepatic iron concentration. There was about ten-fold more iron in the liver than in whole fish. The lowest mean value of hepatic iron concentration was 55% of the highest value (Table 3), while in whole fish the ratio was 76%. These results show that the liver is an important iron storage organ in Atlantic salmon as has also been observed in carp *Tinca vulgaris* (Hevesy *et al.*, 1968) and rainbow trout *Onchorynchus mykiss* (Walker and Fromm, 1976). Further, the hepatic iron concentration is a sensitive parameter in evaluating iron status. This does not apply in cases of vitamin C deficiency when the hepatic iron concentration may be high with concomitant iron deficiency anaemia (Maage *et al* 1990).

The iron concentration in head kidney responded similarly to that of the liver to dietary iron. The rear kidney showed lower iron content than the head kidney, probably due to higher erythropoiesis in the head kidney (Kryvi, 1990).

Response to increasing dietary iron was found neither in white nor in red muscle. With the range of dietary iron used in this experiment it does not seen possible to increase the content of iron in fillets for human consumption. The red muscle had higher iron concentrations due to it containing myoglobin and more mitochondria than the white muscle (Kryvi, 1990).

In Experiment II only samples of whole fish and liver were analyzed for iron. In the fish previously fed the highest dietary iron level the hepatic iron concentration declined rapidly. The significant differences found in Experiment I did also disappear both in liver and whole fish. This indicates that the fish previously fed high iron levels had adapted to

a higher iron excretion, perhaps through the bile (von Dijk *et al* 1975). Further, this indicates that if the dietary iron concentration varies the Atlantic salmon has a significant but time limited ability to store iron. Walker and Fromm (1976), however, reported that iron metabolism in rainbow trout (*Oncorhynchus mykiss*) is a closed, recycling system and the fact that there was no difference observed in gill iron concentrations shows that this organ is probably not active in iron excretion. However, it has been suggested that iron can be absorbed over the gills (Roeder and Roeder 1966).

Haematologic analysis have been used to identify iron deficiency in fish species (Kawatsu 1972, Gatlin and Wilson 1986, Sakamoto and Yone, 1978a and b). The haematologic values found in this experiment are in the range for Atlantic salmon (Sandnes *et al.*, 1988; Maage *et al.*, 1990). There were no differences in values in Experiment I. In Experiment II, however, the fish fed the basal diet without iron supplementation in Experiment I, showed reduction in blood haematocrit. This indicates that the fish fed altogether for 16 weeks on the non-supplemented diet were developing anaemia. Thus, the basal level of 33 mg Fe/kg diet was not sufficient to meet the iron requirements in Atlantic salmon.

In summary, this work shows that liver, head kidney and whole body were sensitive to dietary iron and can be used as iron status indicators. Further, it shows that excess dietary iron is mainly stored in the liver. This iron is readily available when dietary iron becomes scarce. According to this study the requirement of iron for Atlantic salmon is higher than 33 mg/kg dry diet.

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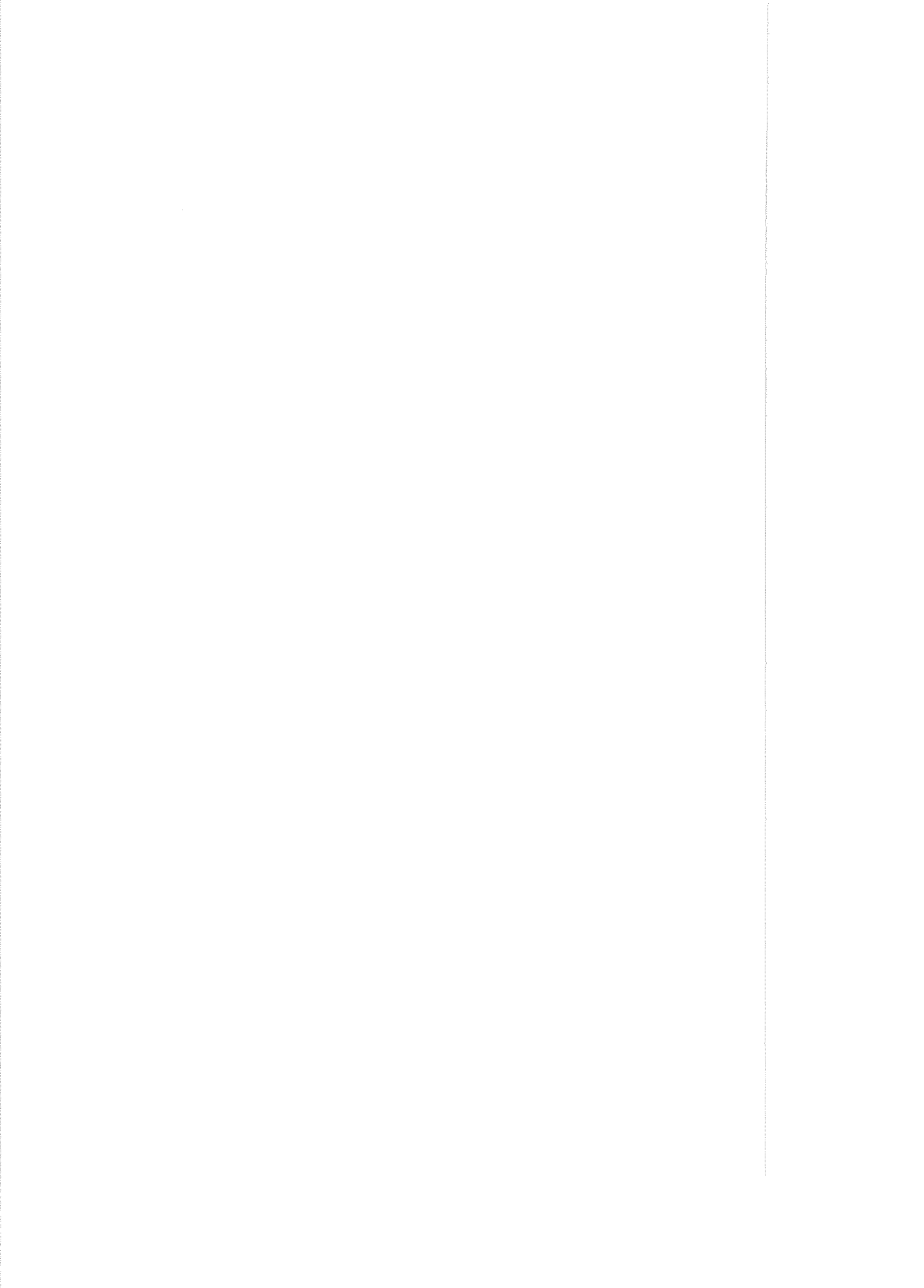
HEALTH ASPECTS OF DIETARY LIPID SOURCES AND VITAMIN E IN ATLANTIC SALMON (*SALMO SALAR*). I. ERYTHROCYTE TOTAL LIPID FATTY ACID COMPOSITION, HAEMATOLOGY AND HUMORAL IMMUNE RESPONSE.

By

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ABSTRACT

Atlantic salmon were given practical diets with three lipid sources: soyabean oil, capelin oil or sardine oil. The oils were used with two levels of vitamin E (57 and 272 mg α-tocopherol kg^{-1}). The oils chosen differed especially in their content of n-3 polyunsaturated fatty acids (PUFA). Analyses after 10 months of feeding showed that erythrocyte total fatty acid composition, vitamin E content and serum vitamin E concentration were influenced by the diets.

In a following vaccination against *Vibrio salmonicida*, the specific antibody level was significantly lower in fish fed the highest level of dietary n-3 fatty acids and vitamin E. Total serum protein concentration was not significantly affected, while total serum antibodies were somewhat higher in vitamin E supplemented groups.

There were minor differences in the haematological status between the groups. Coagulation activity decreased with increasing dietary n-3 fatty acids and high level of vitamin E.

The specific immunity and coagulation time are discussed relative to possible roles of n-3 PUFAs and vitamin E in the eicosanoid metabolism, as substrates and inhibitors.

INTRODUCTION

Health aspects of unsaturated lipids in fish nutrition have mainly been associated with feed rancidity and hazardous *in vivo* lipid peroxidation (Roberts, 1989). Less attention have been paid to the roles of the individual fatty acids in determining the overall membrane structure (membrane fluidity) and, as precursors in the synthesis of eicosanoids. The latter are related to activation processes and functions of immunocompetent cells (Pelus and Strausser, 1977; Erickson *et al.*, 1986; Roitt, 1988; Blazer, 1992).

Dietary lipids and water temperature influenced the fatty acid composition of blood cells in fish (Bly *et al.*, 1986; Leray *et al.*, 1986; Lie *et al.*, 1989). According to Sargent *et al.* (1989) there are higher requirements for n-3 PUFA in cold water species than in warm water species. Changes in fatty acid composition and lipid structure in cell membranes have been considered as an important factor influencing fish immunity at lower water temperatures (Bly *et al.*, 1986; Bly and Clem, 1992).

The requirement for vitamin E is strongly linked to the dietary content of PUFA in several fish species (Watanabe *et al.*, 1981; Greene and Selivonchick, 1987; Sargent *et al.*, 1989; Waagbø *et al.*, 1991). Besides the classical symptoms of vitamin E deficiency, impaired humoral immune responses and depressed macrophage and lymphocyte functions have been reported (Sokol, 1989).

Excessive vitamin E supplementation has been shown to exert positive effects upon the specific immune system (Tengerdy *et al.*, 1981; Sokol, 1989). Studies on salmonids have shown that effects of vitamin E nutrition on immunity are relevant for farmed fish as well (Blazer and Wolke 1984; Leith *et al.*, 1989; Hardie *et al.*, 1990; Ndoye *et al.*, 1990; Verlhac *et al.*, 1993).

The present experiment with Atlantic salmon reports on the influence of dietary lipid sources (with different levels of n-3 PUFA) and vitamin E (high and low levels) on erythrocyte fatty acid composition, haematology and humoral antibody levels following intraperitoneal vaccination. This study is included in a series of experiments using the same diets, in which the influence on growth, organ composition and physiology of Atlantic salmon have been investigated (Waagbø *et al.*, 1991; Waagbø *et al.*, 1993a; Lie *et al.*, 1993). Studies on nonspecific immunity and mineral interactions were performed in the same period of time as the present experiment (Waagbø *et al.*, 1993b; Maage and Waagbø, 1990; Waagbø and Maage, 1992).

Some preliminary data from these studies were presented orally at the Third International Symposium on Feeding and Nutrition in Fish, Toba, Japan, August 28th-September 1st, 1989.

MATERIALS AND METHODS

Fish and diets

The present trial was performed in the period of March 1988 and March 1989 at Matre Aquaculture Research Station (Institute of Marine Research, Bergen, Norway). Atlantic salmon (*Salmo salar*) were fed fish meal based experimental diets containing soyabean oil, capelin oil or sardine

oil. The oils were used either with or without α-tocopheryl acetate supplementation (300 mg α-tocopherol kg^{-1} diet, Rovimix 50E, Roche). The emulsions of the oils (see footnote to Table 1) were added to extruded pellets of the other feed ingredients by spraying amounts corresponding to 160 g kg^{-1} of the finished diets.

Diet compositions are presented in Table 1. By analysis the soyabean oil, capelin oil and sardine oil diets contained 11.2, 20.5 and 35.0% n-3 polyunsaturated fatty acids (n-3 PUFA) in the feed lipid or 19, 35 and 60

Table 1. a) Gross composition and b) total lipid fatty acid composition of the experimental diets.

a) Ingredients	g Kg^{-1}
Fish meal (Norseamink, LT, Norsildmel, Norway)	33
Danpro A Soy concentrate (Aarhus Oliefabrik A/S, Denmark)	29
Extruded wheat	16
Vitamin/mineral mix[1]	6
Lipid sources[2]	16

b)	Diet		
Fatty acid (%)	Soyabean oil	Capelin oil	Sardine oil
14:0	1.4	5.5	6.5
16:0	12.3	13.0	15.8
16:1[3]	1.2	6.0	6.8
18:0	3.5	1.5	2.5
18:1[3]	20.5	11.8	12.1
20:1[3]	1.9	13.4	4.1
22:1[3]	2.9	18.0	4.4
18:2 (n-6)	43.4	6.5	5.1
18:3 (n-3)	5.6	1.3	1.2
20:4 (n-6)	0.1	0.3	1.3
20:5 (n-3)	1.6	7.4	15.3
22:6 (n-3)	3.2	7.7	13.3
Sum saturated	18.3	20.3	25.6
Sum monoenes	26.2	49.6	28.2
Sum (n-3)	11.2	20.5	35.0
Sum (n-6)	43.6	6.9	6.5
Sum polyenes	54.8	27.4	41.5
(n-3)/(n-6)	0.3	3.2	5.4

[1] T. Skretting A/S vitamin and mineral mix exclusive vitamin C, vitamin E and pigments.

[2] Soyabean oil, capelin oil or sardine oil inclusive vitamin C (E.C.), vitamin E (α-tocopheryl acetate, Rovimix 50 E) and astaxanthin (Carophyll pink 5%) correspondent to 500 mg kg^{-1}, 300 mg kg^{-1} (+ E groups only) and 50 mg kg^{-1}, respectively.

[3] Sum of isomers.

g n-3 PUFA kg^{-1} dry feed, respectively. The (n-3)/(n-6) ratios we₁ 3.2 and 5.4. Each oil diet contained either 57 (-E) or 272 (+E) n₁₅ α-tocopherol kg^{-1} (mean analysed values).

Each diet was fed to duplicate indoor flow-through tanks (1.5m x 1.5m). During the first 5 months there were 750 fish tank^{-1}. After then the amount was reduced to 100 fish tank^{-1} for 5 months prior to start of the vaccination experiment (mean body weight in the range of 428 to 459 g). The diets were fed according to standard tables for Atlantic salmon (Austreng et al., 1987). The tanks were supplied with a mixture of filtered sea water and fresh river water adjusted to a salinity of 20 g L^{-1}. The mean monthly water temperatures from January to March 1989 were 10.0, 9.0 and 7.7° C, respectively.

Vaccination

Twenty fish from each tank were weighed, anaesthetized and injected intraperitoneally with 0.2 mL of a commercial vaccine against *Vibrio salmonicida* (Norbio A/S, Norway). Vaccinated fish were marked by cutting the fatty fin. Ten fish were injected with 0.2 mL vaccine medium alone (sham-injected) and marked by cutting the left pelvic fin. Blood was sampled from five fish (initial sample). Seven weeks later ten vaccinated fish, five sham-injected fish and five untreated control fish were sampled from each tank. The fish were weighed, and blood samples collected for determination of serum specific antibodies, total antibodies and total protein (all fish), blood coagulation time (sham and untreated fish) and haematology (untreated fish; blood Hb was recorded in all fish).

Blood sampling

After anaesthetizing the fish (benzocaine-ethanol solution), blood samples were withdrawn with a syringe from the caudal vein, stored at 4° C for approximately 3 hours and then centrifuged at 3000 rpm for 10 min. The serum obtained was stored at -80° C until analysed for total antibody, specific antibody, total protein and α-tocopherol levels. An aliquot (300 μl) of the blood was immediately heparinized for haematological analyses and for determination of erythrocyte total fatty acid composition and vitamin E content. Erythrocytes derived from equal volumes of heparinized blood from five fish were pooled and washed three times in buffered physiological saline solution (PBS) before analyses.

Erythrocyte total lipid fatty acid composition and α-tocopherol and serum α-tocopherol were analysed in the initial sample by the methods described by Lie and Lambertsen (1991) and Lie et al. (pers. comm.), respectively.

Immunological analyses

Specific antibodies against *V. salmonicida* and total antibodies in serum were estimated at vaccination and after 7 weeks, using a standard ELISA procedure (Håvarstein *et al.*, 1990) and a radial immunodiffusion method (Mancini *et al.*, 1965), respectively. In the ELISA procedure microtitre plates (Nunc Immunoplate) were coated with *V. salmonicida* cell suspension (10^6 bacteria well^{-1} for 24h at 4 °C), washed with PBS (including 0.1% Tween 20), blocked with a 3% skimmed milk solution (Molico dry skimmed milk, Nestlè) 1h at room temperature and washed 3 times prior to use. According to a preliminary titration test serum samples were diluted 1:500 (PBS including 0.1% Tween 20 and 0.5% skimmed milk) and added to triplicate wells. The plates were incubated for 24h at 4° C and washed with PBS. Rabbit polyclonal anti-salmon immunoglobulin (obtained from E. Raa Nilsen, University of Bergen, Norway) was used in a dilution of 1:3000 (PBS-Tween-skimmed milk). Finally, Bio-Rad peroxidase conjugated goat anti rabbit immunoglobulin (diluted 1:3000 with PBS-Tween-skimmed milk) was added to the wells and incubated 1h at room temperature. The microtitre plates were washed with PBS-Tween. Substrate (o-phenylenediamine dihydrochloride and H_2O_2) was added and left to react for 10 minutes before adding 5 M sulfuric acid. The absorbance (OD_{492}) was read in an ELISA reader (Titertec Multi-scan MCC 340, Flow Laboratories, Switzerland) in which the absorbance was corrected against blank wells.

In the radial immunodiffusion assay the same polyclonal rabbit anti salmon immunoglobulin (2%) was used in a (1%) barbiturate buffer (0.024 M, pH 8.6)-agar (0.15 mL gel cm^{-2}). Undiluted serum (20 μl) was added to 2.0 mm Ø wells. After incubation (48h at 4° C) the gel was washed and stained with Coomassie Brilliant Blue R 250. The diameter of the precipitation zone was measured. No standard solution of salmon immunoglobulins was available so the results in mm units were evaluated statistically.

Total serum protein was determined colorimetrically according to Sandnes *et al.* (1988).

Haematological analyses

Red blood cell count (Rbc), haematocrit (Hct), haemoglobin (Hb) and leucocrit (Lct) were determined and mean cell volume (MCV), mean cell Hb content (MCH) and mean cell Hb concentration (MCHC) were calculated according to Sandnes *et al.* (1988). The Hct and Lct were measured immediately after blood sampling.

The osmotic fragility test of the red blood cells was performed accu.
to Dacie and Lewis (1975). The salinity ranged from 0 (distilled watei,
to 0.9 g 100 mL^{-1} (0.1 M phosphate buffered NaCl solution, pH 7.4).
The blood cells were incubated in the tubes for one hour at 4° C prior
to centrifugation. The supernatants were measured spectrophotometrically
at 540 nm (Shimadzu UV-240 spectrophotometer). The results are given
as the salinity causing 50% lysis of the blood cells based on a curve (%
haemolysis vs. salinity) for each sample, using the absorbance obtained
with distilled water as 100% haemolysis.

A capillary coagulation test (Poston, 1976) using whole blood was
performed immediately after blood sampling. Four glass capillary tubes
were filled simultaneously with blood as quickly as possible, and after
one minute one tube was broken every 10 seconds. Time was recorded
when a coagulated fibrous strand of blood appeared between the two
broken pieces of the tube. The rest of the tubes were then immediately
tested for coagulation, and the mean coagulation time of the four capillary
tubes was noted.

Table 2. Mean (± SEM, n=4 pooled samples of each 5 fish) total lipid fatty acid composition
of erythrocytes from Atlantic salmon fed diets with three lipid sources differing in levels
of n-3 PUFA for 10 months.

Fatty acid (%)	Soyabean oil	Capelin oil	Sardine oil
14:0	0.4 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
16:0	20.1 ± 0.4	21.8 ± 0.8	22.8 ± 0.7
16:1[1]	0.4 ± 0.2	1.0 ± 0.1	0.8 ± 0.1
18:0	8.6 ± 0.5	6.0 ± 0.2	8.2 ± 0.4
18:1[1]	5.7 ± 0.3	5.6 ± 0.2	5.1 ± 0.2
20:1[1]	0.5 ± 0.1	3.3 ± 0.2	0.7 ± 0.1
22:1[1]	n.d.[2]	1.5 ± 0.1	0.1 ± 0.1
18:2 (n-6)	8.3 ± 0.5	1.1 ± 0.1	1.1 ± 0.1
18:3 (n-3)	0.6 ± 0.1	n.d.	n.d.
20:4 (n-6)	4.5 ± 0.3[a3]	1.8 ± 0.1 [b]	3.5 ± 0.1[c]
20:5 (n-3)	4.6 ± 0.5[a]	9.6 ± 0.2[b]	9.3 ± 0.3[b]
22:6 (n-3)	40.6 ± 0.5[a]	43.7 ± 1.1 [a]	42.5 ± 0.7[a]
Sum saturated	29.4 ± 0.8[a]	29.4 ± 1.0 [a]	32.6 ± 1.1[a]
Sum monoenes	6.6 ± 0.5[a]	11.3 ± 0.2[b]	6.7 ± 0.2[a]
Sum (n-3)	47.6 ± 0.8[a]	55.9 ± 1.1 [b]	55.4 ± 1.2[b]
Sum (n-6)	15.7 ± 0.2[a]	3.0 ± 0.1[b]	4.6 ± 0.2[c]
Sum polyenes	63.3 ± 0.9[a]	58.9 ± 1.2 [b]	60.0 ± 1.2[ab]
(n-3)/(n-6)	3.0 ± 0.0[a]	18.8 ± 0.3 [b]	12.0 ± 0.5[c]

[1] Sum of isomers.
[2] n.d.= not detected
[3] different superscript letters indicate significant differences (p < 0.05).

Statistics

The data were treated statistically using two way analysis of variance and intergroup differences were tested with a Duncan's multiple range test and a non-parametric Mann-Whitney U test (specific antibody level). Spearman rank order correlations were used between individual parameters. All tests were within a CSS Statistica™ statistical program (StatSoft, Inc., USA, 1991).

RESULTS

Lipid fatty acid composition and vitamin E levels

The total fatty acid composition of the erythrocyts at the start of the vaccination trial reflected to some extent the fatty acid pattern in the feeds. Table 2 gives the mean erythrocyte fatty acid composition for the three lipid sources only, as the level of dietary vitamin E did not influence upon the fatty acid composition in the cells.

The fatty acids characteristic for soyabean oil, 18:2 n-6 and 18:3 n-3, were elevated in the erythrocytes. The content of arachidonic acid, 20:4 n-6, was highest in this group (p<0.05). The level of n-6 fatty acids was high (15.7% of total lipid) and the n-3/n-6 ratio was lowest in this group. The content of 20:5 n-3 (EPA) was significantly lower (p<0.05), and 22:6 n-3 (DHA) nearly the same as in the other groups.

The capelin oil feeds contained more monoenes (about 50%) and less polyenes (27%) compared to the other two groups. The erythrocytes from fish fed these diets contained twice as much monoenes (11%) as fish fed the two other lipid sources. The sum of n-6 fatty acids was also low in the capelin oil groups and the (n-3)/(n-6) ratio was therefore high.

The blood cells of fish fed the sardine oil with the highest content of n-3 PUFA (35 %) resembled those from fish fed the capelin oil diets as regards fatty acid composition, except for the lower content of monoenes (6.7%) and a higher content of 20:4 n-6 (3.5%). The sum of saturated fatty acids and the sum of polyenes in the erythrocytes were nearly the same in all groups.

The α-tocopherol contents were higher in the erythrocytes (1.6 to 2.5 times) and serum (2.7 to 3.2 times) of fish fed diets supplemented with vitamin E than in the respective unsupplemented groups (Table 3). The ratio between serum and erythrocyte α-tocopherol contents were lower in both sardine oil groups.

Haematology

The haematological data, including Hb, Rbc, Hct, Lct and the blood indexes MCV, MCH and MCHC (Table 4), showed no differences that

Table 3. Mean vitamin E content in erythrocytes (μg α-tocopherol g^{-1}) and in serum (μg α-tocopherol mL^{-1}) in Atlantic salmon fed diets with different lipid sources (soyabean oil, capelin oil and sardine oil), with (+E) or without (−E) addition of vitamin E for 10 months (2 pooled samples, each of 5 fish).

Diet	Erythrocytes	Serum
Soyabean oil −E	10.7	27.4
Soyabean oil +E	22.1	73.8
Capelin oil −E	10.3	28.2
Capelin oil +E	25.4	85.6
Sardine oil − E	16.0	20.2
Sardine oil +E	24.8	64.7

could be attributed to the feed variables. The lower Rbc and Hb values in fish fed the capelin oil (+E) diet were caused by extremely low values in two individuals.

The erythrocyte osmotic fragility expressed as the salinity causing 50% lysis of the erythrocytes did not differ according to diet, with exception of the sardine oil (-E) diet showing stronger erythrocytes than the other diets ($p < 0.05$).

The capillary blood coagulation time (seconds) increased with increasing dietary content of n-3 PUFA ($p < 0.05$), in fish given the vitamin E supplemented diets (Table 4).

Humoral immune response

Table 5 shows the serum level of specific antibodies against *V. salmonicida* (OD_{492}) in vaccinated fish. Due to individual variations, the table presents the median as well as the lower and upper quartile of the data set. A reduction in the antibody titre was found in vaccinated fish fed high dietary n-3 PUFA (+E) contents. Mean specific antibody levels of fish sampled initially and in sham-injected and untreated control fish were below OD_{492} 0.13 in all dietary groups.

Serum total protein (mean range 38.3 to 44.4 g L^{-1}) and blood Hb (mean range 8.3 - 9.0 g $100mL^{-1}$) did not vary significantly between dietary groups and treatments in the vaccination experiment. Serum total antibody level was not different between vaccinated, sham or control fish. However, combined data from all treatments (Table 5) showed higher total antibody level ($p < 0.05$) in fish fed the vitamin E supplemented diets, an opposite effect to the differences found as regards specific antibodies.

Table 4. Haematological analyses in Atlantic salmon fed diets with three lipid sources (soyabean oil, capelin oil and sardine oil) with increasing level of dietary n-3 PUFA with or without addition of vitamin E (+E or −E) (mean ± SEM; n=10).

	Soyabean oil		Capelin oil		Sardine oil	
	−E	+E	−E	+E	−E	+E
Rbc ($*10^{12}L^{-1}$)	1.16 ± 0.02^{a1}	1.13 ± 0.03^{a}	1.16 ± 0.05^{a}	1.04 ± 0.03^{a}	1.12 ± 0.02^{a}	1.15 ± 0.03^{a}
Hb (g $100mL^{-1}$)	8.9 ± 0.2^{a}	9.0 ± 0.3^{a}	8.8 ± 0.3^{a}	8.2 ± 0.5^{a}	8.4 ± 0.3^{a}	8.7 ± 0.3^{a}
Hct (%)	41 ± 1^{a}	48 ± 1^{b}	41 ± 2^{a}	40 ± 2^{a}	41 ± 1^{a}	43 ± 1^{a}
Lct (%)	0.82 ± 0.07^{a}	0.41 ± 0.08^{b}	0.81 ± 0.04^{a}	0.79 ± 0.06^{a}	0.97 ± 0.08^{a}	0.80 ± 0.03^{a}
MCV ($*10^{-15}L$)	356 ± 7^{a}	426 ± 7^{b}	356 ± 4^{a}	386 ± 9^{a}	364 ± 6^{a}	374 ± 5^{a}
MCH ($*10^{-6}g$)	77 ± 1^{a}	79 ± 1^{a}	76 ± 2^{a}	82 ± 2^{a}	75 ± 2^{a}	76 ± 1^{a}
MCHC (g $100mL^{-1}$)	21.8 ± 0.3^{a}	18.7 ± 0.3^{b}	21.4 ± 0.4^{a}	21.2 ± 0.2^{a}	20.5 ± 0.3^{a}	20.4 ± 0.4^{a}
50% haemolysis (g NaCl $100mL^{-1}$)	0.64 ± 0.02^{a}	0.65 ± 0.01^{a}	0.66 ± 0.01^{a}	0.64 ± 0.01^{a}	0.58 ± 0.01^{b}	0.64 ± 0.02^{a}
Clotting time[2] (seconds)	118 ± 6^{a}	120 ± 3^{a}	123 ± 5^{a}	131 ± 5^{ab}	118 ± 5^{a}	143 ± 5^{b}

[1] Different superscript letters indicate significant differences (p < 0.05).

[2] n=20.

Table 5. Serum specific antibodies against *Vibrio salmonicida* (median values of OD_{492} units and the 25th and 75th quartiles) and total antibodies (mm units) in Atlantic salmon fed three lipid sources with or without addition of vitamin E (+E or -E) 7 weeks post vaccination.

Feed	Specific antibody level Median (25th–75th quartiles)		Total antibody level Mean ± SEM
Soyabean oil − E	0.60[a1]	(0.44– 0.74)	11.2 ± 0.2[a]
Soyabean oil + E	0.40[b]	(0.32–0.49)	13.0 ± 0.1[b]
Capelin oil − E	0.52[a]	(0.40–0.61)	12.3 ± 0.2[c]
Capelin oil + E	0.36[b]	(0.11–0.46)	12.9 ± 0.1[bc]
Sardine oil − E	0.44[b]	(0.27–0.50)	12.5 ± 0.2[bc]
Sardine oil + E	0.06[c]	(0.03–0.09)	12.8 ± 0.1[bc]

[1]Different superscript letters indicate significant differences ($p < 0.05$) according to a non-parametric Mann-Whitney U test.

DISCUSSION

It is assumed that 10 months of feeding of the diets was sufficient for the erythrocyte fatty acid pattern and vitamin E to reach a steady state. The prominent differences between the oils used, high n-6 fatty acids in soyabean oil, high sum monoenes in capelin oil and high sum n-3 fatty acids in sardine oil were reflected in the corresponding erythrocytes but much less pronounced. Despite differences in the sum n-3 and sum n-6 and the ratio between these fatty acid series, the total amounts of membrane polyenes were nearly the same in all groups (Table 2). This is consistent with the suggestion that an optimum level of long chain fatty acids is maintained under given rearing conditions (Greene and Selivonchick, 1990).

Reduced growth, muscular dystrophy and anaemia (red blood cell lysis) caused by oxidative damage of components in the cell membranes were observed in vitamin E deficient fish (Moccia *et al.*, 1984; Cowey, 1986; Furones *et al.*, 1992; McLoughlin *et al.*, 1992). Similar to the present haematological data, Greene and Selivonchick (1990) reported on minor effects of several dietary lipid sources on haematology of rainbow trout (*Oncorhynchus mykiss*). No serious membrane instability of the red blood cells which could be attributed to the dietary n-3 content was observed, apart from an increased osmotic resistance in fish fed sardine oil high in n-3 PUFA -E. Vitamin E protects the erythrocyte membrane lipids from oxidation and thereby improves survival of the red blood cells (Cowey *et al.*, 1981). The low serum vitamin E level in this group may have caused a selection of stronger circulating erythrocytes (i.e. younger cells) following removal of cells damaged by lipid peroxidation. This may

explain the somewhat higher erythrocyte vitamin E levels among the low vitamin E groups, compared to the respective serum levels. According to van den Berg *et al.* (1991) the cell membranes are destabilized when oxidation of fatty acids causes a critical low level of intact phospholipids. In this regard, increased incorporation of n-3 PUFA and vitamin E in the cell membranes may buffer and protect the cell against oxidative damages and lysis (van den Berg *et al.*, 1991).

The requirement for n-3 PUFA in fish have been directly related to their structural role in membrane phospholipids and is suggested to be higher in cold water species (Sargent *et al.*, 1989). A decrease in saturated and monoene fatty acids and a concomitant increase in the polyunsaturated fatty acid (PUFA) fraction was observed in catfish (*Ictalurus punctatus*) peripheral blood cells (Bly *et al.*, 1986) and in cod (*Gadus morhua*) erythrocytes (Lie *et al.*, 1989) at lower water temperature. Accordingly, prophylactic or curative supplementation of n-3 PUFA in diets fed at low water temperatures have been suggested to reduce disease problems in Atlantic salmon (Salte *et al.*, 1988). Improved erythrocyte osmotic fragility was also observed in Atlantic salmon fed increasing levels of dietary n-3 PUFA (Erdal *et al.*, 1991). The mortality in Atlantic salmon, fed the same diets as in the present study, after challenge with *V. salmonicida* at two water temperatures, indicates a structural relation between dietary lipid vitamin E and water temperature (Waagbø *et al.*, 1993b). It was concluded from the rapid response in fluidity to shift in temperature as was seen in an *in vitro* study on carp erythrocytes, that fatty acids play a secondary role in maintaining membrane fluidity (Dey and Farkas, 1992). However, the roles of fatty acids in temperature adaption processes are complex and not fully understood. According to Hazel and Williams (1990) not all membrane functions are directly correlated to the membrane fluidity.

The increase in clotting time observed with increasing levels of dietary n-3 PUFA and vitamin E resemble the classical effects of marine lipids reported in humans and experimental animals, where n-3 PUFA rich diets prevent the development of cardiovascular diseases by decreasing the incidence of thrombosis (Herold and Kinsella, 1986; Weber, 1989). In a comparative study carried out by Doolittle and Surgenor (1962), the coagulation process in teleost fishes showed similar properties as in other vertebrates. Matsumoto *et al.* (1989) demonstrated prostaglandin synthesis from arachidonic acid (20:4 n-6) and EPA (20:5 n-3) in isolated marine fish thrombocytes which may relate thrombocyte aggregation to coagulation reactions in fishes. The present results confirm that dietary supplementation of n-3 PUFA could reduce the intravascular coagulation observed in Atlantic salmon suffering from cold water vibriosis («Hitra disease») (Salte *et al.*, 1987; 1988).

Overall induction of specific antibodies against *V. salmonicida* was seen
7 weeks post vaccination, except in fish fed the sardine (+E) diet.
Immunoglobulins constitute about 5-15% of the total serum protein in
adult Atlantic salmon (Ellis, 1989). Reduced levels of total serum protein
and blood Hb have been found in diseased fish (Waagbø *et al.*, 1988),
which may lead to erroneous interpretations of diverging serum constitu-
ents. Total serum protein was not affected by the present diets or vaccina-
tion. Total serum immunoglobulins levels were elevated in fish fed the
diets supplemented with vitamin E.

A similar immunosuppressive action on antibody production and
specific disease resistance against *Yersinia ruckeri* was seen in Atlantic
salmon fed diets high in n-3 PUFA (Erdal *et al.*, 1991). However,
Henderson *et al.* (1992) did not find any influence of dietary n-3 PUFA
on antibody production in rainbow trout vaccinated with *Y. ruckeri*. In
channel catfish, Sheldon and Blazer (1991) found higher humoral immune
response after immunization with formaline killed *Edwardsiella ictaluri* in
groups of fish fed menhaden oil diet than in fish fed soyabean oil and
beef tallow diets. Besides the species differences, these conflicting results
on the immunomodulatory effects of n-3 PUFA may depend on interacti-
ons with other nutrients, as demonstrated for vitamin E in the present
vaccination study. Immune functions seem to be more sensitive indicators
of vitamin E deficiency than classical signs of vitamin depletion, as
observed in several fish experiments (Blazer and Wolke, 1984; Hardie *et
al.*, 1990; Ndoye *et al.*, 1990; Blazer, 1992; Verlhac *et al.*, 1993). The
present results do not support that immunosupressive effects are caused
by lack of anti-oxidant activity. Thus, several studies have shown minor
effects of oxidized lipids on the specific immune response (Forster *et al.*,
1988; Obach and Laurencin, 1992). However, oxidized fatty acids are
considered harmful in fish feeds. This may be related to the negative
influence on lipid absorption and the interaction with other nutrients.

In contrast to Ndoye *et al.* (1990), Hardie *et al.* (1990) did not find
further positive effects in several immune parameters in Atlantic salmon
when fed 800 mg vitamin E kg^{-1} diet compared to 326 mg kg^{-1}. Recently,
Verlhac *et al.* (1993) showed positive effects of high levels (450 mg kg^{-1})
of vitamin E on mitogen induced lymphocyte proliferation and antibody
production after vaccination against *Y. ruckeri* compared to a vitamin E level
close to the requirement (45 mg kg^{-1}). However, the beneficial effect on
stimulation was time dependent, and after 120 days of feeding no differences
were observed. Despite the general higher level of total immunoglobulins in
fish fed high vitamin E levels, the vitamin E supplementations reduced the
specific immune response synergistically rather than counterweigh immuno-
suppressive effects of n-3 PUFA in the present study.

Different mechanisms for how immune cells are affected by the dietary lipids have been suggested (Adams *et al.*, 1985; Gershwin *et al.*, 1985; Johnston, 1988; Traill *et al.*, 1990). The modulation of B-cell response in mice caused by dietary PUFA seems to be related to a reduced number of specific antibody producing cells rather than the total number of splenic B-cells (Erickson *et al.*, 1986). The modulatory effect of the present dietary variables on coagulation and antibody level seem to be mediated by changes in the prostanoid synthesis. The n-3 fatty acids (mainly 20:5) is competitively inhibitory to eicosanoid synthesis from arachidonic acid and the eicosanoids formed from n-3 fatty acids are relative low-potent compared to the eicosanoids derived from arachidonic acid (Tocher and Sargent, 1987; Matsumoto *et al.*, 1989; Sargent *et al.*, 1989). Several enzymes in the eicosanoid synthesis have been suggested to be negatively modulated by vitamin E (Anonymous, 1986; 1987). Inhibition of eicosanoid synthesis by pharmaceutics reduced antibody synthesis in rainbow trout (Rainger *et al.*, 1992). The interaction between high n-3 PUFA and vitamin E observed in the present experiment fits with this hypothesis. It may be suggested that lipid modulation occurs prior to the stimulatory action of vitamin E in the sequence of lymphocyte activation (initiation).

The interaction between n-3 PUFA and vitamin E should be further studied before these components are used at high dietary levels with the aim to improve fish health.

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HEALTH ASPECTS OF DIETARY LIPID SOURCES AND VITAMIN E IN ATLANTIC SALMON (*SALMO SALAR*). II. SPLEEN AND ERYTHROCYTE PHOSPHOLIPID FATTY ACID COMPOSITION, NONSPECIFIC IMMUNITY AND DISEASE RESISTANCE.

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ABSTRACT

Six practical diets, with three levels of n-3 polyunsaturated fatty acids (PUFA) obtained by using different lipid sources (soyabean oil, capelin oil or sardine oil), each with or without supplementation of vitamin E (α-tocopherol-acetate) were fed to Atlantic salmon in two experiments.

In the first experiment young Atlantic salmon had been on the diets for nearly 12 months. The fatty acid compositions of spleen and erythrocyte phospholipids reflected the fatty acids in the diets, but all phospholipids differently. The contents of n-3 PUFA were not directly related to the content in diet. The erythrocyte phospholipids differed from the spleen phospholipids mainly by higher proportions of n-3 PUFA and lower levels of saturated and monoene fatty acids. Liver α-tocopherol levels varied depending on the supplementation of α-tocopheryl-acetate and n-3 PUFA in the feeds. Liver vitamin A levels increased with increasing dietary n-3 PUFA and vitamin E concentrations. The activity of isolated head kidney macrophages was related to *in vitro* incubation temperature. It seemed also to be related to dietary lipid showing reduced bacterial killing activity in fish fed high n-3 PUFA. Although not statistically significant, macrophage phagocytosis was also somewhat lower in the sardine oil group compared to the soyabean oil group. Minor effects were seen according to vitamin E status. Interleukin-1-like production and serum haemolytic activity were not affected by the diets.

In a second experiment using the same diets, interaction between dietary lipid, vitamin E and water temperature was apparent for disease resistance of Atlantic salmon (50 g) challenged with *Vibrio salmonicida* by injection. Fish fed sardine oil supplemented with vitamin E showed best survival at low water temperature, while fish fed the capelin oil diet with vitamin E was superior to the other groups at 13° C water temperature.

INTRODUCTION

Dietary lipids seem to modulate immune functions in humans as well as in experimental animals (Gershwin et al., 1985; Johnston, 1988; Meydani, 1991; Blazer, 1992; Hinds and Sanders, 1993). Alteration in cell membrane lipid composition caused by the diet may affect the function of immunocompetent cells by changing the physical stability of cell membranes, the chemical environment in the vicinity of receptors, or indirectly by influencing the formation of eicosanoids (Johnston, 1988).

High dietary levels of polyunsaturated fatty acids (PUFA) were shown to modulate the immune system negatively in mice and guinea pigs (Gershwin et al., 1985; Hinds and Sanders, 1993) and salmonids (Erdal et al., 1991; Waagbø et al., 1993). Dietary n-3 PUFA positively influenced immunity (Sheldon and Blazer, 1991) and disease resistance (Salte et al., 1988) in farmed fish.

The oxidation of PUFA in fish feeds with high levels of PUFA have been of great concern and according to Roberts (1989) this may cause significant problems in aquaculture. The requirement for vitamin E in salmonids was shown to increase with increased content of dietary PUFA (Watanabe et al., 1981; Waagbø et al., 1991) and at lower water temperatures (Cowey et al., 1984). High dietary supplements of vitamin E exerted positive effects on the immune system of many terrestrial vertebrates (Tengerdy et al., 1981; Sokol 1989), while vitamin E deficiency impaired immunity (Sokol, 1989). Similar findings were also reported in salmonid fishes (Hardie et al., 1990; Furones et al., 1992; Verlhac et al., 1993).

In the first experiment of this paper nonspecific immune factors were examined and discussed in relation to the fatty acid composition of spleen and erythrocyte phospholipids and the vitamin E status. Analyses of liver vitamin A levels were included as there is evidence that vitamin E interacts with vitamin A metabolism.

In the second experiment Atlantic salmon smolt, fed the same experimental diets from onset of startfeeding, were challenged with *Vibrio salmonicida* by intraperitoneal injection at two water temperatures to evaluate the effect of the diets on disease resistance.

Effects on haematology and antibody production are reported by Waagbø et al. (1993). Some preliminary data from the studies reported here were given orally at the Third International Symposium on Feeding and Nutrition in Fish, Toba, Japan, August 28th–September 1st, 1989.

MATERIALS AND METHODS

Experiment 1 – Fish and diets

Atlantic salmon were fed practical dry feeds with three lipid sources (soyabean oil, capelin oil or sardine oil) at two vitamin E levels for 14 months (March 1988–May 1989) at Matre Aquaculture Research Station (Institute of Marine Research, Bergen, Norway). The diets were fed to duplicate tanks (1.5m × 1.5m) of 100 fish in each tank during the last 9 months of the experimental period.

The indoor flow-through tanks were supplied with a mixture of filtered sea water and fresh river water adjusted to a salinity of 20 g L^{-1}. The mean monthly water temperatures in March, April and May 1989 were 7.7, 8.6 and 9.1° C, respectively. The fish were fed by the use of automatic feeders, and the amount of feed was adjusted daily according to standard tables for Atlantic salmon (Austreng *et al.*, 1987).

Feed composition including proximate and fatty acid analyses of the six test diets are presented in a previous paper (Waagbø *et al.*, 1993). The diets differed mainly in the content of n-3 PUFA (19, 35 and 60 g kg^{-1} dry feed) and vitamin E level (57 mg kg^{-1} and 272 mg kg^{-1}), representing Low, Medium and High n-3 PUFA (± E) groups.

Blood and organ sampling

The fish was anaesthetized and body weight and lenght were recorded. Blood was collected and serum and washed erythrocytes were obtained as previuosly described (Waagbø *et al.*, 1993). Liver, spleen and head kidney were dissected and weighed and hepatosomatic index (HSI) and spleen somatic index (SSI) were calculated (% of body weight). Liver, spleen, erythrocyte and serum samples were stored at − 80° C until analyses of vitamins, lipids and haemolytic activity were performed. Liver, spleen, head kidney and blood samples were collected medio March 1989. Additional head kidney samples were collected in April and May.

Due to limitations in analytical capacity, determinations of macrophage activity were performed on selected samples.

Analytical methods

Liver vitamin E and liver vitamin A analyses, and phospholipid analyses of spleen tissue and erytrocytes were carried out according to methods described by Lambertsen (1983), Lie and Lambertsen (1991) and Lie *et al.* (unpublished).

For the study of *in vitro* killing of *Aeromonas salmonicida* by macro⌐.
head kidney tissue was sampled and kept on Leibovitch-15 (L-15) med.
um. Head kidney macrophages were isolated within 12 hours after
sampling according to the method described by Braun-Nesje *et al.* (1981).
Killing of *A. salmonicida* was studied as described by Graham *et al.* (1988).
The killing assay was carried out with a pathogenic strain (Ft 1942,
A-layer possessing (A+) strain) as well as a non-pathogenic (A-layer
lacking) strain (A-) of the bacterium at different temperatures. The
analyses were run in duplicate, each with macrophages isolated from five
fish per tank (five wells per fish), and the bactericidal activity of the
macrophages (MTT-test) was analysed as described by Graham *et al.*
(1988). In the first sampling macrophages were isolated from fish in the
soyabean oil and sardine oil groups without vitamin E supplementation.
They were incubated with 100 μL of a 10^7 macrophage suspension per
well in 96-well microtitre plates. An *A. salmonicida* (Ft 1942, A+) suspen-
sion was added in the wells (10^6 bacteria well^{-1}) and incubated for 3
hours at 4, 12 and 18° C. In the second sampling head kidney macropha-
ges from the capelin oil and sardine oil groups with or without vitamin
E supplementation were isolated and tested for killing A+ and A- *A.
salmonicida* strains at 12° C (incubated with bacteria for 6 hours) and
18° C (incubated with bacteria for 3 hours). The killing index (KI) was
calculated as the ratio (MTT reduction after incubation/initial MTT
reduction).

The ingestion activity of the isolated macrophages was investigated by
incubating the cells with latex beads (4.8 μm, MP-199 crosslinked polysty-
rene, SINTEF, Norway). The cell suspension was adjusted to 2×10^6
cells mL^{-1} in L-15 medium containing 100 units mL^{-1} penicillin, 0.1
mg mL^{-1} streptomycin and 0.1% Medicult Synthetic Serum Replacement
(Medi-Cult A/S, Denmark). One mL of the suspension was seeded in
24-well culture plates supplied with 12 mm coverslips (Falcon). After 30
minutes the cells were washed three times with L-15 and incubated at
14° C for 48 hours before transferring the plates to 4° C for one hour and
then adding 10^7 latex beads per well. The plates were centrifuged for 4
minutes at 400 g to minimize distance between the cells and the beads,
before incubation for 45 minutes at 4° C. The L-15 medium was removed
and the cells fixed with 1 mL fixation solution (2.5% glutaraldehyde in
0.2 M cacodylate buffer, pH 7.2). After rinsing and staining with May-
Grünwald and Giemsa staining solutions (Merck), the preparations were
air dried and mounted on glass slides. Approximately 100–200 macropha-
ges from each well were studied by use of a microscope. The percentage
of macrophages engulfing latex beads and the mean number of latex beads
engulfed per cell were calculated.

Production of interleukin-1-like (IL-1-like) activity of isolated head kidney macrophages was analysed according to a NOB-1/HT2 IL-1 assay described by Gearing *et al.* (1987).

Serum total haemolytic activity was measured according to a conventional method for complement activity, using sheep erythrocytes (washed three times in PBS) and commercial rabbit antisera raised against sheep erythrocytes (SIFF, Norway) in a 1:2500 dilution (Kwapinski, 1965). The highest serum dilution (%) showing complete haemolysis of the sheep erythrocytes after incubation at room temperature for 30 minutes are reported.

Experiment 2 – Fish and bacterial challenge procedure

This experiment was performed on Atlantic salmon smolt (approx. 50 g) which were fed the same experimental diets as in experiment 1 (Table 1) from onset of startfeeding. After 12 months of feeding, 100 fish were randomly selected from each dietary group and moved to the challenge facilities (1.5m × 1.5m tanks). The mean water temperature in the rearing facilities at this time was 6° C. The fish (40 to 50 fish per feeding group) were acclimatized for one week at low (7° C) and high (13° C) water temperatures (salinity at 10 g L^{-1}). Each fish was then challenged by intraperitoneal injection of 0.2 mL of a 2.5×10^7 bacteria mL^{-1} suspension of *Vibrio salmonicida* (NCMB 2262). Mortality was recorded daily over a period of 20 days. Kidney imprints from dead fish on agar plates confirmed the bacterial infection. All fish in the challenge experiment were weighed.

Table 1. Mean (SEM, n=40) weights, hepatosomatic index (HSI) and spleen somatic index (SSI) in Atlantic salmon fed three dietary lipid sources with or without addition of vitamin E (+ E or − E) for 12 months.

Diet	Length cm	Weight g	HSI %	SSI %
Soyabean oil − E	37 (0)[1]	649 (24)	1.09 (0.02)[a]	0.81 (0.03)[a]
Soyabean oil + E	36 (0)	620 (23)	1.06 (0.01)[ac]	0.76 (0.03)[a]
Capelin oil − E	37 (0)	641 (17)	1.18 (0.02)[b]	0.98 (0.06)[b]
Capelin oil + E	38 (0)	664 (20)	1.21 (0.02)[b]	0.89 (0.03)[ab]
Sardine oil − E	38 (0)	687 (15)	1.02 (0.02)[c]	0.78 (0.03)[a]
Sardine oil + E	37 (0)	623 (17)	1.15 (0.02)[b]	0.88 (0.03)[ab]
ANOVA	n.s.	n.s.	p < 0.001	p < 0.003

[1] SEM < 0.5.

[2] Different superscript letters indicate statistical significant differences (p < 0.05).

Statistics

Statistical evaluation of the zootechnical data was performed using ANOVA and comparison of means by Duncan's multiple range test. Immunological data were examined by using a Kruskall-Wallis ANOVA by ranks and intergroup differences using a non-parametric Mann-Whitney U test. Correlations were evaluated by use of a Spearman correlation test. All tests were within a CSS: Statistica (StatSoft Inc., USA, 1991) statistical software.

RESULTS

Experiment 1

Fish growth was not affected by the dietary regimes (Table 1). The liver and spleen indices (HSI and SSI) varied somewhat according to diet, showing slightly higher values in fish fed capelin oil than in fish fed soyabean oil or sardine oil.

Phosphatidyl-choline (PC), -etanolamine (PE), -serine (PS) and -inositol (PI) constituted 55%, 26%, 16% and 3% of the spleen phospholipids and 67%, 17%, 8% and 8% of the erythrocyte phospholipids, irrespective of dietary regime (Tables 2 and 3). Dietary vitamin E levels did not affect the phospholipid (PL) fatty acid composition.

The most striking differences in spleen PC fatty acid composition were seen in the amounts of PUFA (Table 2). The incorporation of 18:2 n-6 in PC from the soyabean oil groups was mainly balanced by lower levels of 20:5 n-3 and 22:6 n-3. However, an increased level of desaturation and elongation products of 18:2 n-6 (20:2 n-6 and 20:3 n-6, not shown in the table) gave a further increase in sum n-6, giving the highest sum of total PUFA in the soyabean oil groups. Only minor differences in the saturated fatty acids and monoenes were found in PC, represented primarily by 16:0 and 18:1 (accounting for approximately 75% of the total PC fatty acids). The amounts of 20:5 and 22:6 n-3 were nearly the same in the capelin oil and sardine oil groups.

In general, the feed lipids were reflected in the spleen PE in a similar pattern as in PC, although the relative distribution of the fatty acids differed. The 22:6 n-3 fatty acid in spleen PE was at least twice as high as in PC in all groups. The relative amounts of saturated fatty acids in PE were considerably lower than in PC due to a reduced content of 16:0, even though the contents of 18:0 were increased. The monoenes in PE reflected more strongly than PC the feed lipids, demonstrated by the high level of long chain monoenes in the capelin oil groups.

A major PUFA in PS was 22:6 n-3. This PL resembled PC as regards

Table 2. Mean fatty acid composition (%) of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) in spleen tissue of Atlantic salmon fed sources of dietary lipid with increasing levels of n-3 PUFA (n=4 pooled samples, each of 5 fish).

Phospholipid[1]:	Soyabean oil				Capelin oil				Sardine oil			
	PC	PE	PS	PI	PC	PE	PS	PI	PC	PE	PS	PI
14:0	1.1	0.7	0.7	3.4	2.7	0.9	1.8	2.9	3.2	1.3	2.2	3.1
16:0	41.0	15.1	14.1	17.0	38.0	14.1	19.5	18.3	38.9	14.6	20.4	16.3
18:0	5.7	14.4	24.5	25.1	3.2	5.8	13.8	20.4	3.7	9.7	17.4	23.7
Sum saturated	48.4	31.0	42.5	49.4	44.9	21.7	38.2	45.4	47.4	26.8	44.2	47.5
16:1[2]	0.4	0.1	0.3	0.5	2.4	1.7	1.2	3.0	2.1	1.1	2.0	3.6
18:1[2]	20.0	8.3	5.9	11.2	19.3	7.2	5.9	11.0	19.7	6.6	6.8	9.9
20:1[2]	0.1	1.2	1.0	–	1.8	5.9	4.5	3.8	0.6	1.6	1.3	1.5
22:1[2]	–	0.2	0.3	–	0.3	3.3	2.5	3.6	–	0.7	0.6	1.0
Sum monoenes	24.1	12.3	9.9	16.5	29.8	24.4	19.5	26.7	28.9	15.2	15.8	19.8
18:2 n-6	7.9	7.6	3.9	5.2	1.3	1.9	1.8	3.4	1.0	1.7	1.8	2.6
20:4 n-6	1.7	3.6	1.4	16.4	0.8	1.4	0.8	12.9	2.0	4.1	1.6	19.1
20:5 n-3	1.9	2.2	0.7	1.9	4.1	4.2	1.4	2.8	4.1	4.4	1.8	2.5
22:6 n-3	12.4	32.4	35.7	5.4	16.3	36.5	33.8	7.1	14.5	36.0	28.7	6.9
Sum n-6	12.5	15.3	8.2	23.1	2.1	3.9	3.0	16.3	3.1	5.9	3.6	21.8
Sum n-3	14.7	35.7	37.7	7.2	21.1	42.8	37.0	10.4	19.7	43.2	32.8	10.1
n-3/n-6	1.2	2.3	4.6	0.3	10.0	11.0	12.3	0.6	6.4	7.3	9.1	0.5

[1] PC: 55%, PE: 26%, PS: 16%, PI: 3%.

[2] Sum of isomers.

69

Table 3. Mean fatty acid composition (%) of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) in erythrocytes of Atlantic salmon fed sources of dietary lipid with increasing levels of n-3 PUFA (n=4 pooled samples, each of 5 fish).

Phospholipid[1]:	Soyabean oil				Capelin oil				Sardine oil			
	PC	PE	PS	PI	PC	PE	PS	PI	PC	PE	PS	PI
14:0	1.2	0.7	1.7	0.9	1.7	0.9	2.0	1.7	1.7	0.7	1.2	1.0
16:0	31.7	9.5	19.4	8.7	30.8	10.4	21.4	11.4	34.3	10.6	20.6	10.2
18:0	6.1	10.3	24.5	38.4	3.9	7.3	18.4	30.3	4.8	9.7	23.3	35.9
Sum saturated	39.5	20.9	47.1	48.8	37.2	19.1	43.4	44.8	41.7	21.5	46.5	48.0
16:1[2]	1.2	1.0	2.7	1.6	2.0	1.3	2.8	2.8	1.7	1.4	2.5	2.0
18:1[2]	8.2	5.1	5.5	4.5	7.3	5.9	6.1	6.1	7.0	6.1	5.9	5.3
20:1[2]	0.8	1.7	1.4	0.8	2.7	7.1	4.0	2.7	0.9	2.4	1.7	1.4
22:1[2]	0.3	0.6	0.7	0.1	1.8	4.0	1.8	0.7	0.4	0.8	0.8	0.4
Sum monoenes	10.5	8.4	10.3	7.0	13.7	18.3	14.7	12.4	10.0	10.6	10.8	9.2
18:2 n-6	10.0	9.3	3.6	3.4	1.5	1.9	1.7	1.5	1.2	2.0	1.4	1.1
20:4 n-6	1.2	2.9	0.7	23.5	0.4	0.9	0.5	15.4	1.2	2.2	0.6	21.8
20:5 n-3	4.6	6.3	1.4	4.0	9.2	9.0	2.0	10.8	9.9	10.5	2.5	7.5
22:6 n-3	28.7	38.3	28.2	7.1	33.9	43.0	29.1	8.6	32.2	43.9	31.7	8.5
Sum n-6	13.2	18.1	6.6	29.8	1.9	3.2	2.3	17.1	2.4	4.2	1.9	22.9
Sum n-3	35.5	50.2	32.7	12.3	45.1	56.8	33.5	20.8	44.6	61.5	37.7	17.8
n-3/n-6	2.7	2.8	5.0	0.4	23.9	18.3	14.8	1.2	18.6	14.8	19.9	0.8

[1] PC: 67%, PE: 17%, PS: 8%, PI: 8%.

[2] Sum of isomers.

the amounts of saturated fatty acids, although a shift in the relative proportions of 16:0 and 18:0 was found. The contents of monoenes resembled the values found in PE. The fatty acid composition of PI was characterized primarily by the high level of arachidonic acid (20:4 n-6). The sum n-3 PUFA constituted only approximately 10% and was little affected by the dietary lipids. The n-3/n-6 fatty acid ratio was less than 0.6 in the PI from all groups, which is markedly less than in the other phospholipids. PI showed nearly the same level and composition of saturated fatty acids as PS.

The n-3/n-6 ratio was lower in all spleen PL's from fish fed soyabean oil than from fish fed marine oils. Despite the considerable higher dietary n-3 PUFA in the sardine oil diet, all PL fractions from the capelin oil groups showed higher n-3/n-6 ratio due to the higher 20:4 n-6 content in the sardine oil diets.

The fatty acid compositions of erythrocyte PL's (Table 3) were somewhat different from the corresponding spleen PL's, in general showing
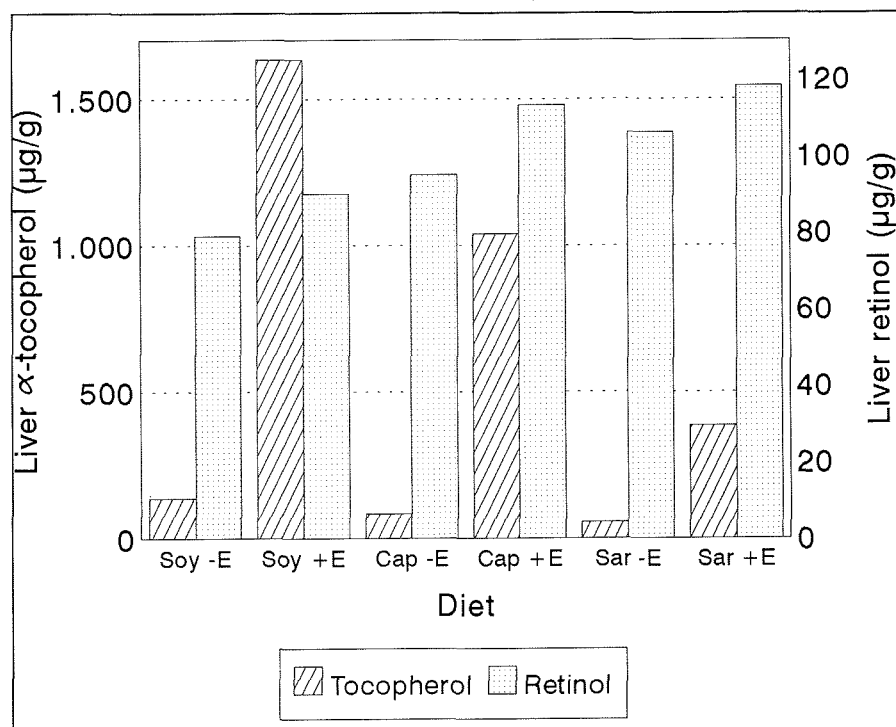

Fig. 1 Mean liver concentrations of α-tocopherol (μg g^{-1}) and retinol (μg g^{-1}) in 2 pooled samples (each of 5 fish) of Atlantic salmon fed diets with lipid sources with increasing levels of n-3 PUFA and with or without addition of vitamin E (+E or –E).

less saturated and monoene fatty acids and higher n-3 PUFA. Erythrocyte PC and PE seemed to reflect the characteristic fatty acids of the dietary lipids to a higher degree than spleen PC and PE. The n-3/n-6 ratio were higher in all erythrocyte PL's compared to spleen.

Liver vitamin E concentrations (α-tocopherol) were lower the higher the amount of dietary n-3 fatty acids (Fig. 1). This was seen both with the high and low dietary vitamin E content. Despite the same level of dietary vitamin A (ranging from 4.4 to 5.5 mg retinol kg^{-1}), for liver vitamin A concentration the opposite was true. Thus there was a negative correlation ($r=-0.920$; $n=6$) between vitamin E and vitamin A in the liver of fish fed the vitamin E supplemented diets. This correlation was closer ($r=-0.996$; $n=6$) in fish fed diets low in vitamin E.

Activities of isolated head kidney macrophages are presented in Table 4a. Comparing macrophages from fish fed soyabean oil −E and sardine oil −E a tendency towards reduced activities in the sardine oil group were seen, as regards the bacterial killing activity (at $18°$ C incubation temperature) and phagocytic activity (% active phagocytes and mean number of engulfed latex beads per phagocyte). No killing of bacteria were observed at $12°$ C (Table 4a) and $4°$ C (data not shown) incubation temperature. The IL-1-like production did not differ among the soyabean oil and sardine oil −E groups (Table 4a). Vitamin E did not affect the IL-1-like production in the soyabean oil and sardine oil groups (data not shown).

Bacterial killing of macrophages from capelin oil and sardine oil ($\pm$ E) groups showed a minor rise in activity when incubated at $18°$ C compared to $12°$ C, using an A-layer lacking *A. salmonicida* strain (Table 4b). A significantly higher killing activity of the A+ strain was seen in the capelin oil groups at $12°$ C than in the sardine oil groups, while no killing activity was detected at $18°$ C. The activity was less affected by dietary vitamin E.

The serum total haemolytic activity was not influenced by any of the dietary parameters, showing serum dilutions causing total haemolysis of sheep red blood cells in the range between 6.6% and 8.6% (range of SEM between 0.7 and 0.9, $n=20$).

Experiment 2

Fish fed the sardine oil diet with vitamin E supplementation showed lower mortality after 10 and 20 days and a prolonged lag time after challenge than all other groups in the cold water ($7°$ C) (Table 5). Challenge at $13°$ C of fish fed the capelin oil diet with vitamin E supplementation showed significantly lower mortality than the other groups at this tempe-rature (Table 5). In both challenge tests fish body weights were signifi-

cantly higher in the capelin oil and sardine oil groups without vitamin E supplementation than the other dietary groups. However, mortality was not correlated to body weights.

The erythrocyte fatty acid composition of fish used in Experiment 2 was similar to that of Experiment 1 (data not shown).

DISCUSSION

The present studies indicate that dietary lipids varying in the contents of n-3 PUFA and vitamin E affect nonspecific immunity and disease resistance in Atlantic salmon. The modes of action are not fully understood, but there seems to be complex interrelationships between effects caused by the dietary components and the water temperature.

The oils used did not affect growth and organ sizes, in accordance with results obtained in other studies (Greene and Selivonchick, 1990; Erdal et al., 1991; Sheldon and Blazer, 1991).

The fatty acid compositions of the spleen and erythrocyte PL's were influenced by the dietary lipids. This was less obvious than in nonpolar storage lipids (Waagbø et al. , 1991). Water temperature also affects the fatty acid composition of membrane phospholipids (Greene and Selivonchick, 1987; Lie et al., 1989; Farkas and Roy, 1989; Hazel and Williams, 1990). The fatty acid composition of the cell membrane is regarded as an important component in determining membrane fluidity (Bly et al., 1986; Hazel and Williams, 1990) and the degree of homeoviscous temperature adaption of immune cell membranes may be related to the function of these cells (Abruzzini et al., 1982; Bly and Clem, 1992). The incorporation of dietary fatty acids reported in the present paper and the temperature adaption involve complex exchange and restructuring of membrane lipids which may go further than achieving optimal fluidity (Hazel and Williams, 1990).

The change in PL fatty acid composition caused by diet as reported in this paper may impose temperature-like effects on immune functions. Thus reduced immunoactivity at low water temperature may be related to the observed increase in n-3 PUFA in the membrane PL's. According to Bly and Clem (1992) the temperature sensitivity is expressed early in cell activation, probably at the receptor level or trans-membrane signalling. However, whether n-3 PUFA's are selectively incorporated into the membrane PL's due to lower water temperature remains open.

Spleen contains haematopoietic and a variety of immune cells. The dietary lipids may affect the composition and activity of these differently

Table 4. Activities of isolated head kidney macrophages sampled from fish from selected dietary groups at a mean water temperature of 9° C.

A. Killing index, phagocytic activity and interleukin-1-like (IL-1-like) production of head kidneys macrophages (mean, SEM)

Diet	Killing index[1] 12° C	Killing index[1] 18° C	% active phagocytes	number of beads per macrophage	IL-1-like production
Soyabean oil − E	1.04 (0.02)[a2]	0.82 (0.06)[a]	36.1 (1.5)[a]	1.57 (0.03)[a]	2.6[a]
Sardine oil − E	1.08 (0.07)[a]	0.98 (0.07)[a]	29.3 (1.6)[a]	1.47 (0.03)[a]	3.1[a]
n	5	5	8	8	3

[1] Macrophage-bacteria incubation time was 3 hours at 12 and 18° C (*A. salmonicida* A+). Killing indexes < 1 indicate bactericidal activity.
[2] Different superscript letters indicate significant differences (p < 0.05).

B. Killing index[1] of head kidney macrophages (mean, SEM, n=5).

A. salmonicida strain	A−	A−	A+	A+
Diet	12° C	18° C	12° C	18° C
Capelin oil − E	1.00 (0.04)	0.90 (0.04)[a]	0.86 (0.03)[a2]	1.24 (0.07)
Capelin oil + E	1.01 (0.05)	0.92 (0.04)[a]	0.83 (0.04)[a]	1.13 (0.04)
Sardine oil − E	1.01 (0.06)	0.92 (0.06)[a]	0.98 (0.01)[b]	1.09 (0.04)
Sardine oil + E	1.06 (0.03)	0.89 (0.04)[a]	1.08 (0.03)[c]	1.00 (0.04)

[1] Macrophage-bacteria incubation time was 6 hours at 12° C and 3 hours at 18° C. Killing indexes < 1 indicate bactericidal activity.
[2] Different superscript letters indicate significant differences (p < 0.05).

Table 5. Mortalities and mean body weights (SEM) in groups of fish from the dietary groups after injection challenge with *Vibrio salmonicida* (5×10^6 bacteria $0.2\ mL^{-1}$ injected per fish).

	Soyabean oil		Capelin oil		Sardine oil	
	−E	+E	−E	+E	−E	+E
Challenge at 7° C						
% mortality after 10 days	87.5	75.0	65.0	33.3	2.6	0.0
% mortality after 20 days	100	100	97.5	92.8	81.5	15.0
Day of first mortality	5	6	7	7	7	17
Mean day to death	8	8	8	11	14	18
Mean body weights (g)	47.2^{ab1} (1.9)	43.7^a (1.1)	52.1^b (1.2)	49.5^{ab} (1.6)	57.7^c (1.9)	46.3^{ab} (1.3)
(n)	40	40	40	40	40	40
Challenge at 13° C						
% mortality after 10 days	60	72	48	33	70	79
% mortality after 20 days	74	90	64	45	84	85
Day of first mortality	6	6	3	7	5	5
Mean day to death	8	8	9	9	8	8
Mean body weights (g)	55.0^a (2.2)	47.8^a (1.6)	61.2^b (2.1)	53.4^a (2.1)	65.8^b (2.0)	52.5^a (1.4)
(n)	43	39	44	42	43	47

[1] Different superscript letters indicate significant differences ($p < 0.05$).

(Adams *et al.*, 1985). This was demonstrated by the differences in PL fatty acid composition of spleen and erythrocytes. The phospholipids PC and PE, which constituted the major part (approx. 80%) of the total, showed fatty acid compositions more influenced by the diet than the composition of PI and PS. In general, PUFA in tissues of marine fish and of salmonids are mainly of the n-3 series. However, 20:4 n-6 (arachidonic acid) is a major component in PI (Tocher and Sargent, 1984; Bell *et al.*, 1985; Lie *et al.* , 1989; Lie and Lambertsen, 1991). As expected, PI was least influenced by the dietary fatty acid composition in this study, showing a characteristic low n-3/n-6 ratio irrespective of dietary regime. Salmon spleen and erythrocyte PI showed low levels of 20:5 n-3 compared to the contents of 20:4 n-6 and 22:6 n-3, which is in contrast to what has been reported in cod spleen and erytrocyte PI's (Lie *et al.*, 1989; Lie and Lambertsen, 1991). A higher n-3/n-6 ratio was found in all PL's from capelin oil fed fish than in PL's from sardine oil fed fish. This was mainly due to the higher content of 20:4 n-6 fatty acid in the sardine oil. The high level of arachidonic acid in PI has been considered to be an important factor in lymphocyte stimulation (Gershwin *et al.*, 1985; Roitt, 1988; Johnston, 1988).

Macrophages from fish fed sardine oil showed reduced bacterial killing activity at 12° C incubation temperatures compared to capelin oil groups (Table 4b). Reduced activities were also found in phagocytosis (although not significant) when macrophages from sardine oil fed fish with soyabean oil fed fish were compared (Table 4a). In contrast, in channel catfish (*Ictalurus punctatus*) the bactericidal activity of macrophages was positively correlated to the dietary level of n-3 PUFA at two temperatures and after vaccination (Sheldon and Blazer, 1991). The discrepancy may be related to dietary level of n-3 PUFA, differences between cold water and warm water fish species, ambient rearing temperature and the analytical conditions. The latter may explain the lack of killing at low incubation temperatures.

Meydani (1990) discussed immunosuppression caused by dietary lipids through reduced production of cytokines. In the present study no effects were found with regard to dietary treatment on IL-1-like production from isolated macrophages, including high and low vitamin E levels (data not shown). Although there are several reports that fish host defences are regulated by a cytokine network analogous to that found in mammals, this area is still poorly understood in fish (Clem *et al.*, 1991; Hamby *et al.*, 1986). In this paper a mammalian assay system has been used which may not be optimal for measuring fish cytokines (Jørgensen, pers. comm.).

Several nutritional studies have shown that resistance to diseases is associated with high serum haemolytic activity (Durve and Lovell, 1984;

Hardie *et al.* , 1990; 1991; Verlhac *et al.*, 1993). Serum haemolytic activity was not influenced by the present diets.

Liver concentration of vitamin E varied according to the dietary content, but was also affected by the content of n-3 PUFA in the feed (Fig. 1). The corresponding liver α-tocopherol level in Atlantic salmon corresponding to the minimum requirement for vitamin E seems to be approximately 50 μg g^{-1} (Hamre and Lie, pers. comm.), thus the vitamin E supply was adequate in all groups. Interactions between hepatic contents of vitamin E, n-3 PUFA and vitamin A were found. Vitamin A was demonstrated to exert positive effects on macrophage functions (Watson and Rybski, 1988), but the experimental approach applied in the present study does not justify further interpretations on the role of vitamin A on immunity in salmon related to PUFA and vitamin E. Further studies should be carried out in this field.

No apparent positive effects of high dietary vitamin E levels were observed on the nonspecific cellular immune reactions examined. This is in accordance with results with other salmonid species (Leith *et al.*, 1989; Hardie *et al.*, 1990; Furones *et al.*, 1992). Verlhac *et al.* (1993) showed, however, a time dependent positive influence of vitamin E on cellular immunity indicating adaption to higher vitamin levels. Similarily, the effect of lipid composition on the immune cells may depend on duration of feeding (Locniskar *et al.*, 1983).

Differences in survival after bacterial infection are probably related to cell membrane structure. The structure may be affected by dietary fatty acid composition, vitamin E and water temperature. A high dietary level of n-3 PUFA gave the best protection at low water temperature. Capelin oil containing a medium level of n-3 PUFA and a high level of monoenes seems to be a preferable dietary lipid source at higher water temperatures (13° C). The relation between n-3 fatty acid levels in erythrocyte from challenged fish (data not shown) and membrane stability is not obvious. However, the importance of vitamin E is demonstrated in the lipid groups with best survival in the challenge experiments. Other experiments with salmonids have shown that low vitamin E status is associated with higher mortality rate than when the status is adequate or high (Hardie *et al.*, 1990; Furones *et al.*, 1992). Of all immune factors examined by Hardie *et al.* (1990), only complement haemolytic activity was compromised by vitamin E deficiency. The higher disease resistance found in their group of vitamin E supplemented fish may be explained by vitamin E as a stabilizing factor in the cell membranes.

Curative effect of n-3 PUFA on cold water vibriosis related to cell membrane stability was suggested by Salte *et al.* (1988). High levels of dietary n-3 PUFA may also reduce microtrombosis caused by the pro-

coagulative activity during the disease (Salte *et al.*, 1987). This aspect of n-3 PUFA was further supported by Waagbø *et al.* (1993).

In summary, the present studies suggest two possible mechanisms for how dietary lipid and vitamin E influence nonspecific immunity in Atlantic salmon. Firstly, there may be a link between cell membrane fatty acid composition and immune cell activity (membrane fluidity or eicosanoid synthesis). Secondly, the physiochemical stability of cell membranes can be affected by dietary fatty acid composition and vitamin E levels in relation to water temperature. These two aspects need to be further studied.

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