LIPID METABOLISM OF HADDOCK (*MELANOGRAMMUS AEGLEFINUS*)

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Dominic A. Nanton

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ABSTRACT

Haddock, a commercially important coldwater marine fish accumulates a high amount of dietary lipid in the liver (>60% lipid, wet weight). Several experiments were conducted to study the dietary lipid utilization by haddock, their lipid transport mechanisms and the biochemical basis of liver lipid deposition and catabolism. Juvenile haddock (6.9g) were fed three isonitrogenous diets containing 14, 16, 19 and 22% lipid to examine the effects of dietary lipid on growth, feed utilization, hepatosomatic index (HSI) and tissue lipid accumulation. Growth and feed efficiency of haddock was not significantly affected by increasing the lipid content of the diet. A significant increase in HSI (9.8-12.1%) and total liver lipid content (63.2-69.0%) was observed in haddock fed 14% versus 22% lipid, however, total muscle lipid content was unaffected. The liver fatty acid (FA) composition mirrored that of the diet. The muscle lipid (1 %) contained a high proportion of polar lipid (84% lipid) and polyunsaturated FA (53% total FA). A dietary lipid level of 14% or less is recommended for use in juvenile haddock grower diets based on the results of this study. A series of liver function tests were also performed on the plasma of cultured haddock grouped on the basis of their HSI. No significant differences were observed between the 11.1, 13.0 and 17.3% HSI groups for any of the liver function parameters tested.

Serum lipoproteins of post-absorptive juvenile haddock showed that HDL (high density lipoprotein) was the predominant lipoprotein class (1517 mg/dL) followed by LDL (low density lipoprotein; 467 mg/dL). A low level of VLDL (very low density lipoprotein; <50 mg/dL) was observed in serum. The concentration of total lipid in the serum averaged 1297 mg/dL. The phospholipid (PL), triacylglycerol (TG), cholesterol ester (CE) and cholesterol (CL) contents of serum were 57, 16, 16 and 11%, respectively. The fatty acid (FA) composition of HDL resembled that of the serum lipid and PL and it contained a higher percentage (51%) of polyunsaturated FA than VLDL (36%). High molecular weight apo B-like proteins were observed in the VLDL and LDL fractions. An apo A-I-like protein was predominant in the HDL fraction. The low level of VLDL (<50mg/dL) circulating in the serum suggests a low level of lipid (triacylglycerol) transport from the liver to the muscle in haddock.

The catabolism of a radiolabelled fatty acid, [1-C^{14}] palmitoyl-CoA, through peroxisomal and mitochondrial ß-oxidation was determined in the liver, red and white muscle of juvenile haddock fed 12, 18 and 24% lipid in the diet. No significant increase in the mitochondrial or peroxisomal ß-oxidation activities in these tissues were observed as the dietary lipid level increased from 12 to 24%. Peroxisomes accounted for 100% of the ß-oxidation observed in the liver, whereas mitochondrial ß-oxidation dominated in the red (91%) and white muscle (97%) of juvenile haddock. Of the tissues tested, red muscle possessed the highest specific activity for ß-oxidation expressed on a per mg protein or per g wet weight. However, these results suggest that white muscle, which forms over 50% of the body mass in gadoid fish, is the most important tissue in juvenile haddock for overall fatty acid catabolism.

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1. INTRODUCTION

1.1 General Introduction

In the past two decades, Atlantic Canada has developed a successful finfish aquaculture industry producing more than 15,000 metric tons of Atlantic salmon, steelhead trout and Arctic char annually (Olin, 2001). An increase in salmon production from Chile and Norway and recent outbreaks of viral and parasitic diseases have reduced the profit margin within this industry. As a result, there is currently much interest in the development of alternate new cold water species for aquaculture (Henry, 1997).

Haddock (*Melanogrammus aeglefinus*) has been identified by the provincial governments of New Brunswick and Nova Scotia as well as the Department of Fisheries and Oceans as a potential species for marine aquaculture (Henry, 1997). Haddock is a round fish like salmon, has relatively fast growth and adapts well to sea cages designed for Atlantic salmon, making it an ideal fish to complement Atlantic salmon.

Nutrition and feeding of haddock have been identified as key areas for research and development (Lall and Nanton, 2002; Henry, 1997). It is important that diets developed for fish culture are nutritionally and economically sound. Feed constitutes the largest single cost item (>50%) in intensive fish production (Lovell, 2002). Unfortunately, information on the nutrient requirements of this species is very limited and the current research approach is to rely on the quantitative nutrient requirement data available for salmonids and other marine fish as well as the feeding techniques used for the closely related gadoid species, cod (*Gadus morhua*).

Gadoid fish store the major portion of the available dietary energy as lipid in the liver (Lie *et al.*, 1986). Recent data obtained on a commercial farm (C. Frantsi, Heritage Salmon, Blacks Harbour, NB; personal communication) show that the hepatosomatic index (HSI; liver to body weight ratio) of haddock may exceed 20% when the diets contain more than 15% lipid. Cultured haddock with fatty livers convert dietary energy into flesh less efficiently and may be more susceptible to stress. This abnormally high liver lipid deposition in cultured haddock is a major constraint for the commercial culture of haddock juveniles to market size.

1.2 Nutrition of Gadoid Fish

1.2.1 Natural Prey

The principal prey for first-feeding gadoid larvae are the nauplii and copepodites of calanoid copepods, particularly *Pseudocalanus* spp. (Last, 1978; McLaren and Avendano, 1995). Marine copepods accumulate large reserves of free amino acids to help maintain their osmotic balance (Fyhn, 1993). At the first-feeding stage, the digestive system (stomach) of cod is not fully functional (Pederson and Falk-Peterson, 1992; Hjelmeland *et al.*, 1993). Thus, copepods provide gadoid larvae with a high proportion of amino acids in the more digestible free form. Copepods may also assist in the digestion process by contributing digestive enzymes, zymogens (enzyme activators) or gut neuropeptides, which stimulate digestion (Kolkovski, 2001). The main prey species of gadoid larvae, *Pseudocalanus* sp., contains high amounts of two highly unsaturated fatty acids (HUFA) in phospholipid that are considered essential for marine fish larvae,

docohexaenoic acid (DHA; 32%) and eicosapentaenoic acid (EPA; 22%) (Fraser *et al.*, 1989).

The natural diet for juvenile or mature haddock (27g – 5.3 kg) and cod (64g – 13.8 kg) on the Scotian Shelf of Atlantic Canada is different. Fish composed 45% of total weight in the stomach of cod (FSRS, 2000a) compared to only 8% in haddock (FSRS, 2000b). Capelin is the most important fish species in cod diet (Pearcy *et al.*, 1978; DeBlois and Rose, 1996). Echindoderms (mainly ophiuroideans) composed the largest proportion (38%) of the stomach contents in haddock (FSRS, 2000b) compared to only 4% in cod (FSRS, 2000a). The whole body lipid composition of capelin (mature, full stomach) ranged from 10 to 35% dry wt. (MacCallum *et al.*, 1969), whereas in ophiuroideans (mature, full stomach) the whole body lipid composition was less than 2% dry wt. (Pomory and Lawrence, 1999). The higher proportion of fish (capelin) in cod diet suggests that their natural diet has a higher energy (lipid) content than haddock diet.

In cod, there was a shift in the prey composition with size. Small cod (<25 cm total length) mainly consumed krill (73-94% of total weight in stomach) and large cod (27-72 cm) mainly capelin or shrimp (*Pandalus borealis*) (Pearcy *et al.*, 1979). The balance of the adult diet in haddock consists mainly of the same food ingested by the smaller haddock (O'Boyle, 1985).

1.2.2 Culture

In early 1980's, much work was directed to develop cod farming in Norway (Holm *et al.*, 1991). Although progress was made in the hatchery and nursery systems for the culture of cod, it did not result in any significant production (Adoff *et al.*, 2002).

Recently the interest in farming cod has been rejuvenated due to both a reduction in supply from the wild fishery and a general increase in demand for white fish species (Adoff *et al.*, 2002; Henry, 1997). Currently, the production of cod in Norway, Canada and the U.K. is a modest 400 tonnes (Maeland, 2001). Haddock has been given priority as an alternative culture species to salmon in Atlantic Canada because of its perceived market potential, proximity of major markets (Boston, New York area) and the decline in the wild fishery (Henry, 1997). Haddock, as an aquaculture species, has been currently described as "precommercial" (Frantsi *et al.*, 2002).

There are four principal methods for the production of cultured cod. These are: 1) ongrowing wild-caught juveniles, 2) extensive system of feeding fry on natural plankton in ponds/ enclosures, 3) semi-intensive methods producing fry onshore or in bags in natural enclosures feeding on natural and cultured plankton, and 4) intensive culture systems where water temperature and the composition of feed may be controlled (Maeland, 2001). The predictability of production and product quality provided by intensive systems, similar to those used for sea bass/ bream, will be necessary for the successful large-scale commercial culture of cod and haddock.

Large sexually mature cod have a lower optimal temperature for growth than smaller cod (50-1000 g). The optimal temperature for growth of large cod may lie within the range 9-12°C, which is lower than the 11-15°C reported for optimal growth of smaller cod (Jobling, 1988; Pederson and Jobling, 1989). Lower growth at lower water temperatures was primarily due to the reduced feeding frequency and feed intake. On a daily basis cod consumed 87, 77 and 54% less food at 8, 4 and 1°C, respectively. After

24 h at the lower water temperatures, the amount of food in the stomach was probably not sufficiently evacuated to cause the return of appetite (Waiwood *et al.*, 1991). The evacuation rate of food in the stomach of cod increased rapidly with an increase in temperature from 2 to 15°C (Tyler, 1970). Feeding stimulants and palatability of the feed for cod is of particular importance at low water temperatures. The food rejection rate increased with decreasing temperature for cod fed dry feeds while it did not change for cod fed capelin. Cod which were fed capelin grew significantly more than those given formulated dry feed. With declining temperatures the activity levels decreased and time before first handling of feed was increased. (Clark *et al.*, 1995).

Lambert *et al.* (1994) observed higher growth rates in cod reared at an intermediate salinity (14 ppt) than either low (7 ppt) or high (28 ppt) salinities during the fall and at lower salinities (7 and 14 ppt) in spring. Higher growth rates at lower salinities resulted from an increase in feed conversion efficiency rather than an increase in feed intake. Increased growth may result from rearing cod at lower salinities in estuaries or land-based systems supplied with brackish water compared to those cultured in 34 ppt. seawater (Lambert *et al.*, 1994).

The following hatchery rearing procedure has been successfully used for the production of haddock fry for commercial culture and experimental purposes at the National Research Council's (NRC) Aquaculture Research Station, Sandy Cove, Nova Scotia, Canada. The buoyant fertilized eggs are collected from the surface of the broodstock tanks containing naturally spawning haddock. These eggs are transferred to incubation tanks (300 L conical black) and held in the dark at ca. 7°C for 14 d. The

newly-hatched haddock larvae are then transferred to the larger larval tanks (3500 L) and cultured at ca. 11°C under a 12:12 h (light: dark) photoperiod. The tank water may be greened with a DHA-rich algae but this is not always necessary. The first-feeding larvae are fed algae-enriched rotifers from 0 to 7 d post-hatch (PH), enriched (commercial product high in DHA) rotifers from 7 to 25 d PH and enriched (commercial product high in DHA) *Artemia* from 25 to 37 d PH. At ca. 37 to 40 d PH, the haddock are weaned from the live food onto a formulated diet. This is achieved by gradually dropping the *Artemia* concentration in the tank to zero, while cofeeding the formulated dry diet over a four day period. Cannibalism can be a major source of mortality during this weaning period. The weaned haddock juveniles are then fed a commercially produced marine fish diet (high protein, low lipid) developed at NRC. Juvenile haddock (3-5 g) are transferred to sea cages at Deer Island in the Bay of Fundy, New Brunswick. These fish are grown to market size (2 to 2.5 kg) in approximately 3 years (Frantsi *et al.*, 2002).

1.2.3 Body and Tissue Composition

Cod and haddock eggs contain approximately 1% lipid and a high amount of water (92%) (Craik and Harvey, 1987). Phospholipid composed 71% of the total lipid in gadoid eggs and phosphatidylcholine is the major lipid class (46% total lipid). DHA comprised 28% and EPA 14% of the fatty acids in the phospholipid of gadoid eggs (Tocher and Sargent, 1984). The free amino acid content of cod eggs was 3.52% (Craik and Harvey, 1987). Vitamin C concentration was 23 ug/g (wet wt.) in the eggs of broodstock fed diet containing 500 mg vitamin C/kg. This is comparable with the level in eggs from wild cod (Mangor-Jensen *et al.*, 1994). The roe from wild cod contained 13

μg/g niacin, 7 μg/g riboflavin, 30 μg/g pantothenic acid and 0.10 μg/g vitamin B_{12} on wet weight basis (Braekkan, 1958).

The endogenous feeding of cod larvae was predominantly fuelled by free amino acids (67%) and lipid (32%) while glycogen accounted for only 1% of the total enthalpy dissipated (Finn *et al.*, 1995a). Over 85% of the free amino acids are catabolized for energy from spawning through the yolk-sac stage in cod (Fyhn and Sergistad, 1987). The lipid content expressed on a dry matter basis of an unfed cod larva was 21% at hatch, 20.5% starved for one week post hatch and 14% starved for two weeks post hatch (Finn *et al.*, 1995b). Phopshatidylcholine was the only lipid class to decline (36%) from fertilization to hatch (Fraser *et al.*, 1988). However, a decrease in both phosphatidylcholine (1.41 μg) and triacylglycerol (0.75 μg) was observed in individual cod for this period (Finn *et al.*, 1995b).

The average lipid content in gutted cod larvae of approximately 111 μg dry weight, feeding primarily on calanoid copepod nauplii, was ca. 27 μg per individual (24% lipid, dry wt.). In first-feeding cod larvae, polar lipid classes dominated but there is a trend towards increasing neutral lipid with time. Polar lipid composed 63-69% of the total lipid in first feeding cod larvae. The phospholipid in first-feeding larvae contained high levels of the EFA, DHA (29%) and EPA (16%), which was similar to that observed in the eggs (Pedersen *et al.*, 1989).

Wild-caught juvenile cod (ave. 605 g) had a whole body proximate composition averaging 5% lipid wet wt., 2% ash wet wt., 16% protein wet wt. and 77% moisture (Holdway and Beamish, 1984). The whole body protein and free amino acid composition

Table I. Whole body protein and free amino acid composition of juvenile haddock (*Melanogrammus aeglefinus*; n=5). Unpublished data from Nanton & Lall (1998).

Amino acid	Haddock PAA (g/100g dry wt.)	Haddock FAA (g/100 g dry wt.)	Haddock PAA (g/100g AA)	Salmon PAA[a] (g/100g AA)
taurine	0.63 ± 0.04	0.892 ± 0.056	-	-
aspartic acid	4.89 ± 0.07	0.054 ± 0.015	10.10	10.10
threonine	2.08 ± 0.05	0.064 ± 0.019	4.20	5.04
serine	1.80 ± 0.08	0.064 ± 0.008	3.39	4.70
glutamic acid	7.13 ± 0.14	0.118 ± 0.017	14.58	14.58
proline	2.28 ± 0.07	0.000 ± 0.000	5.02	4.73
glycine	3.32 ± 0.16	0.094 ± 0.018	7.20	7.55
alanine	3.03 ± 0.10	0.129 ± 0.013	7.04	6.64
cysteine	nd	0.004 ± 0.002	-	-
valine	2.62 ± 0.06	0.037 ± 0.008	5.66	5.18
methionine	1.36 ± 0.13	0.026 ± 0.007	2.80	1.86
isoleucine	2.36 ± 0.05	0.024 ± 0.006	5.12	4.49
leucine	3.89 ± 0.04	0.055 ± 0.015	8.41	7.86
tyrosine	0.90 ± 0.05	0.029 ± 0.010	1.79	3.56
phenylalanine	2.26 ± 0.01	0.027 ± 0.010	4.59	4.44
histidine	1.35 ± 0.05	0.040 ± 0.011	2.88	3.08
lysine	4.54 ± 0.07	0.089 ± 0.028	9.90	9.45
arginine	3.37 ± 0.07	0.058 ± 0.020	7.31	6.73
% EAA	49.92 ± 0.48	22.97 ± 5.17	-	-
Total	47.74 ± 0.63	1.80 ± 0.12	100.00[b]	100.00[b]

[a]Data for Atlantic salmon from Wilson and Cowey (1985).
[b]Total does not include taurine, tryptophan and cysteine.
Abbreviations: PAA=protein amino acids; FAA=free amino acids; EAA=essential amino acids; nd=not determined.

was measured in cultured juvenile haddock (Nanton and Lall, 1998; unpublished data; Table I). Haddock and Atlantic salmon have relatively similar patterns of amino acid composition. Wilson and Cowey (1985) demonstrated that total amino acid composition was similar for a variety of fish species. The amino acids which showed the greatest differences in a comparison of mean values for haddock and Atlantic salmon were methionine (1.0 g/100g AA higher in haddock) and tyrosine (1.7 g/100g AA higher in salmon). This may reflect basic differences in the amino acid requirements of these fish species. The free amino acid composition of the haddock was dominated by taurine (ca. 50% FAA). In adult fishes, taurine has roles in cell volume regulation and bile acid formation (van Waarde, 1988). It is unknown if this amino acid is essential for growth and development in marine fish.

In cultured cod the liver contributes 12 to 17% of total body weight or HSI (Lie *et al.*, 1986), whereas in wild cod 2 to 7% HSI are common (Hemre *et al.*, 1993a). The liver is the major energy storage organ in cod. The liver of juvenile cod (5.5% HSI) contained 8% protein wet wt., 59% lipid wet wt., 0.8 % ash wet wt. and 33% moisture (Holdway and Beamish, 1984). Depending on the condition of wild cod, lipid levels in the liver may range from 15 to 67% (Jangaard *et al.*, 1967; Love *et al.*, 1974). The total liver lipid content of cultured cod fed formulated diets to satiation exceeded 70% (Lie *et al.*, 1986; Jobling *et al.*, 1991; Shahidi and Dunajski, 1994) and triacylglycerol constituted the major fraction of the lipid (>90% lipid). The phospholipid concentration is relatively low (0 to 4%) in cod liver lipid compared to salmonids and other marine fish (DosSantos *et al.*, 1993). Phosphatidylcholine constitutes the major phospholipid

component (54% of the phospholipid) of the liver (Lie and Lambertsen, 1991). The fatty acid composition of the diet has a strong influence on the fatty acid composition of the liver lipid (Lie *et al.*, 1986). The liver lipids of both wild and cultured cod contain a high proportion of monounsaturated fatty acids (>40%), including the long-chain 20:1n-9 and 22:1n-11, which were supplied in high amounts from their natural prey or herring/capelin-based formulated feed (Table II; Dos Santos *et al.* 1993; Shahidi and Dunajski, 1994).

During starvation liver lipid and protein are utilized for energy purposes. The HSI of cod decreased from 14.3 to 8.6 during a nine-week starvation period (Hemre *et al.*, 1993). A salt-resistant triacylglycerol lipase enzyme in the liver of cod was five times more active than the same enzyme in rainbow trout liver (Black *et al.*, 1983). Sargent *et al.* (1989) suggested that salt-resistant lipase may hydrolyze triacylglycerol and mobilize the comparatively larger liver lipid stores in cod. Liver protein may also decline during the starvation. Cod (average weight,1 kg) starved for 107 days showed a decrease in liver protein and DNA level from 145 to 81g and 23 to 13 mg, respectively, which indicates a decrease in the number of liver cells in these fish (Black and Love, 1986).

The natural food organisms consumed by cod contain relatively low amount of glycogen or carbohydrate. Hemre *et al.* (1993b) found that an increase in dietary carbohydrate level from 0.5 to either 10 or 21% caused an increase in liver glycogen level from 5 to 10%, respectively. The liver glycogen content of these fish decreased between 2.8 to 3.8% after four weeks of starvation (Hemre *et al.*, 1993b). An additional 4 weeks of starvation had no significant effect on liver glycogen content (Hemre *et al.*, 1993a).

Table II. Lipid classes (wt.%) and fatty acids (wt.%) of cod muscle and liver (Ackman, 1995).

Fatty Acid	Muscle Total	Muscle PC	Muscle PE	Muscle PS	Muscle CE	Muscle TAG	Liver TAG
% lipid	-	60.0	16.4	4.3	2.2	4.1	-
14:0	1.3	1.4	0.5	0.5	1.5	4.4	3.2
16:0	15.3	23.9	8.8	8.0	8.4	14.1	13.5
16:1	2.6	4.2	1.7	1.4	4.6	12.0	9.8
18:0	2.8	2.6	4.4	18.9	1.6	2.6	2.7
18:1	13.0	12.6	14.4	14.4	10.0	22.4	23.7
18:2n-6	0.3	0.9	0.8	0.7	0.7	0.9	1.4
18:3n-3	1.0	0.2	0.3	0.2	0.4	0.2	0.6
18:4n-3	1.1	0.3	0.3	-	0.2	1.2	0.9
20:1	3.2	1.7	4.0	5.6	3.9	12.1	7.4
20:4n-6	-	2.4	1.6	2.9	1.4	0.5	1.6
20:5n-3	21.2	19.4	10.7	7.5	27.5	7.3	11.1
22:1	-	0.4	0.3	0.5	3.6	11.4	5.1
22:5n-3	2.0	1.6	1.9	1.7	2.2	0.7	1.7
22:6n-3	32.2	27.3	40.5	35.6	30.7	7.4	12.6
Σ SAT	19.4	27.9	13.7	27.4	11.5	21.1	19.4
Σ MONO	18.8	18.9	20.4	21.9	22.1	57.9	46.0
Σ PUFA	57.8	52.1	56.1	48.6	63.1	18.2	29.9

Abbreviations: Total=total lipid, PC=phosphatidylcholine, PE=phosphatidylethanolamine, PS=phosphatidylserine, CE=cholesterol ester, TAG=triacylglycerol, SAT=saturates, MONO=monounsaturates, PUFA=polyunsaturates

Fat soluble vitamins are widely distributed in marine fish liver oils (Lall and Parazo, 1995). The medicinal use of cod liver oil for humans was common long before the discovery of vitamins. In the early 19th century the use of cod liver oil in treating rickets and night blindness was demonstrated (Chabre, 1936). The lipid soluble vitamins (A, D and E) are present at a much higher concentration in the liver compared with the muscle of cod (Table III). The vitamin A and D concentrations in the liver oil increase with age or size in cod. An increase in the lipid content of the liver was accompanied by a decrease in the concentration of lipid-soluble vitamins in the liver oil of cod (Pugsley *et al.*, 1945; O'Keefe and Ackman, 1987). Vitamin E concentration was correlated with the vitamin A and D values in cod liver (O'Keefe and Ackman, 1987). The water soluble vitamins are not deposited to any great extent in the liver of cod (Table III). There was a general decrease of the liver vitamin B concentrations in larger, mature cod (Braekkan, 1958). Cod fed various formulated diets had much lower concentrations of the trace elements in the liver than did salmonids (Table IV). It appears that the concentrations of these trace elements in the liver of cod are independent of the diet and that cod liver is not a storage organ for these elements (Table IV; Lie *et al.*, 1989a).

Gadoids are considered lean, white fish with a fillet consisting mainly of white muscle (Love, 1980). The dark muscle (brown lateral layer) in haddock composes about 8% of the muscle in the tail section, but only about 1% of the middle section (Fraser *et al.*, 1961). The proximate composition of the fillet in wild cod includes 0.7% lipid wet wt., 17% protein wet wt., 1% ash wet wt. and 82% moisture. The lipid increased slightly to 0.9% in the fillet of cod intensively fed 18-20 weeks on formulated diets (Shahidi and

Table III. Vitamin composition of the liver and muscle in cod and haddock.

Vitamins	cod muscle	haddock muscle	cod liver	haddock liver
Fat-soluble vitamins				
Vitamin A (IU 100 g^{-1})	25	50	41,815	-
Vitamin D (IU 100 g^{-1})	-	-	2,301	-
Vitamin E (mg 100 g^{-1})	0.19 (0.15-0.24)	-	15	-
Water-soluble vitamins				
Riboflavin (mg 100 g^{-1})	0.08 (0.02-0.16)	0.10 (0.02-0.16)	0.51 (0.34-0.84)	0.34
Pantothenic acid (mg/ 100 g^{-1})	0.17 (0.08-0.31)	0.25 (0.04-0.29)	0.64 (0.51-0.78)	0.48
Niacin (mg 100 g^{-1})	2 (1.5-2.3)	4 (3.1-4.4)	3 (2.3-3.6)	2
Vitamin B_{12} (μg 100 g^{-1})	1.0 (0.2-1.1)	1.5 (0.5-2.0)	0.013	0.007
Biotin (μg 100 g^{-1})	2.6	2.6 (0.2-4.8)	-	-
Folates (μg 100 g^{-1})	5.0 (1.8-6.7)	0.8	-	-
Thiamin (mg 100 g^{-1})	0.07 (0.05-0.18)	0.07 (0.03-0.10)	-	-
Pyridoxine (mg 100 g^{-1})	0.17 (0.12-0.28)	0.12	-	-

Values in parentheses represent the range of values reported in the literature.
Adapted from Lall and Parazo (1995). Other sources were O'Keefe and Ackman (1987) and Braekkan (1958).

Table IV. Mineral composition of the muscle and liver in cod and the muscle in haddock.

Mineral	cod muscle	haddock muscle	cod liver
Macrominerals			
Calcium (mg $100g^{-1}$)	16 (19-20)	19 (10-30)	-
Magnesium (mg $100g^{-1}$)	23 (20-25)	-	-
Phosphorus (mg $100g^{-1}$)	210 (9-240)	208 (164-320)	-
Potassium (mg $100g^{-1}$)	360 (270-460)	340 (299-434)	-
Sodium (mg $100g^{-1}$)	80 (60-180)	57 (49-67)	-
Microminerals			
Copper (mg kg^{-1})	3.0 (0.2-5.5)	2.0 (0.1-2.4)	2.4 (0.5-6.1)
Iodine (mg kg^{-1})	1.0 (0.2-5.0)	3.0 (0.3-0.6)	-
Iron (mg kg^{-1})	9.0 (3.4-42.9)	8.0 (3.5-14.0)	15 (2.3-49)
Manganese (mg kg^{-1})	0.2 (0-0.5)	0.2	-
Zinc (mg kg^{-1})	32.0 (9.0-52.5)	8.0 (2.7-17.4)	17 (6-37)
Selenium (mg kg^{-1})	0.4 (0.2-0.9)	0.3 (0.2-0.6)	0.55 (<0.02-0.6)
Chromium (mg kg^{-1})	0.1	0.1 (0-0.3)	-
Molybdenum (mg kg^{-1})	-	0.2	-
Fluorine (mg kg^{-1})	3.0 (0.7-7.0)	5.0	-

Values in parentheses represent the range of values reported in the literature.
Adapted from Lall (1995). Source for cod liver micromineral composition was Lie *et al.* (1989).

Dunajski, 1994). Most of the lipid in the whole muscle of cod is membrane or phospholipid (73-78% lipid; Dos Santos *et al.*, 1993). Phosphatidylcholine comprises 60%, or more, of the phospholipid in the muscle of cod (Table II; Lie and Lambertsen, 1991). The muscle lipid of wild and cultured cod contains a high proportion of polyunsaturated fatty acids (ca. 50%), particularly EPA (17-21%) and DHA (25-35%) (Table II; Dos Santos *et al.*, 1993; Shahidi and Dunajski, 1994). In terms of specific muscle tissue, the dark muscle was highest in lipid (1.74-2.14% wet wt.) followed in decreasing order by the white muscle (0.55-0.78% wet wt.) and the connective tissue, myocommata (0.41-0.64% wet wt.) (Love *et al.*, 1975). The tail section of the cod fillet, which has a higher proportion of dark muscle (Fraser *et al.*, 1961), was higher in lipid (1.13% wet wt.) than the middle (0.93% wet wt.) or head sections (0.96% wet wt.) (Dambergs, 1963). In general, pelagic continuously swimming fish contain a higher amount of dark muscle (Love, 1970) and muscle lipid (up to 17% wet wt. in salmonids; Bell, 1998) than the less active demersal gadoids. The white muscle in the whole body musculature is used for quick acceleration, whereas the dark muscle near the tail is more aerobic and used for cruising with lipid as a fuel (Love, 1980). Glycogen levels in the muscle of cod fed diets containing 0.5, 10 and 21% carbohydrate were similar (0.3 – 0.4% wet wt.). There was no significant decrease in muscle glycogen after a four week fast (Hemre *et al.*, 1993b). Glycogen levels in the dark and white muscles of rested cod were similar, but decreased only in the more anaerobic white muscle during exercise (Fraser *et al.*, 1966). In cod, the filaments of actin and myosin comprise about 75% of the total protein in the muscle (Bailey, 1954). A highly significant negative correlation

exists between muscle protein and muscle water concentration in cod (Love, 1970). In starved cod, the timing and level of an increase in fillet water depends on the initial liver energy reserves (HSI) in the prefasting fish (Love *et al.*, 1980; Hemre *et al.*, 1993a). The muscle protein concentration in starved cod rose to a maximum (with protein mobilized from liver; Love, 1980) after three to four weeks of fasting, followed by a steady decline below initial values after six weeks of fasting (Robertson *et al.*, 1967). Cod starved for four weeks had a total weight loss of 12-17%. The percent of the weight loss contributed by the muscle was 81-87% (Hemre *et al.*, 1993b). In comparison with the liver, the muscle of cod contained a much lower concentration of lipid-soluble vitamins (A and E; Table III) However, the range of water-soluble vitamin concentrations (Table III) and microminerals (Table IV) in the liver and muscle of cod were similar.

Free fatty acids averaged 1542 μmole/L plasma in wild adult haddock and 1283 μmole/L plasma in wild adult cod. The plasma of adult gadoid fish contained a higher concentration of free fatty acids, in comparison with other fish species. In gadoids, a large transport of free fatty acids from the main lipid stores in the liver may be needed to provide the very lean muscle with enough free fatty acids to catabolize for energy (Larsson and Fange, 1977). The effects of temperature on various blood parameters were examined in cod. At 12 °C, the average red blood cell count was 1.1 X 10^{12}/L, hematocrit was 28% and haemaglobin was 5.25 g/100 mL. These parameters were significantly lower at 8°C than at 12 or 16°C. This was probably an adaptation of the cod to its increased metabolic rate and the reduced oxygen tension in the water at higher temperatures. The proportion of polyunsaturated fatty acids increased in the erythrocyte

phospholipid classes as water temperature decreased (Lie *et al.*, 1989). The free amino acid levels peaked at ca. 12 h in the plasma of juvenile cod fed whole sand eels at a temperature of 8-11°C. In general, the appearance of absorbed amino acids in the hepatic portal blood reflected that of the food. The exceptions were the nonessential amino acids, asparagine and aspartic acid, which were plentiful in the food but poorly represented in the hepatic portal blood (Lyndon *et al.*, 1993). Plasma glucose peaked 4 hours post-feeding (5.5-6.3 mmol/L) in juvenile cod fed a moist diet containing 10 or 21% carbohydrate dry wt. There was a significant decline in plasma glucose levels (2.9-3.5 mmol/L) measured 4 days post feeding (Hemre *et al.*, 1993b).

1.2.4 Nutrient Requirements

In order to formulate the haddock diet for either research or commercial use, basic information is required on the quantitative nutrient requirements, the organoleptic properties of feed in relation to acceptability and the ability to utilize major nutrients from potential feed ingredients or feed supplements. Nutrient requirements of haddock are virtually unknown and there is limited information in other areas related to nutrition and feeding. Haddock, like other fish species and terrestrial animals, have dietary requirements for energy, protein and amino acids, lipids, minerals and vitamins for growth, reproduction and health. Deficiencies of these substances can reduce growth, produce deficiency signs and make fish susceptible to pathogens.

Protein and amino acids

The natural diet of haddock and other marine fish is rich in protein. The protein requirement of most marine fish species ranges between 50-60% of the diet (NRC, 1993).

Recently the requirement for juvenile haddock has been estimated to be 53.8% dry wt. (Kim and Lall, 2001). The protein requirement in gadoids (cod) is high because its retention is low (26-29%; Lie *et al.*, 1988) compared to other species such as rainbow trout (35%; Reinitz *et al.*, 1978) or carp (50%; Murai *et al.*, 1984).

Fish do not require protein per se, but require essential amino acids. Ingested proteins are hydrolyzed by proteolytic enzymes in the gastrointestinal tract to release peptides and amino acids and distributed to different tissues to synthesize appropriate proteins or to be catabolized for energy. These amino acids contribute to the maintenance and growth of fish. It has been shown that most fish cannot synthesize the following amino acids at a sufficient rate to meet the requirement for growth: arginine, lysine, tryptophan, methionine, leucine, isoleucine, histidine, phenylalanine, valine and threonine. The quantitative amino acid requirements of haddock remains to be established.

A supply of free amino acids in the diet of cultured gadoid larvae similar to the high concentration and pattern found in their major prey item, calanoid copepods (*Pseudocalanus* sp.; Jeffries and Alzara, 1970) has been recommended. A high level of amino acids in the more available free form was required by the first-feeding gadoid larvae because their digestive system (stomach) was not fully functional (Pederson and Falk-Peterson, 1992; Hjelmeland *et al.*, 1993). A large amount of free amino acids was required for both protein synthesis and oxidation as a fuel to provide the larvae with energy needed for the anabolic processes during early development (Ronnestad, 1999). Free amino acids also act as a feed attractant for marine fish larvae (Knutsen, 1992).

Artemia is currently used as a live food for culturing gadoid larvae (Maeland, 2001). The free amino acid concentration in unenriched *Artemia* nauplii was lower than that observed in wild zooplankton (45 vs. 116 umol/g wet wt.; Naess *et al.*, 1995). *Artemia* nauplii as a diet for marine fish larvae are thought to be deficient in free methionine (Conceicao *et al.*, 1997). However, it is possible to enrich *Artemia* experimentally with free amino acids (Tonheim *et al.*, 2000).

Lipid

Dietary lipid supplies both energy and essential fatty acid (EFA) in fish diets. Most vertebrates, including fish cannot synthesize the polyunsaturated fatty acids 18:2n-6 and 18:3n-3 *de novo* and either one or both of these fatty acids are required in the diet (NRC, 1993). Most freshwater species can desaturate and elongate 18:2n-6 to 20:4n-6 and 18:3n-3 to 20:5n-3 and 22:6n-3 (Henderson, 1996; Fig. 1). However, marine fish have an absolute dietary requirement for these highly unsaturated fatty acids (20:4n-6, 20:5n-3 and 22:6n-3). Studies tracing the fate of radiolabelled fatty acids in cultured cell lines indicated that underlying this requirement in marine fish was either a deficiency in fatty acyl delta-5 desaturase or C18-20 elongase activity. A deficiency in the desaturation/elongation pathway was clearly identified at the level of the delta-5 desaturase in gilthead seabream (Tocher and Ghioni, 1999). However, low C18-20 elongase and high delta-5 desaturase activity was observed in turbot cells (Tocher *et al.*, 1989). Most fish have a preponderance of 20:5n-3 and 22:6n-3 in the fatty acids of their cell membrane phospholipids, which is reflected in their high requirement for these fatty acids. Fish larvae, particularly of marine species, have a particularly high requirement for

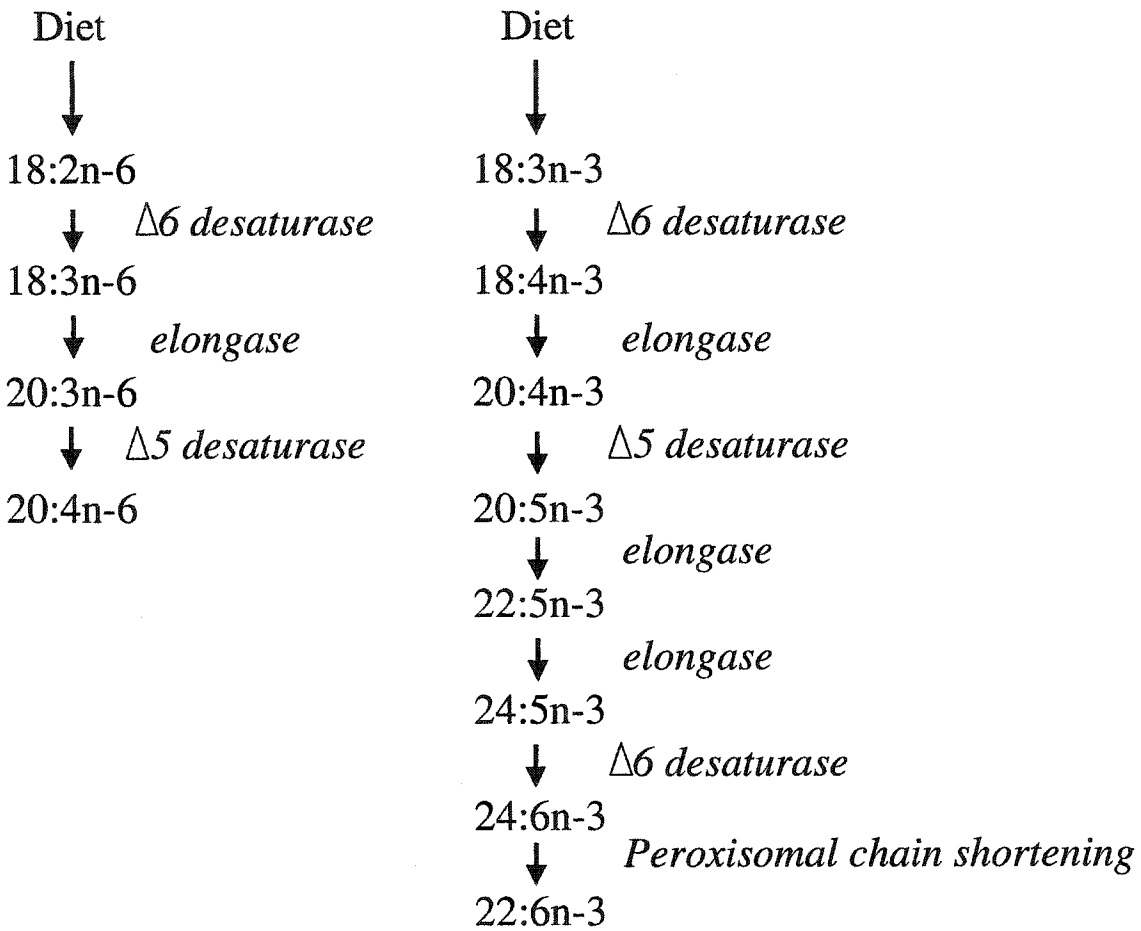

Fig. 1. Postulated pathways of polyunsaturated fatty acid conversions involving Δ5 and Δ6 desaturases. Modified from Henderson (1996).

22:6n-3. This fatty acid is required for the proper development and functioning of neural and retinal cell membranes. Another essential fatty acid, 20:4n-6, is the major precursor for production of the biologically active eicosanoid compounds. Eicosanoids are involved in "stress reactions" ranging from blood clotting to inflammatory reactions and the immune response (Sargent *et al.*, 1995).

All fish generally display reduced growth and poor feed efficiency when fed essential fatty acid deficient diets. Other signs of essential fatty acid deficiency in fish may include swollen pale or fatty liver, fin erosion, degeneration of gill epithelium, shock syndrome, swimming disorders, poor reproductive performance, high mortality etc. (Tacon, 1996).

Lipid levels in cod or gadoids diets should be kept low to reduce fat accumulation in the liver. The HSI of cod was positively correlated with dietary lipid levels (Jobling *et al.*, 1991). A dietary lipid level of about 9% (dry wt.) or less was necessary to produce HSI in juvenile haddock of 7%, similar to that of wild gadoids (Kim and Lall, 2000). In this study, haddock were fed isonitrogenous diets containing 14 to 22% lipid. A dietary lipid level of 14% or less was recommended for juvenile haddock. Growth did not decrease significantly, whereas the HSI and liver lipid were comparatively lower in haddock fed 14% lipid (Nanton *et al.*, 2001). The highly unsaturated fatty acids DHA, EPA (NRC, 1993) and to a lesser extent arachidonic acid (Castell *et al.*, 1994) are required in the diet of marine fish. Based on the published literature (Tucker, 1998; NRC, 1993, n-3 highly unsaturated fatty acid content of 2%, including at least 1% EPA plus 1% DHA may be a reasonable estimate for cold-water marine fish such as cod. Lie

et al. (1986) reported that diets containing either peanut, cod liver or Greenland halibut oil (making up 48% of the available energy) were well accepted by juvenile cod. However, the peanut oil diet gave slightly lower feed consumption, feed conversion and growth rates. This was probably due to a deficiency in the essential fatty acids. It is obvious that for optimal performance, vegetable oils should be supplemented with marine fish oils in the diet of juvenile cod and other gadoids to meet essential fatty acid requirements.

There is little information on the lipid requirements of gadoid broodstock. Lie *et al.* (1993a) investigated the effects of diets containing 90 g per kg of soybean oil, capelin oil or sardine oil in cod for 24 months prior to maturation. The level of essential fatty acids for marine fish, EPA and DHA, were higher in both the liver and eggs of cod broodstock fed the marine fish oils compared with fish fed soybean oil. However, there were no differences in egg production, mean fertilization or egg size between the groups. The high amount of DHA in gadoid eggs, larvae and natural prey suggests a large requirement for DHA in their initial diet.

Larvae of Pacific cod (*Gadus macrocephalus*) fed enriched *Artemia* which were highest in DHA (1.6 –2.1%) showed the best growth performance (Zheng *et al.*, 1996). The occurrence of dropsy was reduced in these cod larvae when the DHA content of their rotifer prey was increased (Zheng *et al.*, 1997). The concentration of EPA markedly decreased in both the neutral and polar lipids of unfed cod larvae 15 days post hatch, whereas the level of DHA remained constant (Zheng, *et al.*, 1995). This conservation of

DHA over EPA demonstrates the importance of DHA for normal growth and development of gadoid larvae.

It was also demonstrated that vitamin E should be presented as free tocopherol rather than tocopherol acetate (the major form in fish feed; NRC, 1993) in the live food of Pacific cod larvae for maximum absorption (Zheng *et al.*, 1997). Thus, vitamin E in the free form is necessary to protect the highly unsaturated DHA against oxidation in both the enriched live prey and cod larvae.

Phospholipids are indispensable for sustaining growth and survival of fish larvae (Kanazawa, 1993). Phospholipid acts as a feed attractant increasing ingestion rates, as an emulsifier in the digestive tract of the fish larvae aiding digestion, and is used for lipoprotein (lipid transport) and membrane synthesis during rapid growth of the larvae (Kanazawa *et al.*, 1985; Koven *et al.*, 1993). The effect of phospholipid in enhancing dietary fatty acid incorporation decreases with the age of the larvae (Koven *et al.*, 1993). Diets containing phospholipid from marine fish eggs high in assimilable EFA (DHA:EPA ratio of ca. 2:1) are superior to soybean phospholipid for marine fish larvae, but not markedly so (Kanazawa *et al.*, 1985; Sargent *et al.*, 1997). Large vaccuoles were observed in the enterocytes of cod larvae fed experimental weaning diets. This blockage of lipid transport (lipoprotein synthesis) out of the intestine suggests a phospholipid deficiency (Baskerville-Bridges and Kling, 2000). A phospholipid-rich diet containing a DHA:EPA ratio of ca. 2:1 along with arachidonic acid (this fatty acid is a major prostaglandin precursor), may be optimal for marine fish larvae (Sargent *et al.*, 1997).

Gadoid eggs are rich in phospholipid with a DHA:EPA ratio of 2:1 (Tocher and Sargent, 1984) and free amino acids (Craik and Harvey, 1987). Therefore, gadoid eggs/roe have been utilised as a major dietary component to meet the nutritional requirements for gadoid larvae (Molvik *et al.*, 1984; Garatun-Tjeldsto *et al.*, 1987; Ringo *et al.*, 1991). Although not reared through metamorphosis due to high mortality, cod larvae initially fed formulated diets containing cod roe had higher growth rates than those fed rotifers (Ringo *et al.*, 1991) and other artificial diets (Molvik *et al.*, 1984; Garatun-Tjeldsto *et al.*, 1987). The stomach and pyloric caeca develop comparatively late in cod starting at about 15 mm larval length (Pederson and Falk-Peterson, 1992). A sharp increase in the stomach protoleolytic enzyme, pepsin, was observed at this stage of development (Hjelmeland *et al.*, 1993). This late development of the digestive system may explain the low survival in cod larvae initially fed on artificial diets.

Carbohydrates

Haddock and cod are carnivorous marine fish and their natural prey mainly provides protein and fat (Jobling, 1982). Increased digestible carbohydrate in the diet (0 to 35% dry wt.) did not reduce protein ingestion in cod, suggesting an inefficient utilization of carbohydrate as an energy source to spare protein. There was a linear decrease (40 to 26%) in carbohydrate digestibility, as the digestible carbohydrate content of the diet increased (11 to 35% dry wt.). This indicates that cod have a limited ability to digest starch (Hemre *et al.*, 1989). There was a 96 h delay before basal levels were reached after an intraperitoneal injection of diluted glucose, suggesting poor utilization of absorbed glucose in cod (Hemre *et al.*, 1997). The inclusion of carbohydrate at the levels

employed in commercial feeds may lead to negative effects in cod, such as a lower ability to withstand handling stress (Hemre *et al.*, 1991). Practical limits for good quality, relatively digestible carbohydrates are in the range of 10-20% for strict carnivores such as cod (Tucker, 1998). Chitin (a homopolymer of N-acetylglucosamine) is a major component in the exoskeleton of crustaceans and is highly digestible in cod (90%; Danulat, 1987). Cod contain a high level of chitinase activity in the stomach and intestine (Danulat and Kausch, 1984). Thus, chitin may prove to be superior to other forms of carbohydrate in the diet of cod.

The utilization of dietary carbohydrate by broodstock cod has been investigated to a limited extent by Hemre et al (1995). Maturing cod were fed increasing levels, from 5 to 28%, of starch in a dry pellet. The level of starch did not influence feed conversion, growth or gonadal development. Glycogen, protein and lipid did not vary in the eggs of broodstock fed different levels of starch. Very low glycogen levels were observed in the eggs (0.3-0.4 mg/g wet wt.), demonstrating its insignificance as an energy reserve in the eggs of gadoid fish.

Energy

Dietary lipid, carbohydrate and protein serve as sources of energy for fish. It is generally accepted that cultured marine fish rely to a large extent on lipid and protein rather than on carbohydrate for energy. Whether protein calories are used for catabolic or anabolic purposes depend on the availability of fat and carbohydrate calories to spare the protein for tissue production and on the quality of protein for fish. Available energy values of feedstuffs for fish have been determined on a digestible energy (DE) and

metabolizable energy (ME) basis (Cho *et al.*, 1982; Cho and Kaushik, 1985). ME is a more exact measure of the energy value for a complete diet that becomes available for the metabolism by the animal. However, it is difficult to measure quantitatively the urinary and branchial ammonia excretions to determine the ME measurement in fish. For practical feed formulations, the digestible energy data of conventional feed ingredients is considered adequate.

Lipid is a more energy-dense nutrient (9.44 kcal/g) in comparison with protein (5.64 kcal/g) and carbohydrate (4.11 kcal/g). Lipids are the major non-protein energy source for fish. Dietary carbohydrates, particularly for cold-water marine species, are poorly utilized in fish and are often ignored. It is very important to use an optimal protein to lipid ratio in fish diets. Protein is the most costly of the energy-yielding nutrients. A diet deficient in lipid will cause expensive dietary protein to be catabolised for energy. A diet containing excessive lipid will reduce feed consumption, thus decreasing the intake of protein as well as other essential nutrients (NRC, 1993). High fat diets containing 34 to 41% lipid are being used for salmon culture to minimize protein catabolism and increase growth performance.

Although several studies have been conducted to determine the requirements of energy yielding nutrients, lipid, protein and carbohydrate for cod (Lied and Braaten, 1984; Holdway and Beamish, 1984; Lie *et al.*, 1988; Dos Santos and Jobling, 1988; Hemre *et al.*, 1989, Foster *et al.*, 1993; Hemre and Kahrs, 1997), the energy metabolisms of gadoids and marine fish have not been fully investigated. Most studies have been directed to utilization of natural food organisms and the bioenergetics of wild fish

(Jobling *et al.*, 1991). Lie *et al.* (1988) found that optimal growth of cod may be achieved by formulating a diet that supplies available energy concentrations of 60%, 25% and 15% from protein, fat and carbohydrate, respectively. Jobling *et al.* (1991) observed the greatest weight gain of large cod fed diets containing protein-energy to be from total energy ratios of 0.4-0.45. Gadoids store a large amount of lipid in their liver (Love, 1980). The increase in liver lipid and liver weight is closely associated with the amount of energy supplied by the dietary lipid in cod (Lie *et al.*, 1986, 1988; Jobling *et al.*, 1991; Grant *et al.*, 1998). Thus, the amount of energy in the form of lipid should be minimized in the diet of gadoids.

Unlike fish species that retain a higher amount of lipid in their muscle (salmonids store up to 17% lipid; Bell *et al.*, 1998), cod and haddock muscle contains less than 1% lipid (Shahidi and Dunajski, 1994; Nanton *et al.*, 2001). Therefore the potential of using lipid as a source of energy in gadoid diets is limited. A recent study conducted in our laboratory showed that diets containing high levels of protein (55%) and low levels of lipid (10%) produced optimal growth and feed utilization in juvenile haddock (Tibbetts *et al.*, 2001). In juvenile cod, an extruded diet containing 48% protein and 16% lipid appeared to provide the best compromise between growth, feed utilization and cost (Morais *et al.*, 2001). Protein is preferentially used as an energy source over lipid (retention 50-60%) in cod (Lie *et al.*, 1988).

Vitamins and minerals

The quantitative vitamin and mineral requirements of marine fish including cod and haddock have not been investigated (NRC, 1993), but the distributions of water and

fat-soluble vitamins (Table III), as well as minerals (Table IV) in their tissues have been reported.

Based on mortality, coagulation and haematology measurements, it was suggested that juvenile cod have a low dietary requirement for vitamin K (<0.2 mg/kg diet). Growth (1.7g initial weight) may have been reduced in cod fed higher levels of vitamin K in the form of menadione sodium bisulphite (21.5 compared with 9.8 and 6.5 mg/kg diet) (Grahl-Madsen and Lie, 1997).

At least part of the requirement of cod and other marine fish for certain minerals such as calcium, cobalt, iron, magnesium, potassium, sodium, zinc and others can be obtained directly from the seawater (Lall, 2002). Juvenile haddock require 9.6 mg phosphorus/g of diet for optimum growth, feed utilization and bone mineralization and their requirement is much higher than salmonids. Deficiency symptoms in haddock include reduced growth, vertebrae ash, vertebrae phosphorus, vertebrae calcium, serum phosphate and increased whole body lipid (Roy and Lall, 2002).

Although the importance of broodstock nutrition in gonadal development, egg quality and the survival of larvae is widely recognized, few studies have been conducted to define the role of vitamins and trace elements in reproduction of gadoids. Mangor-Jensen *et al.* (1994) studied the effects of different vitamin C levels (0, 50 and 500 mg/kg L-ascorbic acid equivalents) in cod broodstock diets for three months prior to maturation. Growth, gonad and liver sizes were not affected by vitamin C levels. There was a significantly higher level of vitamin C incorporation in the eggs of cod broodstock fed the highest level of vitamin C (500 mg/kg), which is comparable with levels in wild cod (23

μg/g wet wt.). The egg strength of cod fed 500 mg vitamin C /kg diet was also significantly higher than those fed lower amount of ascorbic acid (0 and 50 mg/kg). However, there were no significant effects of dietary vitamin C on egg production, fertilization rate or survival rate of offspring.

1.2.5 Fatty Liver Research

In contrast with salmonids, the main energy storage organ in gadoids (haddock and cod) is the liver and the lipid content of the muscle rarely exceeds 1.0% of the wet weight (Ackman, 1967; Dos Santos *et al.*, 1993). Lipid levels in the liver of cod fed formulated diets can exceed 70% wet weight (Jobling *et al.*, 1991; Shahidi and Dunajski, 1994). Farmed cod are generally fed to satiation so it is expected that they would accumulate fairly large liver lipid reserves. However, the HSI of farmed cod fed formulated diets exceeded 12% which was at least twice the level found in wild fish (Eliassen and Vahl, 1982; Shahidi and Dunajski, 1994). The HSI of cod was directly related to dietary lipid level and intake (Lie *et al.*, 1988; Jobling *et al.*, 1991). The HSI has also been positively correlated with growth rate in wild (Holdway and Beamish, 1984) and cultured cod (Dos Santos *et al.*, 1993). A high retention (60%) of dietary lipids was found in the liver of cod fed formulated feeds containing different oils which made up 48% of the available energy. However, the retention of fatty acids in the liver was generally non-specific (Lie *et al.*, 1986). During starvation the major part of the lipid in cod (initial HSI of 8.6 or 14.3) was utilized within nine weeks of starvation (Hemre *et al.*, 1993a).

About 26 to 29% of the dietary protein compared with 50 to 60% of dietary lipid is retained in cod, which demonstrates the preferred use of protein over lipid as an energy source. Increasing the protein energy to total energy ratio in the diet of juvenile cod from 0.41 to 0.75 reduced the HSI from 11.9 to 7.3%. (Lie *et al.*, 1988). An increase in the protein level from 45 to 65% in isoenergetic diets significantly decreased the HSI of juvenile haddock from 10.7 to 7.3% (Kim and Lall, 2001).

Formulated feeds, when compared to whole natural prey, were emptied more rapidly from the stomach in juvenile cod. This resulted in an overloading of the intestinal digestive capacity and decreased digestibility of the formulated diets (Dos Santos and Jobling, 1988). It was hypothesized that this intestinal nutrient overload in excess of immediate requirement may lead to fat accumulation in the liver of gadoids fed formulated diets (Jobling, 1988). Interestingly, cod fed a mixed diet of coarsely chopped herring four days per week and a formulated wet diet three days per week had a high accumulation of lipid in the liver (>70%), similar to livers in those cod fed seven days per week on moist diets. However growth, feed utilization efficiency and protein efficiency ratio were highest in cod fed the chopped herring and wet feed versus moist diets (Jobling *et al.*, 1991).

Due to this high level of energy (lipid) storage in the liver of gadoid fish fed formulated diets, less frequent feeding may improve feed utilization and reduce fatty liver. Maeland (2001) suggested feeding cod three times per week is better than feeding six times per week or one day per week. However, cod juveniles fed (ration size ca. 8% body weight) on alternate days compared to daily feeding had reduced growth rates

(Foster *et al.*, 1992). Feeding cod juveniles ad libitum once every three days compared to once a day had no effect on feed conversion whereas weight gain was reduced. The HSI and liver lipid levels were reduced in cod fed once every three days compared to once a day (Lie *et al.*, 1988). Growth rate has been positively correlated with HSI in gadoids (Dos Santos *et al.*, 1993). Thus, attempting to reduce the abnormally high HSI or liver lipid storage levels by reducing feeding frequency can negatively affect growth in gadoids fed formulated diets.

The histological features of the liver in wild fish may differ considerably from those of cultured fish. Cultured juvenile red drum hepatocytes contained more lipid and were several times larger (hypertrophic) than those of wild red drum (Tucker *et al.*, 1997). The livers from farm-raised red drum had higher levels of cellular lipid which was correlated with higher levels of lipid peroxides and a reduction in cytochrome P-450 enzyme activity (Flood *et al.*, 1996).

The development of fatty livers has been associated with specific nutrient deficiencies (choline, inositol, phosphorus and essential fatty acids) and toxicities (oxidized fish oil) in fish (Tacon, 1992). Choline, incorporated as phosphatidylcholine, forms the outer monolayer of the very low density lipoprotein particle (VLDL), which is a major transporter of lipid (triacylglycerol) out of the liver in the plasma of animals (Zeisel and Blusztajn, 1994). Liver lipid was significantly reduced when dietary choline was supplied at or above requirement in channel catfish (Wilson and Poe, 1988), lake trout (Ketola, 1976) and hybrid striped bass (Griffin *et al.*, 1994). However, the liver lipid level was not significantly reduced with increased dietary choline in rainbow trout

(Rumsey, 1991) and increased dietary choline or lecithin (phosphatidylcholine) in yellow perch (Twibell and Brown, 2000a). Dietary factors such as methionine, betaine and folic acid are involved in methyl group donation or transfer for the synthesis of the trimethyl-amino group in choline. Supplementation of these nutrients can reduce the dietary choline requirement in animals (Zeisel and Blusztajn, 1994). An increase in the amount of methionine supplemented to a choline deficient diet significantly reduced the liver lipid in channel catfish (Wilson and Poe, 1988). Inositol may be another lipotropic nutrient for fish (Steffens, 1989). Inositol deficiency caused a numerical increase in liver lipid and significantly decreased plasma triacylglycerol in Atlantic salmon (Waagbo *et al.*, 1998).

Red sea bream fed a phosphorus deficient diet (0.2%) had increased liver lipid and decreased serum triacylglycerol levels. However, the amount of lipid deposited in the liver of red sea bream is relatively low (14% lipid in fish fed a phosphorus deficient diet) compared with gadoids. This suggests that lipoprotein (phospholipid) synthesis and release in the liver was also impaired by phosphorus deficiency (Sakamoto and Yone, 1980). Essential fatty acid deficiency in red sea bream was associated with increased deposition of lipid in the liver. The liver lipid level increased when the diet was deficient in either EPA or DHA (Takeuchi *et al.* 1990). In fish, these essential fatty acids were concentrated in the lipoprotein membranes (Lee and Puppione, 1972; Lie *et al.*, 1993) and it appeared that a minimum level in the diet may be required for their synthesis.

Alpha-tocopherol fed to rainbow trout in excess (1%), significantly increased the total lipid content of rainbow trout liver. Excess dietary alpha-tocopherol promoted lipid

accumulation in the form of lipid droplets in the liver. However, liver function was not impaired (Tokuda and Takeuchi, 1995). Channel catfish fed diets containing oxidized fish oil without supplemetation of the antioxidants, alpha-tocopherol (25 mg/kg) or ethoxyquin (100 mg/kg), had a significantly higher level of liver lipid. The diets without supplemented antioxidants had higher peroxide and thiobarbituric acid (TBA) levels and lower levels of polyunsaturated fatty acid levels at the end of the 16 wk feeding trial (Murai and Andrews, 1974). This increase in liver lipid due to peroxidation of fatty acids in fish may be due to a deficiency in essential fatty acids. Juvenile red sea bream fed essential fatty acid (EPA and DHA) deficient diets had higher hepatosomatic indices and liver lipid contents (Takeuchi *et al.* 1990). Ceroid deposition has also been observed in the liver of fish fed oxidized fish oil (Tacon, 1992). Ceroid deposition may occur when the lipid oxidation product, malondialdehyde, crosslinks with the amine groups in protein and phospholipids (Brody, 1994).

Carnitine has been added to experimental fish diets to reduce tissue lipid deposition and improve growth. Carnitine is required for the transport of long-chain fatty acids into the mitochondria, which is the site of ß-oxidation. Carnitine increases the fatty acid oxidation activity in fish (Bilinski and Jonas, 1970; Ji *et al.*, 1996). The effects of carnitine supplementation on the accumulation of lipid in fish is variable (Twibell and Brown, 2000b). For example, dietary carnitine reduced the lipid content of the liver and muscle of tilapia (Jayaprakas *et al.*, 1996), muscle, liver and plasma of sea bass (Santulli and D'Amelio, 1986), the muscle of Atlantic salmon (Ji *et al.*, 1996) and the liver and muscle of channel catfish (Burtle and Liu, 1994). However, carnitine supplementation

did not reduce tissue lipid concentrations in striped bass (Twibell and Brown, 2000b; Gaylord and Gatlin, 2000), rainbow trout (Rodehutscord, 1995) or red sea bream (Chatzifotis *et al.*, 1995). Carnitine supplementation may only reduce lipid deposition in fish, when the diet is deficient in carnitine or its precursor amino acids, lysine and methionine (Gaylord and Gatlin, 2000).

Dietary medium chain triacylglycerols (MCT) have been investigated for their potential to reduce lipid deposition and increase nutrient digestibility and nitrogen retention in fish (Nordrum *et al.*, 2000). In mammals, so-called medium chain fatty acids (6 to 12 carbon chain length) are highly absorbable, enter the mitochondria independent of the carnitine transport system, are preferentially oxidized and can reduce tissue lipid deposition (Papamandjaris *et al.*, 1998). Supplementation of MCT in fish diets can have a negative effect. Reduced growth was observed in red drum (Craig and Gatlin, 1995; Davis *et al.*, 1999) and Nile tilapia fed MCT (Takeuchi *et al.*, 1983), as well as channel catfish fed short or medium chain fatty acids (Stickney and Andrews, 1972). However, supplementation of dietary MCT (addition of 2-6% MCT to commercial diet) did not reduce growth, although tissue lipid accumulation was suppressed in ayu (Nematipour *et al.*, 1989; Nakagawa and Kimura, 1991; Mustafa *et al.*, 1991). In Atlantic salmon fed a restricted ration of diets containing 0 compared with 10% MCT, fat retention was reduced from 58 to 40% and nitrogen retention increased from 52 to 66% (Nordrum *et al.*, 2000). In a similar study, the muscle lipid was reduced from 6.1 to 4.4% wet wt., but liver lipid (ca. 4.5%) was not significantly different in Atlantic salmon fed 0 versus 10% MCT. Triacylglycerol levels tended to increase (54 vs. 41%; not significant) and ß-

oxidation activity of palmitic acid decreased in the liver of these Atlantic salmon fed MCT. It was hypothesized that the MCT were broken down and the fatty acids reesterified into basically longer chain triacylglycerols in the liver, thus inhibiting ß-oxidation (carnitine palmitoyl transferase I) through the production of malonyl coA (Rosjo *et al.*, 2000). This possible resynthesis of longer chain triacylglycerols in the liver of fish fed MCT would work against reducing liver lipid accumulation.

Dietary supplementation of conjugated linoleic acid reduced the accumulation of adipose tissue in mice, rats, pigs and humans. CLA are a group of polyunsaturated fatty acids that exist as positional and stereoisomers of 18:2. CLA are typically found in beef, lamb and dairy foods (Belury, 2002). The potential of dietary CLA for reducing lipid accumulation in gadoids or other fish species remains to be investigated.

1.3 Lipid Metabolism in Fish

1.3.1 Lipid Absorption

In many teleost fish species, the main site of lipid digestion is in the anterior intestine where the pyloric cecae are located. Some lipid digestion occurs in the lower intestine and rectum, particularly in strict carnivores with short digestive tracts (Koven *et al.*, 1997). Lipid when administered alone or in a mixture routinely gives digestibility values of 85 to 95% for fish (NRC, 1993). In rainbow trout, the digestibility of individual fatty acids decreased with increasing chain length up to C18 and then increased with further increases in chain length to C22; digestion of unsaturated fatty acids was greater than their saturated counterparts (Austreng, 1980). Recent studies suggest that the major digestive lipase in teleosts is bile salt-dependent and nonspecific,

while the 1,3 specific lipase is at lower levels or nonexistent (Lie and Lambertsen, 1985; Koven *et al.*, 1997). The process of lipid digestion yields free fatty acids, fatty alcohols from wax esters (marine species), glycerol, 2-mono-acylglycerol, sterols and lysophospholipids (Higgs and Dong, 2000). These products of lipid digestion are absorbed across the brush border of the anterior intestine. The majority of glycerols, monoacylglycerols and lysophospholipids are then re-esterified with free fatty acids in the enterocyte to form triacylglycerols and phospholipids, respectively (Leger, 1985).

1.3.2 Lipid Transport

The inter-organ transport of lipids in fish is similar to that of mammals (Sheridan, 1988; Fig. 2). Part of the free fatty acids absorbed in the intestinal mucosa are released as albumin complexes directly into the portal blood without esterification (Robinson and Mead, 1973; Sargent *et al.*, 1989). However, a majority of the free fatty acids are reconstituted into triacylglycerol and released from the intestinal mucosa as chylomicrons and very low density lipoprotein (VLDL) particles (Sire *et al.*, 1981). These lipoprotein particles are transported to the liver via the lymphatic system and ultimately the blood (Leger, 1985). Uptake of lipid from the lipoproteins in the blood occurs through the lipoprotein lipase enzyme, which has been observed in the liver, muscle and adipose tissue of fish (Sheridan, 1988). In the liver, exogenous lipid removed from the circulation are repackaged together with endogenously synthesized lipid and transported to extra-hepatic tissues in the form of VLDL. The liver also actively modifies this lipid through desaturation and elongation enzymes located in the microsomes (Greene and

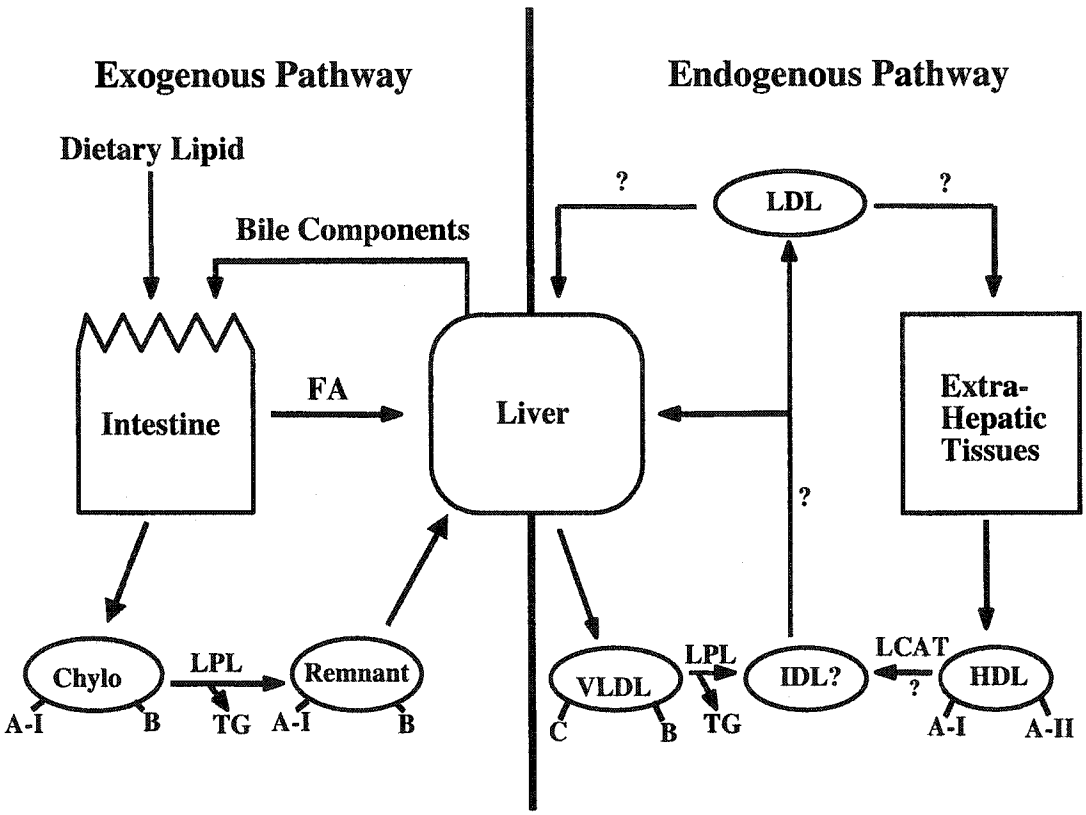

Fig. 2. Exogenous and endogenous lipid transport pathways in fish (Sheridan, 1988). Abbreviations: Chylo=chylomicrons; FA= fatty acid; VLDL=very low density lipoprotein; IDL=intermediate density lipoprotein; LDL=low density lipoprotein; HDL=high density lipoprotein; LCAT=lecithin cholesterol acyltransferase; LPL=lipoprotein lipase; TG=triacylglycerol. Apolipoproteins A-I, A-II, B and C are shown associated with the lipoproteins.

Selivonchick, 1987). Triacylglycerols in the VLDL of fish plasma appear to be deposited in a similar manner to mammals, i.e., hydrolysis via lipoprotein lipase to free fatty acids and glycerol followed by absorption and re-esterification within the cell (Sargent *et al.*, 1989).

High density lipoprotein (HDL) is the major lipoprotein class in the plasma of most teleost fish species (Chapman, 1980; Babin and Vernier, 1989). HDL is responsible for reverse cholesterol transport or movement of cholesterol from extrahepatic tissues to the liver (Sheridan, 1988). Although HDL was the dominant lipoprotein class, as a general trend, fish species which store higher amounts of lipid in the muscle tended to have a higher level of VLDL triacylglycerol in the plasma (Ando and Mori, 1993).

1.3.3 Lipid Synthesis

The pathways of lipid biosynthesis in fish are similar to those of other vertebrates. Enzyme studies have clearly demonstrated the presence and functionality of enzymes such as acetyl coA carboxylase, fatty acid synthetase, glycerol-3-phosphate acyltransferase and CDP-choline-1,2-diglyceride choline phosphotransferase in various fish species. The liver is the main site of fatty acid synthesis in fish, and unlike mammals comparatively little takes place in the adipose tissue (Greene and Selivonchick, 1987; Sargent *et al.*, 1989). High fat diets have been shown to reduce the activity of lipogenic enzymes in Atlantic salmon (Arnesen *et al.*, 1993) and seabass (Dias *et al.*, 1998). High carbohydrate diets stimulated enzymes for fatty acid synthesis in channel catfish (Likimani and Wilson, 1982) and sturgeon (Fynn-Aikins *et al.*, 1992). Glucose-6-phosphate dehydrogenase, malic enzyme and acetyl coA carboxylase were the key

regulatory enzymes in the lipogenic pathway in seabass. Glucose-6-phosphate dehydrogenase was the main NADPH generating enzyme (Dias *et al.*, 1998). For inhibition of fatty acid synthesis in fish, a dietary lipid level above 10% is needed (Henderson and Sargent, 1981; Brauge *et al.*, 1995). Several weeks are required to obtain a dietary response of lipogenic enzyme activities in fish (Lin *et al.*, 1977).

1.3.4 Lipid Catabolism

On a per mg protein basis, the red muscle and heart of rainbow trout (Bilinski and Jonas, 1970) and mackerel (Fjermestad *et al.*, 2000) had a greater ability to oxidize fatty acids than the white muscle or liver. Oxidation of palmitoyl-L-carnitine was also higher in red muscle compared to the liver or white muscle in Atlantic salmon, when expressed on a per g wet tissue basis (Froyland *et al.*, 1998; Froyland *et al.*, 2000).

There are two subcellular systems for the ß-oxidation of fatty acids in both mammals (Osmundsen *et al.*, 1991) and fish (Froyland *et al.*, 2000), one located in the mitochondria and the other in the peroxisomes. The tricarboxylic acid cycle and electron transport chain contained in the mitochondrial matrix can further catabolize the acetyl CoA units produced through mitochondrial or peroxisomal ß-oxidation (Figs. 3 and 4). In the mitochondria of rainbow trout muscle, fatty acids were oxidized to carbon dioxide and this oxidation was greatly stimulated by coenzyme A and carnitine. This observation suggested that fatty acid oxidation in fish mitochondria occurs via the ß-oxidation pathway observed in mammals (Bilinski, 1970). Carnitine palmitoyltransferase acts as a carrier for acyl groups into the mitochondrial matrix where ß-oxidation occurs. As in mammals, carnitine palmitoyltransferase-I activity was inhibited by malonyl CoA

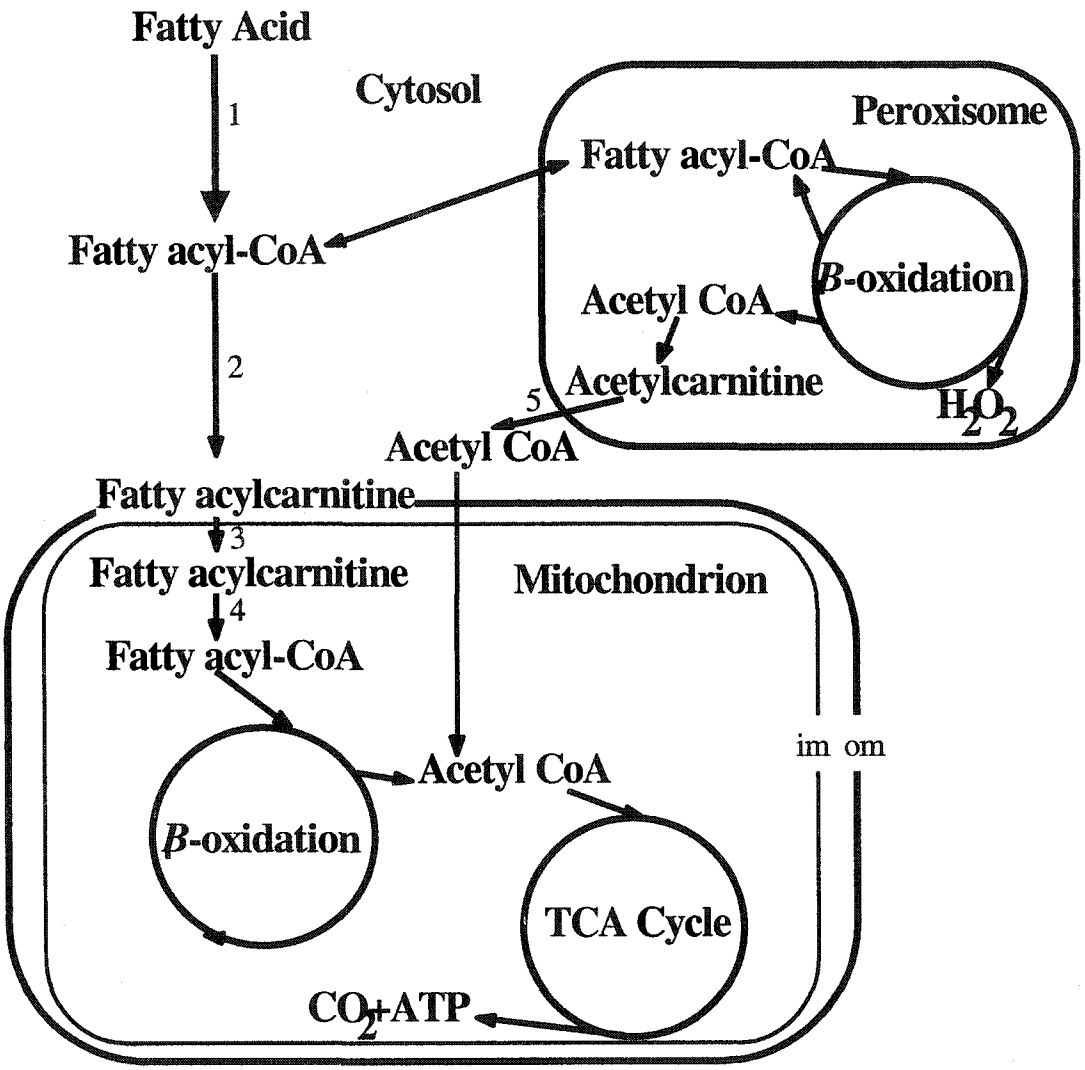

Fig. 3. Activation and transport of fatty acids for ß-oxidation in mitochondria and peroxisomes. Modified from Sargent *et al.*, (1989). Enzymes numbered are: 1=Acyl coA synthetase; 2=carnitine palmitoyltransferase I; 3=acyl carnitine translocase; 4=carnitine palmitoyltransferase II; 5=carnitine acetyl transferase. Abbreviations are: TCA=tricarboxylic acid; im=inner mitochondrial membrane; om=outer mitochondrial membrane. For ß-oxidation pathways in mitochondria and peroxisomes see Fig. 4.

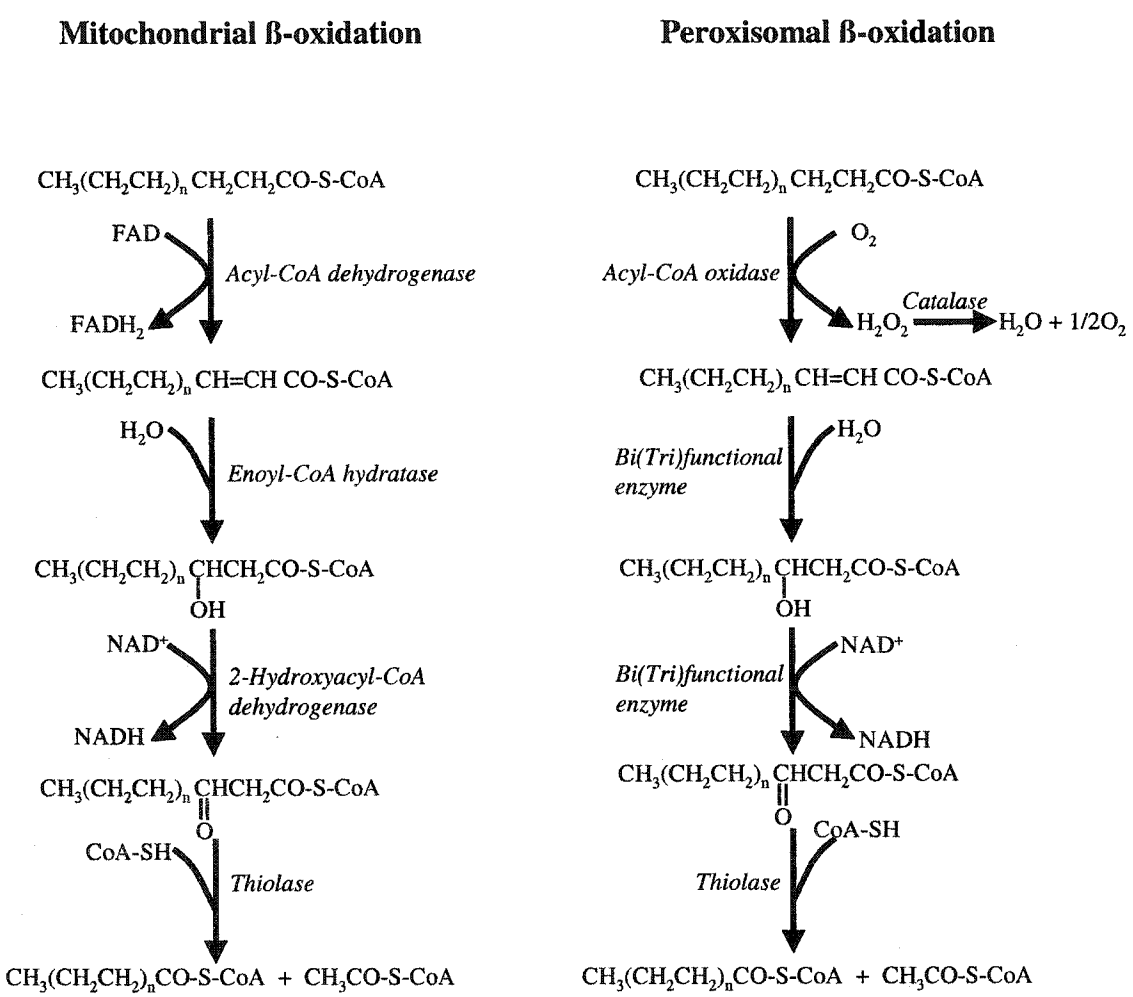

Fig. 4. Pathways for fatty acid ß-oxidation in mitochondria and peroxisomes. Modified from Osmundsen *et al.* (1991).

(intermediate product of fatty acid synthesis) in Atlantic salmon (Froyland *et al.*, 1998) and striped bass (Rodnick and Sidell, 1994). Carnitine palmitoyltransferase I is considered to exert control over the rate of ß-oxidation in mitochondria (McGarry and Foster, 1980).

The mitochondria of rainbow trout can utilize a broader range of fatty acids than observed in mammals (Henderson and Sargent, 1985). The essential fatty acids, 18:2n-6 and 18:3n-3, had a lower ß-oxidation rate than other non-essential fatty acids in the mitochondria of rainbow trout red muscle (Kiessling and Kiessling, 1993). The polyunsaturated fatty acid, 22:6n-3, was inferior to 18:1 as a substrate for mitochondrial ß-oxidation in common carp, Japanese eel, Zilli's tilapia, rainbow trout and red sea bream (Murata, 1979).

In fish, the peroxisomal ß-oxidation enzyme, palmitoyl CoA oxidase, was first reported in several goldfish tissues by Small and Connock (1981). Peroxisomal ß-oxidation is relatively more important in the liver than the white or red muscle of fish (Froyland *et al.*, 2000). The peroxisomes may account for 30-50% (Crockett and Sidell, 1993a,b) up to 100% (Froyland *et al.*, 2000) of ß-oxidation activity in the liver of marine teleost fish. Mammalian peroxisomes, in contrast with mitochondria, ß-oxidize a wide range of substrates including fatty acids, dicarboxylic acids, prostaglandins and various fatty acid analogues. Chain-shortening, particularly of very-long chain fatty acids, rather than complete ß-oxidation is the prominent feature of peroxisomal ß-oxidation in mammals (Osmundsen *et al.*, 1991).

As in mammals, substrate selectivity of peroxisomes in fish is also thought to be broader than that of mitochondria. In the liver of a marine teleost fish, all polyunsaturated fatty acids yielded higher activities with the peroxisomal enzyme, acyl CoA oxidase, than the mitochondrial enzyme, carnitine palmitoyl transferase (Crockett and Sidell, 1993b). Peroxisomal ß-oxidation in the livers of both the rat (Christiansen *et al.*, 1979) and rainbow trout (Henderson and Sargent, 1984) was induced by feeding hydrogenated fish oil. In rats, feeding diets higher in lipid also increased peroxisomal ß-oxidation activity (Neat *et al.*, 1980).

Free fatty acids, but not ketone bodies, are an important fuel during starvation in teleost fish (Zammit and Newsholme, 1979). A key enzyme in ketone body production in mammals, 3-hydroxybutyrate dehydrogenase, is lacking in the liver of teleost fish (Zammit and Newsholme, 1979). Regardless of tissue or species, triacylglycerols, the predominant form of reserve lipid are always mobilized before phospholipid during starvation (Sargent *et al.*, 1989). The presence of lipase enzyme capable of hydrolyzing the stored triacylglycerols has been observed in the muscle of rainbow trout (Bilinski and Lau, 1969), plaice, seabass and mackerel (Zammit and Newsholme, 1979). A salt-resistant triacylglycerol lipase enzyme in the liver of cod was ca. five times more active than the same enzyme in rainbow trout liver (Black *et al.*, 1983). The more active salt-resistant lipase may be used by the cod to mobilize its comparatively larger liver lipid stores (Sargent *et al.*, 1989). Longer-chain free fatty acids (>10C) released into the plasma may be transported on albumin-like carrier proteins in teleost fish (Sheridan, 1988).

1.3.5 Lipid and Fish Health

The requirements (NRC, 1993) and deficiency signs (Tacon, 1996) of several marine and freshwater fish species for n-3 and n-6 essential fatty acids have been documented. However, the functional role of n-3 and n-6 essential fatty acids in the nonspecific and specific immunity in fish has not been clearly defined (Lall, 2000). The specific defence system involves T and B (specific antibodies) lymphocytes; nonspecific defence mechanisms include phagocytes or natural killing cells and several humoral components (eg., lysozymes and complement) that facilitate the activity of phagocytes (Higgs and Dong, 2000).

Dietary fatty acids may affect disease resistance and the immune system in fish by influencing the cell membrane lipid composition. Many immune responses are based on leucocyte membrane interactions such as phagocytosis, antigen-antibody binding and activation steps involving cytokine production (Balfry and Higgs, 2001). Channel catfish fed higher levels of n-3 polyunsaturated fatty acids showed enhanced immune function, especially phagocytic capacity, at a lower water temperature. Increased incorporation of the n-3 polyunsaturated fatty acids into cells involved in immune response will result in increased membrane fluidity at lower water temperatures. Membrane fluidity is important during the ingestion stage of phagocytosis (Lingenfelser, 1995).

The composition of fatty acids in the diet of fish may also affect immune response by influencing the production of the immunoregulatory eicosanoid compounds. Eicosanoids including prostaglandins, leukotrienes and lipoxins are produced by enzymatic action on n-3 or n-6 polyunsaturated fatty acids liberated from cell membrane

phospholipids (Knight and Rowley, 1995). Diets containing different levels of n-3 and n-6 fatty acids from fish or vegetable oils modified the fatty acid composition of the membrane phospholipids in fish. This change in membrane fatty acid composition influences the production of eicosanoids. A higher level of eicosanoids derived from arachidonic acid (20:4n-6) were observed in fish fed a diet higher in n-6 fatty acids (Bell *et al.*, 1992; Bell *et al.*, 1993). Although fish contain much higher levels of eicosapentaenoic acid (20:5n-3) in their cell membrane phospholipids, arachidonic acid is a major substrate for eicosanoid synthesis in fish (Tocher and Sargent, 1987) and fish cells (Tocher et al., 1996). Arachidonic acid produces the pro-inflammatory 2-series of prostaglandins and 4-series of leukotrienes and lipoxins (Hummel, 1993; Higgs and Dong, 2000). An increase in the ratio of n-3 to n-6 fatty acids in the diet counters the action of these pro-inflammatory eicosanoids derived from arachidonic acid. An increase in the ratio of dietary n-3 to n-6 fatty acids will reduce the level of arachidonic acid substrate in the tissues directly or through the inhibition of elongation and desaturation of 18:2n-6. Also, the increased n-3 polyunsaturated fatty acids can inhibit enzymes involved in eicosanoid synthesis directly or through competition with arachidonic acid. Eicosanoids synthesized from eicosapentaenoic acid are less bioactive than those of arachidonic acid (Bell *et al.*, 1996).

Due to the complex nature of this field, published reports on the effects of dietary n-3 versus n-6 polyunsaturated fatty acids on immune response and disease resistance in fish are variable and often conflicting (Lall, 2001). Further research may allow diets to be formulated which provide optimal levels of n-3 and n-6 polyunsaturated fatty acids for

boosting immunity in fish (Higgs and Dong, 2000). Studies on the effects of dietary fatty acids on immune response in fish will become increasingly important as alternative vegetable and animal lipid sources are used to replace marine fish oils.

1.4 Practical Feeds and Feeding of Gadoids

On the basis of current knowledge of the nutrient requirement of gadoids and other fish, diets containing a high amount of protein (48-60 %) and low levels of carbohydrate (10-14%) and lipid (<15 %) with a sufficient amount of n-3 long chain HUFA (1 % EPA and DHA for juvenile fish), as well as being fortified with vitamins and trace elements have been found suitable for initial feed formulations of haddock and cod growout diets (Lall, unpublished data). Our recent research on haddock (50-250 g) suggests that high energy diets containing 15-24% lipid showed a lower feed conversion ratio but growth rate was not improved. There was no real advantage of incorporating more than 15% lipid, however, the growth rate declined when dietary lipid was reduced below 12% (Lall and Nanton, 2002). A recent study on cod suggests that extruded feed containing 48 % protein and 16 % lipid would be desirable in terms of growth, feed utilization and overall cost of the diet (Morais *et al.*, 2001).

Feed attractants are an important component in the diet of gadoid fish. The substitution of ca. 100g of squid or prawn per kg moist diet for saithe fillet nearly doubled food consumption and growth in juvenile cod (Lie *et al.*, 1989c). Cod were also attracted to aqueous extracts of various marine invertebrates including *Nereis*, *Loligo*, *Mytilus* and *Arenicola*. The stimulatory properties were found in an aqueous fraction containing the amino acids glycine, serine, alanine, glutamic acid, valine, leucine and

threonine. Only the free amino acids glycine and alanine evoked a strong response in cod, when tested individually (Pawson, 1977).

Several forms of moist and dry commercial and experimental feeds have been used for feeding cod. Dry extruded pellets have several advantages because they ensure a continuous availability and uniformity of feed, ease of transport, storage and feeding. The processing and production of moist feeds show wide variations. Moist pellets contain a variable amount of fish tissue (ground whole fish, fish and crustacean waste) or fish silage. Dry ingredients (fish and crustacean meal, wheat by-products, corn gluten meal, soybean meal etc.) mixed with vitamins, mineral supplements, a binding agent etc, can be extruded through meat grinders or more elaborate cold extruders. Raw fish and fishery-products must be pasteurized to destroy pathogens and thiaminase enzyme found in fish tissues. Improper and/or long-term storage of these diets can adversely affect the stability of vitamins, cause oxidation of lipid and increase in bacterial and fungal contamination. The overall acceptability of moist diets by cod and haddock remains high as compared with dry extruded feeds for fish reared in Eastern Canada where average annual seawater temperature is between 6-8° C (range 0-18 ° C) (Lall and Nanton, 2001).

Large sexually mature cod have a lower optimal temperature for growth than smaller cod (50-1000 g). The optimal temperature for growth of large cod may lie within the range 9-12°C, which is 2-3°C lower than the temperature range (11-15°C) found to promote optimal growth in smaller cod (Jobling, 1988; Pederson and Jobling, 1989). Attractiveness or palatability of the feed for cod is of particular importance at lower water

temperatures. Food rejection rate increased with decreasing temperature for cod fed formulated feeds, however it remained constant for cod fed capelin (Clark *et al.*, 1995).

Due to the high level of energy (lipid) storage in the liver of gadoid fish fed formulated diets, less frequent feeding may improve feed utilization and reduce fatty liver. Feeding cod three times per week may be better than feeding six times per week or one day per week (Maeland, 2001). Feeding cod juveniles *ad libitum* once every three days compared to once a day reduced the HSI and liver lipid levels. However, weight gain was also reduced in cod fed once every three days (Lie *et al.*, 1988). Growth rate has been positively correlated with HSI in gadoids (Dos Santos *et al.*, 1993), thus attempting to reduce the abnormally high HSI or liver lipid storage levels by reducing feeding frequency can negatively affect growth in gadoids fed formulated diets.

Growth, feed utilization efficiency, protein efficiency ratio and fillet to body weight ratio was highest in cod fed a combination of chopped herring and wet feed compared with moist or wet diets. The level of lipid in the liver of cod fed a mixed diet of coarsely chopped herring for four days and a formulated wet diet three days per week was relatively high (70.3%). However, these cod had a slightly lower liver lipid level compared to those fed seven days per week on moist diets (72.6 to 75.6%) (Jobling *et al.*, 1991).

Cannibalism can be a major source of mortality for the fry of gadoids weaned from live to formulated diets, accounting for 3 to 76% of the total mortality in cod (Ottera and Lie, 1991). Therefore, gadoids require highly attractive and digestible diets during weaning of a similar composition to their natural copepod prey or larval diets, as

described above. Moist compared to dry diets gave better survival rates during weaning of cod (Ottera and Lie, 1991). Clearly, there is a need to further improve the weaning, growout and broodstock diets and feeding practices for haddock, cod and other gadoids.

1.5 Objectives

The main objectives of this thesis research were to:

1) Determine the effect of dietary lipid level on growth and pattern of lipid deposition.
2) Evaluate lipid transport capabilities by characterizing the serum lipoproteins.
3) Measure the effect of dietary lipid level on the lipid catabolic enzyme activity in the muscle and liver.
4) Examine the effect of HSI on liver function parameters.

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2. Effects of Dietary Lipid on Liver and Muscle Lipid Deposition in Juvenile Haddock, *Melanogrammus aeglefinus* L.

2.1 Introduction

Haddock (*Melanogrammus aeglefinus* L.) has been identified as a potential species for marine aquaculture in Atlantic Canada. Like Atlantic salmon, haddock is a round fish that grows rapidly and adapts well to cage-culture making it an ideal alternative new fish species for aquaculture in Atlantic Canada. The development of an efficient grower diet for haddock is essential because feed will account for the major cost (>50%) of production. Little information is currently available in the literature on the nutritional requirements of haddock. Fatty liver condition has been observed in haddock fed high-energy, lipid-rich formulated feeds. From field observations, it is obvious that enlarged fatty livers affect somatic growth and health of fish.

The primary site of lipid storage in cod (*Gadus morhua*), a closely related gadoid species, is the liver (Dos Santos *et al.*, 1993; Lie *et al.*, 1986). A high retention (60%) of absorbed dietary lipids was observed in the liver of cod fed diets containing different oils which made up 48% of the available energy (Lie *et al.*, 1986). In cultured cod fed lipid-rich diets to apparent satiation, the liver lipid can surpass 70% (Shahidi and Dunajski 1994). The lipid in the liver consists mainly (>90%) of triacylglycerols (Dos Santos *et al.*, 1993), and is strongly influenced by the fatty acid composition of the diet (Lie *et al.*, 1986). The hepatosomatic index (HSI) in cultured cod often exceeds 12% (Jobling, 1988; Shahidi and Dunajski, 1994), whereas in wild cod a HSI of 2-6% is considered normal (Jobling, 1988). A positive linear relationship has been observed in cod between dietary lipid intake and HSI (Lie *et al.*, 1988; Jobling, 1988). Growth rate has also been

correlated with HSI (Dos Santos *et al.*, 1993; Jobling, 1988). Cod are lean fish that store low amounts of lipid (<2%) in the muscle (Dos Santos *et al.*, 1993; Ackman, 1967). Phospholipids or membrane lipids comprise a large proportion (>70%) of the total muscle lipid (Dos Santos *et al.*, 1993).

The development of haddock diets that promote high growth rates, while reducing abnormally high fat storage levels in the liver, is of primary importance. The objective of this study was to determine the effect of increasing levels of dietary fish oil on growth performance and biochemical composition of juvenile haddock.

2.2 Materials and Methods

Experimental conditions

Feeding trials for haddock fingerlings (initial weight 6.88±0.37 g) were conducted at the NRC Aquaculture Research Station at Sandy Cove, Halifax, Nova Scotia, where the fish were hatched and reared to attain their initial size at the start of the experiment. Fifty fish were randomly allotted into each of 12 tanks and adapted to experimental conditions for 3 weeks before the feeding trial started. Fish were hand-fed experimental diets to satiation three times daily on weekdays and twice daily on weekends during the 63 d experimental period. Filtered and UV-treated seawater (salinity, 30 ppt) was supplied to each tank at a rate of 4 L/min in a flow-through system. Water volumes in each tank were maintained at 250 L (holding capacity: 320 L) to ensure a renewal rate of approximately once every hour. Fish were held on a 12 h dark : 12 h light photoperiod with the light intensity at the water surface ranging between 40 and 60 lux. Dissolved oxygen levels and water temperature were measured every morning and averaged 11 mg/L and 11.5°C, respectively. Fish were bulk-weighed and counted at the

beginning and at 3 week intervals during the experiment. Fish were fasted for 24 hours prior to weighing and counting.

Diet preparation

Experimental diets were formulated using practical ingredients, of which, herring meal made up the major protein source (Table I). Increasing amounts of herring oil were incorporated to provide levels of 12, 15, 18 and 21% lipid in isonitrogenous diets. Fish meal and wheat were ground and passed through a fine screen using a Fitz mill (Fitzpatrick Co., Ill., USA) before mixing with other ingredients. Supplemental casein was added to provide 25% of the dietary protein in each diet. All dry ingredients, except the choline chloride, starch and fish oil, were weighed and mixed in a large Hobart mixer (Model H600T, Rapids Machinery Co., Iowa, USA) for 15 minutes. The choline chloride and cornstarch were then added and the ingredients were homogenized for 15 more minutes before adding the fish oil and continuing to mix for an additional 15 minutes. Diets were steam pelleted into 1.5 mm and 2.4 mm pellets using a laboratory pellet mill (California Pellet Mills, San Francisco), dried in an air-convection drier at 30 °C and screened prior to feeding. The diets were stored in a freezer until needed.

The compositions of the diets are presented in Table I. The protein was maintained at a level of ca. 55% dry wt. whereas the lipid level was increased from 13.8 to 21.5% dry wt. in the diet. The fatty acid composition of the diets were similar with respect to the essential fatty acids: docosahexaenoic acid (DHA) comprising 12-14%, and eicosapentaenoic acid (EPA) comprising 9-11%, of the total fatty acids. The diets had an approximate 1:1 DHA/EPA ratio. The long-chain monounsaturated fatty acids 20:1n-9

(4-6%) and 22:1n-11 (4-7%) characteristic of oil from Atlantic herring (*Clupea harengus*) were also prevalent in the dietary lipid (Table II).

Sampling and analytical methods

At the beginning of the feeding trial, 10 fish were sampled and sacrificed with an overdose of TMS (tricaine methanesulfonate). These 10 initial fish were individually weighed and dissected to remove the liver. Five of these initial fish were used for muscle and liver lipid analysis and 5 were freeze-dried and used for proximate analysis of the whole body. At the end of the experiment, 5 fish from each tank were sampled and killed in a similar manner. After measuring individual weights of whole body and liver, the livers and carcasses were recombined and freeze-dried for subsequent proximate analyses of the whole body. Chemical compositions of the experimental diets and freeze-dried carcass were determined using the following AOAC (1990) procedures: dry matter by drying in an oven at 110°C for 24 h; crude fat after acid hydrolysis; crude ash by incineration in a muffle furnace at 550°C for 24 h; calcium by a wet ash method and titration with $KMnO_4$; phosphorus by a spectrophotometric method using molybdovanadate reagent; crude fiber by digestion with 1.25 % H_2SO_4 and 1.25 % NaOH solutions. Nitrogen-free extract was calculated by subtracting protein, lipid, ash and fiber from total dry matter. Crude protein (%N x 6.25) was measured using a Leco Nitrogen Determinator (Model FP-228, Leco Corporation, St. Joseph, MI). Energy contents were determined using an adiabatic bomb calorimeter (Model 1261, Parr Instruments, Moline IL). Lipids were extracted using the method of Bligh and Dyer (1959). Fatty acid compositions of the diets were estimated from the fatty acid methyl ester (FAME) derivatives of the transesterified lipids. The FAME were prepared using

7% boron trifluoride in methanol and heating to 100°C for 1 h (Christie, 1982). The FAME were separated on a gas chromatograph (Hewlett Packard 6890 GC system, Wilmington, DE) equipped with a flame ionization detector on an Omegawax 320 capillary column (30m length x 0.32mm diameter x 0.25 μm film thickness; Supelco, Bellefonte, PA). Split injection was used with a front inlet temperature of 250°C. The carrier gas was helium (2 mL/ min). The oven temperature was initially held at 185°C for 8 min, increased at a rate of 3°C / min to a final temperature of 230°C and held for an additional 10 min. The FAME were identified by comparison of retention times with those of known standards (Supelco 37, Menhaden Oil; Supelco, Bellefonte, PA).

Nitrogen and energy utilization parameters were calculated from the intake, nitrogen and energy composition of the feed as well as the weight, nitrogen and energy composition of the haddock at the start and finish of the trial. The values for nitrogen intake, nitrogen gain, nitrogen retention, protein efficiency, energy intake, energy gain and energy retention were estimated from these values using the equations given in Table VI.

Total lipid (Bligh and Dyer, 1959) from the liver or muscle of the haddock at the end of the experiment was extracted and measured from five randomly sampled fish for each diet and for the initial fish. The muscle lipid from four haddock fed 14 or 22% lipid dry wt. diets were separated into neutral and polar fractions using a silica gel column. The column consisted of a pasteur pipette plugged with glass wool, a thin bottom layer of anhydrous sodium sulphate and silica gel (40 μm flash chromatography packing; J.T. Baker Inc., Phillipsburg, NJ). Chloroform followed by methanol were used to elute neutral and polar lipid classes, respectively (Christie, 1982). Fatty acid compositions of muscle neutral

and polar lipids were determined according to the same procedure described above for the dietary lipid.

Statistical analysis

Analyses of variance (ANOVA) with pairwise comparisons (Tukeys HSD) were performed at the $p<0.05$ level to determine significant differences. Percentage values were square root, arcsine transformed prior to analysis. Statistical analyses were performed using the Systat 5.1 software package (Systat, Evanston, IL).

2.3 Results

The juvenile haddock exhibited rapid growth rates for all of the dietary lipid levels. The fish at the end of the nine week experimental period had more than a six-fold increase in weight compared to the initial fish. Specific growth rates (2.8%) of the haddock were high for all of the experimental diets. There was a small, non-significant increase in both specific growth rate and weight gain as the lipid level in the diet was increased. The feed conversion ratio was low (<0.7) for these rapidly growing juvenile fish and a small but non-significant decrease was observed as the lipid level in the diet increased from 14 to 22% dry wt. (Table III).

The HSI was high for all diets and increased significantly from 9.8 to 12.1% with the increasing lipid in the diet. (Table III). The total lipid composition of the liver also increased significantly from 63.2 to 69.0% as the lipid level in the diet was increased from 14 to 22% dry wt. However, no significant differences were observed in the low levels of muscle lipid (1.0%) as the lipid in the diet was increased. The neutral or storage lipid in the muscle was also low (14-15%) and did not increase as dietary lipid level was increased (Table IV).

The whole body composition of the haddock at the end of the 9 week study was also analyzed. Moisture content was significantly higher for the haddock fed the lowest level of dietary lipid (14% dry wt.; Table V). Protein content was significantly lower in haddock fed the highest level of dietary lipid (22% dry wt.). No significant differences were observed in the ash content of haddock. The energy content of the fish increased significantly from 6.03 to 6.39 kcal/g dry wt. as the lipid level was increased from 14 to 22%. There were no significant differences in the nitrogen utilization parameters (nitrogen intake, nitrogen gain, nitrogen retention or protein efficiency ratio) for the fish fed different levels of lipid. A significant increase in energy gain was found as the dietary lipid was increased from 14 to 22% dry wt. (Table VI).

The fatty acid compositions of the total lipid in the livers of haddock fed the four levels of dietary lipid were similar (Table VII) and reflected the fatty acid composition of the diet. The essential fatty acids, DHA and EPA, ranged from 6.2 to 7.3% and 7.6 to 9.3%, respectively. The total polyunsaturated fatty acids ranged from 27.2 to 30.8%. Monounsaturated fatty acids made up a large proportion of the haddock liver lipid ranging from 41.2-46.1% of the total fatty acids. There was a significant increase in the liver levels of the long-chain monounsaturated fatty acids (20:1n-9, 22:1n-11) from the dietary herring oil. The range for 20:1n-9 was 4.8 to 8.7%, and for 22:1n-11 was 4.2 to 8.8%, as the lipid in the diet was increased from 14 to 16% dry wt. (Table VII). The muscle lipid was comprised mainly of membrane phospholipids (>85%) and polyunsaturated fatty acids were prevalent, comprising 51.9 to 53.5% (Table VIII). The essential fatty acids, DHA (22.0-22.6%) and EPA (16.3-18.1%), were present in high amounts. The monounsaturates made up only 20.8 to 23.9% of the total fatty acids in

muscle. As was the case in the liver, there was a significant increase of the long-chain monounsaturated fatty acids 20:1n-9 (1.8 to 3.5%) and 22:1n-11 (0.6 to 1.3%) in the muscle lipid, mostly as the lipid in the diet was increased from 14 to 16% dry wt. (Table VIII). The fatty acid composition of the neutral (Table IX) and polar lipid (Table X) fractions in the muscle was also analyzed for 14 and 22% dry wt. of lipid in the diet. Compared to the polar lipid, the neutral lipid had a fatty acid composition more closely resembling the storage lipid in the liver with higher levels of monounsaturated (36.8 compared to 21.3%) and lower polyunsaturated fatty acids (36.7 compared to 54.9%). The long-chain monounsaturated fatty acids, 20:1n-9 and 22:1n-11, were incorporated at higher levels in the neutral lipid compared with the polar (membrane) lipid but a similar significant increase of these fatty acids was observed in both the neutral and polar fractions as the lipid in the diet increased from 14 to 22% dry wt.

2.4 Discussion

Information on either the growth or chemical composition of juvenile haddock fed practical diets is limited. It is clear from this study that juvenile haddock are capable of achieving high growth rates over a range of dietary lipid levels (13.8 to 21.5% dry wt.). An increase in dietary lipid from 13.8 to 21.5% dry wt. was associated with a small, non-significant increase in the growth rate of the haddock. A positive correlation between HSI and growth was demonstrated in cod (Dos Santos *et al.*, 1993). An increase in the lipid content of whole captured cod has also been positively correlated with specific growth rate (Holdway and Beamish, 1984). In this study, significant increases in both HSI and liver lipid were also associated with the increase in dietary lipid. The HSI in these cultured haddock increased from 9.8 to 12.1% as dietary lipid was increased from 13.8 to

21.5% dry wt. Thus, in juvenile haddock fed isonitrogenous diets (55% dry wt.) it is possible to use a lower level of lipid (13.8% dry wt.) in the diet to significantly reduce HSI, while not significantly affecting the growth. The hepatosomatic indices reported in this study for haddock are much higher than the 2 to 6% level reported for "normal" wild cod (Jobling, 1988). Although the hepatosomatic indices were relatively high, histological analyses of the cultured haddock livers did not reveal any overt pathology.

Wild cod had liver lipid levels of 60% and pen-held cod fed a moist herring-based diet had liver lipid levels averaging 76% (Shahidi and Dunajski, 1994). Haddock fed diets containing increasing lipid levels significantly increased their liver lipid content from 63.2 to 69.0%. Cod efficiently retain large amounts of absorbed lipid (60%) in the liver when fed formulated feeds containing different oils which made up 48% of the available energy (Lie *et al.*, 1986). Presumably, the increase in the lipid content of the liver in haddock is mainly due to dietary lipid deposition, since lipogenesis in fish is inhibited at dietary lipid levels above 10% (Henderson and Sargent, 1981). The muscle lipid remained consistently low (1.0%) as the dietary lipid was increased. This suggests that the haddock, unlike salmonids, has little or no ability to increase the storage of lipid in the muscle as dietary lipid is increased. This consistently low level of muscle lipid as the levels of dietary lipid are increased has been noted in studies with cod (Dos Santos *et al.*, 1993; Morais *et al.*, 2001). This pattern of lipid deposition in gadoids contrasts with cultured salmon that are capable of greatly increasing their lipid stores in the muscle (values may reach 17%) when fed high-energy, lipid-rich diets (Bell *et al.*, 1998).

Our preliminary observations of the lipoprotein composition in cultured haddock showed relatively low concentrations (<50mg/dL serum) of very low density lipoprotein

(VLDL) in the serum (unpublished results). This is typical of lean fish containing low levels of muscle lipid. The concentration of VLDL triacylglycerol in the plasma of various marine fish species has been positively correlated with muscle lipid levels (Ando and Mori, 1993). The VLDL is thought to be a major vehicle for transporting lipid out from the liver to extrahepatic tissues (i.e., muscle) in fish (Sheridan, 1988).

A general non-specific retention of fatty acids in the liver was observed in cod fed oils with different fatty acid compositions. In other words, there was little alteration or selectivity of the dietary fatty acids incorporated into the liver (Lie *et al.*, 1986). This trend was also observed in the haddock. The long-chain monounsaturated fatty acids 20:1n-9 and 22:1n-11, were prevalent in the dietary herring oil and high percentages of these fatty acids were also observed in the liver. Presumably, these fatty acids are absorbed from the diet, transported and deposited in the liver with little alteration. Monounsaturated fatty acids are preferentially oxidized over saturated and polyunsaturated fatty acids in carp liver mitochondria (Murata and Higashi, 1980). Marked catabolism of 22:1n-11 was also observed in the liver of rainbow trout fed diets rich in 22:1 (Henderson *et al.*, 1982). As the lipid level in the diet was increased from 13.8 to 16.1% dry wt. there was approximately a 2-fold increase in both 20:1n-9 and 22:1n-11 in the liver lipids. This suggests that the haddock liver was not able to oxidize the higher concentrations of these dietary long-chain monounsaturates. The increased percentage of the long-chain monounsaturates in the liver was also paralleled by significantly higher percentages in the muscle lipid, particularly in the neutral lipid fraction, as the dietary lipid level was increased. Although 20:1n-9 and 22:1n-11 increased significantly in the muscle total lipid, there was little incorporation of these

fatty acids (particularly 22:1n-11) in the polar (membrane) lipid fraction. However, the essential fatty acids 22:6n-3 and 20:5n-3 were incorporated at high concentrations in the muscle polar lipid which is typical of the fatty acid incorporation pattern in fish (Sargent *et al.*,1989; Murata and Higashi, 1980).

Optimal growth for cod may be achieved with a feed containing 60%, 25% and 15% of available energy from protein, fat and carbohydrate, respectively. A HSI below 10% may be obtained by using feeds with 25% or less of available fat energy (Lie *et al.*, 1988). Recently, Kim and Lall (2001) observed that the protein requirements for juvenile haddock may be less than 45% based on growth rate (protein requirement of 53.8% based on nitrogen gain). The HSI was reduced from 10.7 to 7.3% as the crude protein (mg) to gross energy (kJ) ratio was increased from 19.2 to 29.5. Protein is the most expensive component of carnivorous fish diets and its use should be minimized. It appears that the juvenile haddock are capable of utilizing increased amounts of lipid to maintain growth as the protein level is reduced. The present study demonstrates that in isonitrogenous diets, increasing dietary lipid in haddock diets causes an increase in HSI and liver lipid content. Thus, there may be a tradeoff between the economic need to minimize dietary protein and the potential for inducing fatty liver syndrome in haddock fed lipid-rich diets.

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Table I. Formulation and composition[1] of experimental diets

	Dietary lipid level (%)			
Ingredients	12	15	18	21
Fish meal[2]	38.5	39.0	39.3	40.1
Krill meal[3]	2.0	2.0	2.0	2.0
CPSP-G[4]	5.0	5.0	5.0	5.0
Casein[5]	13.9	13.9	13.9	13.9
Fish oil[6]	5.1	8.1	11.1	14.2
Ground wheat[7]	19.8	17.5	16.3	12.6
Whey[8]	7.0	7.0	7.0	7.0
Vitamin mixture[9]	1.5	1.5	1.5	1.5
Mineral mixture[10]	0.5	0.5	0.5	0.5
Choline chloride[4]	0.2	0.2	0.2	0.2
Cornstarch[11]	6.5	5.3	3.2	3.0
Chemical analysis (% dry wt.)				
Moisture	13.3	13.0	12.8	12.7
Protein	54.3	54.8	54.8	55.1
Lipid	13.8	16.1	18.9	21.5
Fiber	0.9	0.8	0.6	0.5
Ash	6.9	6.9	7.0	6.9
Nitrogen-free extract[12]	24.1	21.4	18.7	16.1
Calcium	0.9	0.9	1.0	1.1
Phosphorus	1.4	1.3	1.3	1.3

[1] Average of two replicates.
[2]Composed of 75% of herring meal (Sea Life Fisheries Inc., Canada) and 25% of Norse-LT 94 (Silfas Karmsund AS, Norway)
[3]Specialty Marine Products Ltd., West Vancouver, BC
[4]Hydrolyzed fish meal, Sopropêche, France
[5]US Biochemical, Cleveland, OH
[6]Stabilized with 0.06% ethoxyquin, Comeau Seafood, Saulnierville, NS
[7]Dover Mills Ltd., Halifax, NS
[8]Farmers Co-operative Dairy Ltd., Truro, NS
[9]Vitamin added to supply the following (per kg diet): vitamin A, 8000 IU; vitamin D_3, 4500 IU; vitamin E, 300 IU; vitamin K_3, 40 mg; thiamine HCl, 50 mg; riboflavin, 70 mg; d-Ca pantothenate, 200 mg; biotin, 1.5 mg; folic acid, 20 mg; vitamin B_{12}, 0.15 mg; niacin, 300mg; pyridoxine HCl, 20 mg; ascorbic acid, 300 mg; inositol, 400 mg; BHT, 15 mg; BHA, 15 mg
[10]Mineral added to supply the following (per kg diet): manganous sulfate (32.5% Mn), 40 mg; ferrous sulfate (20.1% Fe), 50 mg; copper sulfate (25.4% Cu), 10 mg; zinc sulfate (22.7% Zn), 75 mg; cobalt chloride (24.8% Co), 5 mg; sodium selenite (45.6% Se), 1 mg; sodium fluoride (45.2% F), 4 mg
[11]Pregelatinized (National 1215), National Starch and Chemical Co., Bridgewater, NJ
[12]Nitrogen-free extract = 100 – (protein + lipid + fiber + ash)

Table II. Fatty acid composition of experimental diets[1]

	Dietary lipid level (% dry wt.)			
Fatty acid	14	16	19	22
14:0	4.5	4.8	4.1	4.4
16:0	18.3	17.6	19.3	19.3
16:1 n-7	5.6	6.4	5.6	6.0
18:0	2.5	2.4	2.2	2.4
18:1 n-9	10.0	9.0	9.2	8.9
18:1 n-7	3.1	3.0	3.2	3.1
18:2 n-6	12.7	10.8	12.2	9.9
18:3 n-3	1.5	1.6	1.6	1.4
18:4 n-3	1.4	1.7	1.4	1.6
20:1n-9	3.6	6.0	4.9	5.0
20:4 n-6	0.8	0.7	0.7	0.7
20:4 n-3	0.5	0.5	0.4	0.5
20:5 n-3	11.0	9.1	9.2	9.7
22:1n-11	3.8	7.5	5.0	5.6
22:5 n-3	1.0	1.0	1.0	1.0
22:6 n-3	12.7	11.9	13.9	14.3
Σ SAT	26.1	25.5	26.1	26.4
Σ MONO	27.3	32.6	28.7	29.1
Σ PUFA	44.8	40.3	43.3	41.6
Σ n-3	28.6	26.2	28.0	29.0
Σ n-6	13.8	11.7	13.3	10.6
DHA/EPA	1.2	1.3	1.5	1.5

[1]Data expressed as area % of FAME, n=2.

Table III. Growth, feed utilization and hepatosomatic index of haddock fed graded levels of dietary lipid for 9 weeks[1]

Lipid level[2] (%)	Wt. gain[3] (g/fish)	SGR[4] (%)	Feed conversion ratio[5]	HSI[6] (%)
12 (13.8)	30.6 ± 3.2^{a}	2.7 ± 0.05^{a}	0.69 ± 0.05^{a}	9.8 ± 0.59^{a}
15 (16.1)	32.3 ± 1.6^{a}	2.8 ± 0.07^{a}	0.66 ± 0.02^{a}	10.8 ± 0.51^{ab}
18 (18.9)	32.7 ± 3.9^{a}	2.8 ± 0.13^{a}	0.68 ± 0.06^{a}	11.3 ± 0.35^{bc}
21 (21.5)	33.0 ± 1.0^{a}	2.8 ± 0.06^{a}	0.65 ± 0.03^{a}	12.1 ± 0.69^{c}

[1]Values (means ± SD of three replicate tanks) in the same column containing different superscripts were significantly different ($p<0.05$).

[2]Values within brackets are % lipid in diet on dry matter basis.

[3]Average initial weight was 6.9 g.

[4]Specific growth rate (%) = 100 x (ln(final wt.)-ln(initial wt.))/duration (d).

[5]Feed intake (expressed as dry matter)/wet wt. gain.

[6]Hepatosomatic index (%) = 100 x wet liver wt./body wt.; HSI of initial fish was 6.82

Table IV. Lipid composition of juvenile haddock fed graded levels of lipid for 9 weeks[1]

Lipid[2] level (%)	Liver lipid[3]	Muscle lipid[4]	Muscle NL[5] (%)
14	63.2 ± 2.4[a]	0.95 ± 0.03[a]	14.5 ± 1.6[a]
16	64.3 ± 2.0[a]	1.05 ± 0.09[a]	-
19	67.0 ± 1.3[ab]	1.05 ± 0.10[a]	-
22	69.0 ± 2.4[b]	1.01 ± 0.04[a]	14.0 ± 0.6[a]

[1]Values (means ± SD) in the same column containing different superscripts were significantly different ($p<0.05$).
[2] Dietary lipid level (% dry wt.)
[3] n = 5; Initial fish liver lipid was 54.05%.
[4] n = 5; Initial fish muscle lipid was 1.40%.
[5] n = 4; Neutral lipid (NL) measured as % total lipid.

Table V. Whole body composition of haddock fed graded levels of lipid for 9 weeks[1]

	Dietary lipid level (% dry wt.)			
Composition	14	16	19	22
Moisture (%)	77.5 ± 0.4[a]	76.0 ± 0.2[b]	75.6 ± 0.4[b]	75.7 ± 0.2[b]
Protein[2] (%)	13.8 ± 0.4[a]	13.9 ± 0.2[a]	14.0 ± 0.2[a]	13.1 ± 0.3[b]
Ash[2] (%)	2.2 ± 0.1[a]	2.2 ± 0.1[a]	2.3 ± 0.0[a]	2.2 ± 0.1[a]
Energy (kcal/ g DM)	6.0 ± 0.1[a]	6.2 ± 0.1[b]	6.3 ± 0.1[b]	6.4 ± 0.1[b]

[1]Values (means ± SD of three replicate tanks x two fish/ tank) in the same row containing different superscripts were significantly different ($p<0.05$).
[2]Wet weight basis

Table VI. Nitrogen (N) and energy (E) utilization of fish fed different levels of lipid for 9 weeks[1]

	Dietary lipid level (% dry wt.)			
Parameter	14	16	19	22
N intake[2] (g/ fish)	1.8 ± 0.1[a]	1.9 ± 0.0[a]	1.9 ± 0.1[a]	1.9 ± 0.0[a]
N gain[3] (g/fish)	0.7 ± 0.0[a]	0.7 ± 0.0[a]	0.8 ± 0.1[a]	0.7 ± 0.0[a]
N retention[4] (%)	37.9 ± 2.3[a]	39.5 ± 2.0[a]	38.7 ± 3.7[a]	37.4 ± 2.3[a]
PER[5]	2.7 ± 0.2[a]	2.8 ± 0.1[a]	2.7 ± 0.2[a]	2.8 ± 0.1[a]
E intake[6] (kcal/fish)	113.5 ± 6.0[a]	118.3 ± 2.8[ab]	125.5 ± 4.5[b]	123.8 ± 1.2[ab]
E gain[7] (kcal/fish)	43.1 ± 5.6[a]	50.9 ± 2.9[ab]	53.0 ± 4.6[ab]	54.2 ± 2.2[b]
E retention[8] (%)	37.9 ± 3.1[a]	43.0 ± 1.4[a]	42.2 ± 2.4[a]	43.8 ± 2.1[a]

[1]Values (means ± SD of three replicate tanks x two fish/ tank) in the same row containing different letter superscripts were significantly different ($p<0.05$).
[2]Feed intake per fish x N content of feed.
[3]N in whole body of final fish – N in whole body of initial fish.
[4]100 x N gain/ N intake.
[5]Protein efficiency ratio = wet wt. gain/ protein (N x 6.25) intake.
[6]Feed intake per fish x E content of feed.
[7]E in whole body of final fish – E in whole body of initial fish.
[8]100 x E gain/ E intake.

Table VII. Fatty acid composition of total liver lipid from haddock fed graded levels of dietary levels of lipid for 9 weeks[1,2]

		Dietary lipid level (% dry wt.)			
Fatty acid	Initial	14	16	19	22
14:0	3.5 ± 0.2	3.6 ± 0.2^{a}	4.0 ± 0.2^{ab}	4.4 ± 0.2^{b}	4.3 ± 0.3^{b}
16:0	15.4 ± 0.8	18.5 ± 0.6^{a}	17.4 ± 0.9^{ab}	16.8 ± 0.6^{b}	15.4 ± 0.8^{c}
16:1 n-7	8.5 ± 0.5	6.8 ± 0.4^{a}	7.2 ± 0.4^{a}	8.0 ± 0.2^{b}	8.2 ± 0.2^{b}
18:0	3.2 ± 0.3	4.2 ± 0.4^{a}	3.7 ± 0.5^{ab}	3.3 ± 0.2^{b}	3.1 ± 0.4^{b}
18:1 n-9	16.1 ± 1.7	19.4 ± 0.9^{a}	15.9 ± 0.8^{bc}	14.6 ± 0.2^{cd}	14.1 ± 0.5^{d}
18:1 n-7	5.4 ± 0.4	4.4 ± 0.2	4.1 ± 0.3	3.9 ± 0.2	3.8 ± 0.3
18:2 n-6	5.1 ± 0.7	6.1 ± 0.2^{a}	4.9 ± 0.2^{b}	4.4 ± 0.1^{b}	3.5 ± 0.0^{c}
18:3 n-3	1.2 ± 0.1	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.1 ± 0.0^{a}	1.0 ± 0.1^{a}
18:4 n-3	1.7 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	2.3 ± 0.1
20:1 n-9	6.1 ± 0.9	4.8 ± 0.2^{a}	8.7 ± 0.6^{b}	9.0 ± 0.4^{b}	9.5 ± 0.6^{b}
20:4 n-6	0.8 ± 0.2	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.1^{a}
20:4 n-3	0.8 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
20:5 n-3	10.3 ± 1.4	9.3 ± 0.5^{a}	7.6 ± 0.5^{b}	8.4 ± 0.3^{a}	9.2 ± 0.6^{a}
22:1n-11	4.6 ± 1.2	4.2 ± 0.5^{a}	8.8 ± 1.0^{b}	8.7 ± 0.7^{b}	9.0 ± 1.0^{b}
22:5 n-3	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	0.9 ± 0.3
22:6 n-3	8.4 ± 1.7	7.0 ± 0.3^{a}	6.2 ± 0.3^{a}	6.6 ± 0.4^{a}	7.3 ± 0.9^{a}
Σ SAT	23.0 ± 0.8	27.0 ± 0.8^{a}	25.7 ± 1.1^{ab}	25.1 ± 0.7^{b}	23.4 ± 0.7^{c}
Σ MONO	42.4 ± 3.2	41.2 ± 1.0^{a}	46.1 ± 0.7^{b}	45.4 ± 1.0^{b}	45.7 ± 2.1^{b}
Σ PUFA	33.7 ± 3.5	30.8 ± 1.2^{a}	27.2 ± 1.2^{b}	28.7 ± 0.8^{a}	29.7 ± 1.6^{a}
Σ n-3	24.4 ± 3.3	21.1 ± 1.0^{a}	18.9 ± 0.9^{a}	20.5 ± 0.7^{a}	22.0 ± 1.7^{a}
Σ n-6	6.4 ± 0.7	7.2 ± 0.2^{a}	5.9 ± 0.2^{ab}	5.4 ± 0.1^{ab}	4.5 ± 0.1^{b}
DHA/EPA	0.8 ± 0.1	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}

[1]Values (means ± SD; n=5) in the same row containing different superscripts were significantly different ($p<0.05$). Statistical analysis did not include initial value.
[2]Data expressed as area % of FAME.

Table VIII. Fatty acid composition[1,2] of muscle total lipid from haddock fed graded levels of dietary levels of lipid for 9 weeks

		Dietary lipid level (% dry wt.)			
Fatty acid	Initial	14	16	19	22
14:0	2.2 ± 0.2	1.7 ± 0.4^{a}	2.0 ± 0.4^{a}	2.1 ± 0.1^{a}	2.1 ± 0.2^{a}
16:0	18.4 ± 0.4	17.0 ± 1.0^{a}	16.4 ± 0.7^{a}	15.9 ± 0.4^{a}	16.1 ± 0.4^{a}
16:1n-7	4.1 ± 0.4	2.8 ± 0.4^{a}	3.0 ± 0.5^{a}	3.1 ± 0.2^{a}	3.0 ± 0.3^{a}
18:0	3.8 ± 0.3	4.3 ± 0.1^{a}	3.6 ± 0.4^{b}	3.4 ± 0.1^{b}	3.4 ± 0.3^{b}
18:1n-9	9.6 ± 0.2	11.5 ± 0.8^{a}	11.6 ± 1.2^{ab}	11.1 ± 0.5^{b}	9.9 ± 0.4^{b}
18:1n-7	3.7 ± 0.1	3.2 ± 0.1	2.9 ± 0.2	2.9 ± 0.1	2.8 ± 0.0
18:2n-6	4.9 ± 0.3	5.7 ± 0.3^{a}	5.4 ± 0.3^{ab}	4.6 ± 0.2^{c}	3.6 ± 0.2^{d}
18:3n-3	0.9 ± 0.0	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}	0.8 ± 0.0^{a}	0.7 ± 0.1^{a}
18:4n-3	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
20:1n-9	2.6 ± 0.3	1.8 ± 0.3^{a}	3.5 ± 0.5^{b}	4.0 ± 0.2^{b}	4.0 ± 0.4^{b}
20:4n-6	1.6 ± 0.1	1.3 ± 0.1^{a}	1.3 ± 0.2^{a}	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}
20:4n-3	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
20:5n-3	15.7 ± 0.1	16.8 ± 0.7^{a}	16.3 ± 0.7^{a}	17.0 ± 0.4^{a}	18.1 ± 0.3^{b}
22:1n-11	1.6 ± 0.4	0.6 ± 0.1^{a}	1.3 ± 0.2^{b}	1.8 ± 0.3^{bc}	2.0 ± 0.2^{c}
22:5n-3	1.9 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.2 ± 0.0	2.4 ± 0.1
22:6n-3	20.2[illegible] ± 0.9	22.6 ± 1.9^{a}	21.6 ± 2.0^{a}	21.3 ± 0.7^{a}	22.0 ± 1.1^{a}
Σ SAT	25.2 ± 0.7	23.8 ± 0.9^{a}	22.8 ± 0.6^{ab}	22.2 ± 0.4^{b}	22.3 ± 0.6^{b}
Σ MONO	23.1 ± 1.0	20.8 ± 1.8^{a}	23.3 ± 2.5^{ab}	23.9 ± 0.8^{b}	22.7 ± 1.2^{ab}
Σ PUFA	50.2 ± 0.6	53.4 ± 2.1^{a}	51.9 ± 2.2^{a}	51.9 ± 0.6^{a}	52.9 ± 0.8^{a}
Σ n-3	41.1 ± 0.5	44.1 ± 2.3^{a}	43.2 ± 2.4^{a}	43.9 ± 0.8^{a}	45.9 ± 1.1^{a}
Σ n-6	7.8 ± 0.3	8.3 ± 0.3^{a}	7.7 ± 0.2^{b}	6.9 ± 0.0^{c}	5.8 ± 0.2^{d}
DHA/EPA	1.3 ± 0.1	1.4 ± 0.1^{a}	1.3 ± 0.1^{ab}	1.3 ± 0.0^{ab}	1.2 ± 0.1^{b}

[1]Values (means ± SD; n=5) in the same row containing different superscripts were significantly different ($p<0.05$). Statistical analysis did not include initial value.
[2]Data expressed as area % of FAME.

Table IX. Fatty acid composition[1,2] of muscle neutral lipid from haddock fed graded levels of dietary levels of lipid for 9 weeks

	Dietary lipid level (% dry wt.)	
Fatty acid	14	22
14:0	3.4 ± 0.9[a]	4.3 ± 0.4[a]
16:0	16.8 ± 0.8[a]	13.9 ± 1.0[b]
16:1n-7	5.5 ± 1.2[a]	6.4 ± 0.5[a]
18:0	3.8 ± 0.2[a]	2.7 ± 0.3[b]
18:1n-9	13.2 ± 0.5[a]	9.5 ± 0.3[b]
18:1n-7	3.3 ± 0.1	2.5 ± 0.0
18:2n-6	5.5 ± 0.5[a]	3.0 ± 0.2[b]
18:3n-3	0.8 ± 0.1[a]	0.7 ± 0.1[a]
18:4n-3	1.3 ± 0.3	2.0 ± 0.4
20:1n-9	4.2 ± 1.3[a]	9.6 ± 1.0[b]
20:4n-6	1.0 ± 0.3[a]	1.2 ± 0.6[a]
20:4n-3	0.6 ± 0.1	0.6 ± 0.0
20:5n-3	13.0 ± 2.0[a]	10.9 ± 1.2[a]
22:1n-11	4.2 ± 1.7[a]	11.2 ± 1.2[b]
22:5n-3	1.4 ± 0.1	1.4 ± 0.1
22:6n-3	13.4 ± 1.5[a]	11.2 ± 1.8[a]
Σ SAT	24.7 ± 1.5[a]	21.7 ± 0.8[b]
Σ MONO	32.2 ± 4.4[a]	41.4 ± 2.6[b]
Σ PUFA	39.8 ± 4.1[a]	33.7 ± 2.9[a]
Σ n-3	30.7 ± 3.0[a]	27.3 ± 2.7[a]
Σ n-6	7.5 ± 0.6[a]	4.8 ± 0.7[b]
DHA/EPA	1.0 ± 0.1[a]	1.0 ± 0.1[a]

[1]Values (means ± SD; n=4) in the same row containing different superscripts were significantly different ($p<0.05$).
[2]Data expressed as area % of FAME.

Table X. Fatty acid composition[1,2] of muscle polar lipid from haddock fed graded levels of dietary levels of lipid for 9 weeks

	Dietary lipid level (% dry wt.)	
Fatty acid	14	22
14:0	1.7 ± 0.4[a]	1.8 ± 0.2[a]
16:0	16.8 ± 0.6[a]	15.6 ± 0.8[a]
16:1n-7	2.7 ± 0.5[a]	2.8 ± 0.2[a]
18:0	4.6 ± 0.4[a]	3.5 ± 0.3[b]
18:1n-9	11.7 ± 0.9[a]	10.2 ± 0.4[b]
18:1n-7	3.2 ± 0.2	2.9 ± 0.1
18:2n-6	5.6 ± 0.3[a]	4.4 ± 0.7[b]
18:3n-3	0.7 ± 0.1[a]	0.8 ± 0.0[a]
18:4n-3	0.7 ± 0.1	0.9 ± 0.1
20:1n-9	1.7 ± 0.2[a]	3.2 ± 0.8[b]
20:4n-6	1.4 ± 0.2[a]	1.3 ± 0.1[a]
20:4n-3	0.8 ± 0.1	1.0 ± 0.1
20:5n-3	16.5 ± 0.4[a]	18.8 ± 0.4[b]
22:1n-11	0.4 ± 0.1[a]	0.7 ± 0.2[b]
22:5n-3	2.2 ± 0.0	2.6 ± 0.1
22:6n-3	22.6 ± 2.8[a]	23.4 ± 1.4[a]
Σ SAT	23.6 ± 0.3[a]	21.3 ± 0.8[b]
Σ MONO	21.1 ± 1.8[a]	21.4 ± 1.6[a]
Σ PUFA	53.6 ± 2.2[a]	56.1 ± 2.1[a]
Σ n-3	43.8 ± 3.0[a]	48.1 ± 1.6[b]
Σ n-6	8.7 ± 0.5[a]	7.3 ± 1.0[b]
DHA/EPA	1.4 ± 0.1[a]	1.2 ± 0.1[a]

[1]Values (means ± SD; n=4) in the same row containing different superscripts were significantly different ($p<0.05$).
[2]Data expressed as area % of FAME.

3. Serum Lipoproteins in Juvenile Haddock, *Melanogrammus aeglefinus*.

3.1 Introduction

In contrast to most farmed fish, the main energy storage organ in gadoids such as haddock and cod (*Gadus morhua*) is the liver (Dos Santos *et al.*, 1993; Shahidi and Dunajski, 1994; Nanton *et al.*, 2001). Our preliminary results showed that the liver lipid content (63.2 – 69%) of haddock was directly related to the dietary lipid intake, and the total muscle lipid content did not exceed 1.0% (Nanton et al, 2001). These observations suggest that the transport of lipid out of the liver to the muscle for storage in gadoids is limited. Lipoproteins are major carriers of lipid through the circulatory system in vertebrates (Chapman, 1980). Studies on the distribution and characterization of plasma lipoproteins in fish such as rainbow trout (Babin and Vernier, 1989), sea bass (Santulli et al, 1996) and red sea bream (Iijima *et al.*, 1995) have shown that the density interval of each lipoprotein class and the distribution of their lipids and apolipoproteins generally resemble those observed in mammals. Fish lipoproteins are classified, similarly to mammals, as very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL) (Iijima *et al.*, 1995; Babin and Vernier, 1989; Chapman, 1980).

The prevalence of fatty liver condition in farmed haddock fed commercial feeds is a major constraint in aquaculture development of gadoids. VLDL is a major transporter of lipid, in particular triacylglycerol, out of the liver to peripheral tissues such as the muscle in fish and other vertebrates (Sheridan, 1988). Fatty liver occurs in many avian

species when the increase in lipogenesis and deposition in the liver exceeds the synthesis and secretion of VLDL for export (Hermier, 1997). In several marine fish species, low plasma VLDL triacylglycerol concentrations were related to low muscle lipid levels (Ando and Mori, 1993). The aim of this study was to characterize the lipoproteins, lipid class and fatty acid composition of serum that will be useful in further investigating the fatty liver condition and lipid metabolism of haddock. The gas chromatographic (GC) lipid profiling technique (separation of lipid classes based on carbon number) of Kuksis *et al.* (1978) was used to evaluate the lipid classes in haddock serum.

3.2 Materials and Methods

Blood was collected from the caudal vein of six 24 h fasted, juvenile haddock in non-heparinized tubes and left at 0°C for 30 min. The serum was then obtained from the blood by low-speed centrifugation. The serum was stored at 4°C and analyzed within 24 h. Lipoprotein classes were separated by density ultracentrifugation for three haddock juveniles. The serum was adjusted to a density of 1.210 g/mL and to a final volume of 3.0 mL by the addition of potassium bromide (KBr) solution (aq). A discontinuous density gradient was then constructed in a Beckman ultracentrifugation tube for a Beckman SW41 swinging bucket rotor. The layers of graded density were introduced to the tube using the Buchler Auto Densi Flow II C apparatus equipped with a peristaltic pump. Two mL of 1.240 g/mL KBr (aq) was pumped into the bottom of the tube at a rate of 1 mL/min. The following solutions were then over-layered in order from bottom to top: 3 mL of serum sample adjusted to a density of 1.210 g/mL, 2.0 mL of 1.063 g/mL KBr (aq), 2.5 mL of 1.019 KBr (aq) and 2.2 mL of 1.006 g/mL KBr (aq). Determinations

of density were determined at the same temperature (15°C) used for centrifugation. The gradient was then centrifuged at $5.6X10^8 g_{max}$/min for 44 h at 15°C in a Beckman XL70 ultracentrifuge. No braking was used at the end of the run.

After centrifugation, the lipoprotein distributions and lipid compositions were examined over the entire gradient. Successive fractions of 0.4 mL were aliquotted from the meniscus downwards using a narrow-bore Pasteur pipette. Individual lipoprotein fractions were analyzed by the packed column GC lipid profiling technique of Kuksis *et al.* (1978). The protein content of the individual fractions was measured using a modified Lowry procedure (Markwell *et al.*, 1978).

Lipid and lipid class concentrations in haddock whole serum and the aliquotted density gradient fractions could not be accurately assessed using this GC lipid profiling method without first separating the total lipid into their lipid classes. There was overlapping of the chromatographic peaks between lipid classes due to the substantial amount of long-chain fatty acids present in these marine lipids (Fig. 1a). The lipids were first extracted from the whole serum or individual density gradient fractions using the method of Folch *et al.* (1957). The lipid was applied to a 40 μm silica gel column (J.T. Baker, Phillipsburg, NJ) and lipid classes were separated using solvents of increasing polarity. Cholesterol esters (Fig. 1c), triacylglycerols (Fig. 1d) and phospholipids (Fig. 1b) were eluted with 2% diethyl ether in hexane, chloroform and methanol, respectively (Christie, 1982).

Lipids were derivatized for injection on the GC. Polar lipids were first digested with phospholipase C, which converted phophatidylcholine, lysophosphatidyl choline and

sphingomyelin to diacylglyerol, monoacylglycerol and ceramide, respectively. The hydrolysis products were then converted to volatile trimethylsilyl ether derivatives using BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide; Supelco, Bellefonte, PA) (Kuksis *et al.*, 1978). The GC (model no. 5890, Hewlett Packard) was equipped with a silanized glass column (61cm X 2mm ID) containing 3% SP-2100 on 100-120 mesh Supelcoport (Supelco) and a flame ionization detector (FID). Samples were injected on column. The inlet temperature was held constant at 320°C. Helium (33 mL/min) was used as the carrier gas. The initial column temperature was held at 200°C for 1 min, increased at a rate of 5°C/min to a final temperature of 350°C and held for a further 8 min. The temperature of the FID was a constant 350°C. The absolute amounts of serum lipid were quantified by means of an internal standard (tridecanoylglycerol) added to the serum at the time of extraction at a relative proportion of 10-20% of the total.

Apolipoproteins of the principal classes of haddock serum lipoproteins were electrophoresed under reducing conditions on sodium dodecyl sulphate (SDS) polyacrylamide slab gels by the method of Laemmli (1970). Linear gradients of 5-19% and 4-20% (161-1195, Biorad, Hercules, CA) polyacrylamide were used for separation of proteins. Standard proteins, 85059 and 85611 (Biorad), of known molecular weight were run simultaneously. The protein bands on the gels were stained with Coomassie Blue R-250.

The commercial diet was evaluated following procedures described in AOAC (1990). The dry matter was determined by drying in an oven at 110°C for 24 h and crude ash by incineration in a muffle furnace at 550°C for 24 h. Crude protein (%N x 6.25)

was measured by the Dumas method (Ebling, 1968) using a Leco Nitrogen Determinator (Model FP-228, Leco Corporation, St. Joseph, MI). Total lipid was extracted (Bligh and Dyer, 1959) and determined gravimetrically.

Fatty acid composition of the diet, serum, serum lipid classes and serum lipoprotein classes were estimated from the methyl ester (FAME) derivatives of the fatty acids. The FAME were prepared using 7% boron trifluoride in methanol and heating to 100°C for 1h (Christie, 1982). The FAME were separated by gas chromatography (Hewlett Packard 6890 GC system equipped with a flame-ionization detector) using an Omegawax 320 capillary column (Supelco) as described in Chapter 2. FAME were identified by comparison of retention times with those of known standards (menhaden oil, Supelco-37).

Lipid classes from haddock whole serum were analyzed for fatty acids. Total lipid was first extracted using the method of Folch *et al.* (1957). The lipid classes were then separated on a 20 X 20 cm TLC plate (Silica Gel 60; Merck, Darmstadt, DE) developed in an hexane:diethylether:acetic acid 85:15:1 solvent system. Known lipid class standards were run simultaneously. Bands containing lipid were visualized by spraying the plates with 0.1% 2',7'-dichlorofluoroscein in methanol and observing under UV light. The identified bands were then scraped from the plate and transesterified to the FAME for GC analysis as described above. A known amount of 23:0 fatty acid standard was added to quantify the fatty acids present in a particular lipid class.

3.3 Results

The haddock were fed a commercial haddock feed (Ziegler Bros. Inc., Gardners, PA) based on herring meal and oil as a major source of protein and lipid, respectively. The diet contained 19% lipid and 50% protein on a dry matter basis (Table I). The essential fatty acids for cold-water marine fish were provided by herring meal and oil. The dietary fatty acids included docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at 11 and 10% of the total, respectively (Table I).

The whole serum from juvenile haddock was analyzed for lipid class composition (Fig. 2). The concentration of total lipid in the serum averaged 1297 ± 93 mg/dL (n=6). The phospholipids (57%) were the major lipid class transported in the serum followed by lesser amounts of triacylglycerol (16%), cholesterol ester (16%), and free cholesterol (11%) (Fig. 2). The whole serum lipid classes (phospholipid, triacylglycerol and cholesterol esters) were further subdivided and quantified according to the carbon number (C = total carbon number excluding glycerol moiety) of the molecule using the Kuksis *et al.* (1978) GC lipid profiling technique (Fig. 1). The 38C (36%) and 36C (24%) molecules were the predominant phospholipids (Fig. 3). The 49C (51%) and 47C (25%) molecules comprised a large part of the cholesterol esters (Fig. 4). The 50C (11%), 52C (17%), 54C (21%), 56C (21%) and 58C (15%) molecules formed the highest proportion of the triacylglycerols (Fig. 5). The cholesterol esters had the highest percentage (76%) of molecules containing long-chain fatty acids (defined here as a fatty acid with a chain length averaging 20C or greater) followed by the phospholipids (26%) and triacylglycerols (12%).

The fatty acid composition of the whole serum and individual lipid classes was also evaluated (Table II). The fatty acid composition of whole serum lipid closely resembled that of the phospholipid. The DHA content of the serum lipid and phospholipid were 25% and 28%, respectively. Cholesterol ester contained the highest proportion of DHA (36%) and triacylglycerol the lowest (10%). The EPA content of the serum lipid and phospholipid were 12 and 11%, respectively. Cholesterol ester also contained the highest amount of EPA (15%), whereas triacylglycerol (10%) and free fatty acid (9%) contained the lowest. Of the lipid classes, cholesterol ester had the highest proportion of polyunsaturated fatty acids (65%) followed in decreasing order by phospholipid (50%), free fatty acids (34%) and triacylglycerol (33%). Cholesterol ester also contained the lowest amount of saturated fatty acids (12%). Triacylglycerol (38%) and free fatty acids (32%) contained the highest proportion of monounsaturated fatty acids followed in descending order by phospholipid (25%) and cholesterol esters (22%). In this respect, the triacylglycerol fatty acid composition most closely resembled that of the free fatty acid, its hydrolysis product.

The lipoprotein composition of juvenile haddock was also analyzed through density gradient ultracentrifugation (Fig. 6; Table III). The lipoproteins comprised 2005 mg/dL serum. The high density lipoprotein (HDL) class predominated in haddock (1517 mg/dL serum) and peaked at a density of 1.097 g/mL. HDL consisted mainly of protein (51%) and phospholipid (32%). Triacylglycerol (6%) was a minor component of the HDL. The very low density lipoprotein (VLDL) class had a density of less than 1.017 g/ mL and was present at low concentrations (21 mg/dL serum) in the haddock serum.

Triacylglycerol comprised a much larger proportion (25%) of the VLDL, whereas protein (11%) and phospholipids (31%) were present at much lower levels in the VLDL compared to HDL. The low density lipoprotein (LDL) class peaked at a density of 1.056 g/mL. Similar to HDL, the LDL contained a large proportion of protein (48%). However, the lipid class composition of LDL was very different. The proportion of cholesterol ester (26% compared with 12% lipid) and triacylglycerol (25% compared with 11% lipid) were much higher in the LDL than HDL and phospholipid lower (39% compared with 64% lipid).

The fatty acid composition of the lipoprotein classes, VLDL and HDL, were evaluated (Table IV). The HDL fatty acid composition resembled that of the whole serum and differed considerably from VLDL. The proportion of polyunsaturated fatty acids was higher in HDL (51%) and whole serum (48%) than VLDL (36%). The higher level of polyunsaturated fatty acids in HDL compared with VLDL is reflected in its increased level of EPA (12 vs. 9%) and particularly DHA (27 vs. 15%). The proportion of saturated fatty acids was higher in VLDL (35%) compared to HDL (20%) and whole serum (23%).

The apolipoprotein components of VLDL, IDL, LDL and HDL lipoprotein classes in haddock serum were analyzed by gradient SDS polyacrylamide gel electrophoresis (Fig. 7). A large apo B-like protein was observed in the VLDL fraction. Other apolipoproteins, which may have been present in VLDL, were not observed due to their very low concentration in the serum. An apo-B like protein was also present in the LDL fraction. The IDL in haddock contained two species of apo B-like proteins. An apo-AI-

like protein, which was observed in LDL, was the predominant protein in the HDL fraction. HDL also contained substantial amounts of an apo A-II-like protein (Fig. 7b). This apo A-II-like protein was not observed in the lower density lipoproteins. An apo A-IV-like protein was present in both LDL and HDL fractions.

3.4 Discussion

HDL was the predominant lipoprotein class in haddock serum (1517 mg/dL) which was similar to other teleost fish (Babin and Vernier, 1989), as well as rodents (Lima *et al.*, 1998). Human and primate plasma (*Callithrix jacchus* sagüi) contains a higher proportion of LDL and LDL cholesterol ester than either rodents or haddock. This may be due to the presence of an active cholesterol ester transfer protein (CETP) in primates which catalyzes the heteroexchange and net mass transfer of cholesterol esters and triacylglycerol between HDL and apoB-containing lipoproteins (Lima *et al.*, 1998).

A relatively high concentration of lipid (1297 mg lipid /dL) was transported in haddock serum. This is typical of teleost fish, which are considered hyperlipidemic animals (Babin and Vernier, 1989). In contrast with mammals, fish preferentially utilize lipids rather than carbohydrates as their main source of energy (Watanabe, 1982), which may explain the comparatively higher levels of lipid and HDL transported in the serum of fish compared to mammals.

Phospholipid (57% lipid; 739 mg/dL) comprised a major proportion of the serum lipid in post-absorptive haddock. Over 70% of the muscle lipid in gadoids was phospholipid (Nanton *et al.*, 2001; Dos Santos *et al.*, 1993). Thus, a large proportion of phospholipid in the serum of haddock may be used to supply the membrane lipids of the

muscle. In teleost fish, the dominance of HDL as a lipoprotein class is reflected in the high levels of circulating phospholipid in the serum (Ando and Mori, 1993; Chapman, 1980). The level of triacylglycerol (16% serum lipid; 214 mg/dL serum) transport in the whole serum of haddock was low compared to the phospholipid concentration. The serum triacylglycerol concentration ranged from 46 to 339 mg/dL (6 to 31% serum lipid) in five marine fish species (Ando and Mori, 1993). Fish with the lowest muscle lipid levels (ca. 1%) had the lowest level of serum triacylglycerol (Ando and Mori, 1993). The concentration of total cholesterol (343 mg/dL) and percent esterified cholesterol (59%) in haddock serum were lower than the values reported for adult haddock (513 mg/dL, 69%) (Larsson and Fange, 1977).

In a comparison of percent lipid in the major lipoprotein classes, VLDL contained the highest level of triacylglycerol (28%), LDL the highest cholesterol ester (26%), and HDL the highest phospholipid (64%). A similar pattern of lipoprotein lipid composition is conserved across many animal species (Chapman, 1980; Lima *et al.*, 1998). Of the lipoprotein classes, VLDL contained the lowest proportion of protein (11%) and HDL the highest (51%). In five marine fish species, the protein in VLDL ranged from 10-29% and HDL from 49-57% (Ando and Mori, 1993). LDL in haddock contained a higher level of protein (48%) compared to other teleost fish species (20-35%; Chapman, 1980).

VLDL had a density of less than 1.017 g/mL. The density of LDL ranged from 1.050-1.085 g/mL and HDL from 1.085-1.169 g/mL. This density cutoff (1.085 g/mL) between LDL and HDL was also observed in rainbow trout lipoproteins (Babin, 1987). There were relatively low concentrations (<50mg/dL) of VLDL in the serum of cultured

juvenile haddock. This would be expected in a lean fish, which stores the majority of its lipid in the liver and contains a low amount in the muscle. The concentration of VLDL triacylglycerol in the serum of various marine fish species has been positively related to the lipid levels in the muscle (Ando and Mori, 1993). VLDL is a major vehicle for transporting storage lipid (triacylglycerol) out from the liver to extrahepatic tissues (i.e., muscle) in fish (Sheridan, 1988). This is consistent with our observation of the larger proportion of triacylglycerol in the VLDL (25%) compared to the other lipoprotein classes. In a previous study, the liver lipid levels increased significantly whereas the low levels of muscle lipid (aver. 1.0%) did not increase in juvenile haddock in response to increased amounts of lipid in the diet (14 to 22% lipid dry wt.), (Nanton *et al.*, 2001). Both the consistently low level of muscle lipid as dietary lipid increased, and the low concentration of serum VLDL triacylglycerol (<12 mg/dL serum) suggest that gadoid fish have little or no ability to transport the large amounts of deposited lipid (triacylglycerol; Dos Santos *et al.*, 1993) out of the liver to the muscle for storage. Although HDL was the major lipoprotein class it contained low amounts of triacylglycerol (6%). This indicates that HDL is not a major transporter of triacylglycerol (on a per particle basis) compared to VLDL in post-absorptive haddock.

The fatty acid composition of the whole serum lipid in haddock resembled that of the serum phospholipid, HDL (major lipoprotein class) and muscle (Nanton *et al.*, 2001). The serum (57%), HDL (64%) and muscle (82%; Nanton *et al.*, 2001) lipids all contained a high proportion of phospholipid. Polyunsaturated fatty acids were high in serum (48%), serum phospholipid (50%), HDL (51%) and muscle (53%). These

polyunsaturates were comprised mainly of the essential fatty acids (EFA) for marine fish (NRC, 1993), eicosapentaenoic acid (EPA) and, in particular, docosahexaenoic acid (DHA). In comparison with the proportion in the diet, these EFA (DHA) were concentrated in the serum. Quantitatively, a major part of these EFA are present in the serum as phospholipid in HDL particles. These observations suggest that a high proportion of EFA (phospholipid) are transported as HDL and transferred to the muscle with little alteration for the maintenance or construction of cell membranes. HDL has the ability to exchange phospholipid, mainly phosphatidylcholine (PC), with the plasma membranes. This may serve as an important mechanism for the turnover and renewal of the membrane phospholipids (Illingworth *et al.*, 1973; Eisenberg, 1984). In carp, HDL seems to play a central role for the transport and supply of dietary lipids (cholesterol, phospholipid and triacylglycerol) to the muscle and other tissues from the intestinal mucosa (Iijima *et al.*, 1990), instead of chylomicrons and VLDL in mammals (Davis, 1991). This has been proposed as a reason for the high HDL levels in fish (Iijima *et al.*, 1990).

Cholesterol esters contained the highest levels of polyunsaturated fatty acids (65%) and DHA (37%) of the lipid classes tested in the whole serum. In cod muscle, cholesterol esters also contained a higher level of polyunsaturates (65%) than the phospholipids and a high proportion of DHA (31%) (Addison *et al.*, 1968). In addition to phospholipids, cholesterol esters may also serve as an important transporter of EFA (DHA) in the serum to or from the muscle in gadoids. The high concentrations of polyunsaturated fatty acids or DHA in the cholesterol esters of haddock serum could be

explained by the specific activity of lecithin cholesterol acyl transferase (LCAT). LCAT is responsible for converting cholesterol and fatty acids from phospholipid to cholesterol esters on the surface of HDL. LCAT activity has been observed in the plasma of teleost fish (Black *et al.*, 1985; Dannevig and Norum, 1979). The LCAT present in HDL (human) demonstrates substrate specificity for the fatty acid in the *sn*-2 acyl position of the phospholipid. (Jonas, 2000). The polyunsaturated fatty acids, particularly EPA and DHA, are concentrated in the *sn*-2 acyl position of phospholipids in cod muscle (Bell and Dick, 1991).

Serum triacylglycerol and VLDL (lipoprotein class containing highest proportion of triacylglycerol) contained lower levels of polyunsaturated fatty acids (33%) and DHA (10%) than serum phospholipids and HDL, respectively. Serum triacylglycerol contained a higher proportion of the monounsaturated fatty acids (38%), including 20:1n-9 (4%) and 22:1n-11 (3%), compared to the other lipid classes. Long chain monounsaturated fatty acids, 20:1n-9 and 22:1n-11, are characteristic of herring oil-based diets (Table I). A high level of the long-chain monoenes are stored in the liver of gadoid fish as triacylglycerol (Nanton *et al.*, 2001; Dos Santos *et al.*, 1993). However, these long-chain monounsaturated fatty acids which are stored in the liver and transported out as serum triacylglycerol (VLDL), are incorporated at low levels in the muscle lipid. Haddock (n=6) fed the same standard diet contained only 0.2% 22:1n-11 and 14% monounsaturated fatty acids in the muscle lipid (Nanton and Lall, 2001; unpublished results). Dietary monounsaturated fatty acids (Murata and Higashi, 1980), particularly

22:1n-11 (Henderson *et al.*, 1982), are preferentially catabolized as an energy source, whereas the polyunsaturated EFA are conserved in fish (Murata, 1979).

This is the first study to use the GC lipid profiling technique of Kuksis *et al.* (1978) for estimation of lipid classes in a fish lipid. The presence of the long-chain fatty acids in fish lipids caused overlapping of the chromatographic peaks, particularly phospholipids with cholesterol esters and cholesterol esters with triacylglycerol (Fig. 1). Therefore, the lipid classes were separated before GC analysis. This problem was not encountered when using the Kuksis *et al.* (1978) method to measure the lipid profiles of mammalian species. See GC chromatograms depicted in Lima *et al.* (1998).

Oshima *et al.* (1983) used a modified technique of Kuksis *et al.* (1978) to analyze the components, based on total carbon number, in phosphatidylcholine (PC) isolated from cod muscle. PC is the major phospholipid class (69% of phospholipid) in cod muscle (Lie and Lambertsen, 1991). The pattern, in terms of total acyl carbon number, was similar for PC in the cod muscle compared with phospholipid in haddock serum. The most prominent components in the PC of cod muscle were also those having total acyl carbon numbers of 38 and 40 respectively (Oshima *et al.*, 1983). Molecular species of PC in the muscle of cod have been further determined by high performance liquid chromatography (HPLC) of its 1,2 diacylglycerol 3,5- dinitrobenzoyl derivatives. 16:0/22:6 (38C) and 16:0/20:5 (36C) were the major molecular species of PC in cod muscle (Bell and Dick, 1991). These molecular species should also be predominant in haddock serum phospholipid. The fatty acid composition (Lie and Lambertsen, 1991) and total acyl carbon number components (Oshima *et al.*, 1983) of cod muscle PC is

similar to that of phospholipid in haddock serum. PC is the predominant phospholipid class in both fish HDL (Chapman, 1980) and cod muscle (Lie and Lambertsen, 1991). The PC exchange between HDL and plasma membranes is greater than that for any other phospholipid class (Illingsworth *et al.*, 1973). These observations suggest that the phospholipid (PC) molecular species present in serum of gadoid fish as HDL closely match those species exchanged and incorporated into the muscle membranes.

Little data has been published to date on the molecular species of triacylglycerol in fish oil (Perona and Ruiz-Gutierrez, 1999). Complete chromatographic resolution of the molecular species of triacylglycerol in a fish oil is difficult to obtain because it is a complex mixture of different fatty acids ranging between 14 and 24 carbon atoms containing from 0 to 6 double bonds (Laasko *et al.*, 1990). The GC lipid profiling technique of Kuksis *et al.* (1978) separates the molecular species of triacylglycerol on the basis of their total acyl carbon number. This analysis provides information on the composition of the triacylglycerol molecules as a whole, rather than of their pooled constituent fatty acids. The 50C (11%), 52C (17%), 54C (21%), 56C (21%) and 58C (15%) molecules formed the highest proportion of the triacylglycerols in haddock serum. Wada *et al.* (1979) used a similar GC lipid profiling technique to analyze the triacylglycerol composition in the muscle of black cod. A similar triacylglycerol composition was observed in black cod muscle compared with haddock serum. The 50C (11%), 52C (23%), 54C (26%), 56C (21%) and 58C (11%) molecules were the major triacylglycerols observed in the muscle of black cod.

The situation in fish clearly resembles that in humans, in which the B apolipoprotein is the major component of VLDL and LDL, while the A apolipoproteins predominate in HDL (Chapman, 1980). In cod serum, the HDL proteins gave electrophoretic patterns essentially identical to that in humans (Skinner, 1983). Apo B is the main structural component of the low density lipoproteins (Davis, 1991). Haddock contained high molecular weight apo B-like proteins in the VLDL, IDL and LDL fractions. No other apolipoproteins were visualized in the VLDL fraction because of its low concentration in the serum. Low molecular weight apo C-like proteins (6-11 kDa) have been observed in the VLDL of other fish species such as red sea bream (Iijima *et al.*, 1995) and rainbow trout (Babin, 1987). Two apo B-like protein species were observed in haddock IDL, which was similar to rainbow trout (Babin, 1987). Only the smaller apo B-like protein species was present in haddock LDL, which was also comparable to rainbow trout (Babin, 1987). The presence of an apo A-I-like, but not the apo A-II-like protein was observed in haddock LDL. Both apo A-I and apo A-II-like proteins were present in the VLDL and LDL fractions of sea bass (Santulli *et al.*, 1996). The apo A-I-like protein was also found in VLDL of four marine fish species (flounder, red sea bream, amberjack and striped jack), but the VLDL from the flounder and red sea bream lacked the apo A-II-like proteins (Ando and Mori, 1993). The apo A-I-like protein was the major protein in the HDL of haddock. The smaller apo A-II-like protein (hepatic lipase activator; Dolphin, 1985) was also present in haddock HDL. Similar to haddock, red sea bream HDL contained both apo A-I-like and apo A-II-like proteins. A minor protein species at 45 kDa was also found in red sea bream HDL (Iijima *et al.*,

1990). An apolipoprotein of comparable size was present in haddock HDL and LDL. This protein in haddock LDL and HDL may be analagous to the apo A-IV protein (46 kDa), which is responsible for LCAT activation (apo A-I is the main activator) in human HDL (Dolphin, 1985). In general, the protein composition of the lipoprotein classes in haddock serum is similar to that of other fish species as well as humans. This suggests a similar functional role of the various lipoprotein classes.

Histological analysis of farmed juvenile cod liver revealed a high level of vacuolation in the hepatocytes similar to that observed in well-fed wild cod. The majority of vacuolation in cod appeared to be due to lipid accumulation and there were no other substantial changes; bile ducts appeared normal and there was no significant inflammation (Morais *et al.*, 2001). An electron microscopical study on turbot liver during the weaning period from live prey to dry food revealed that lipid deposition (steatosis) was located in the cisternae of the endoplasmic recticulum (ER). An overcharging of the transfer capacity for lipoproteins from the ER to Golgi apparatus may explain the lipid accumulation in the ER (Segner and Witt, 1990). This step in the metabolic pathway of lipoproteins in mammals is known to be rate limiting and its blockage leads to the observed phenomena (Chao *et al.*, 1986). This bottleneck for the synthesis of lipoproteins may contribute to the high lipid accumulation in the liver (Nanton *et al.*, 2001) and low VLDL concentration in the serum of cultured haddock.

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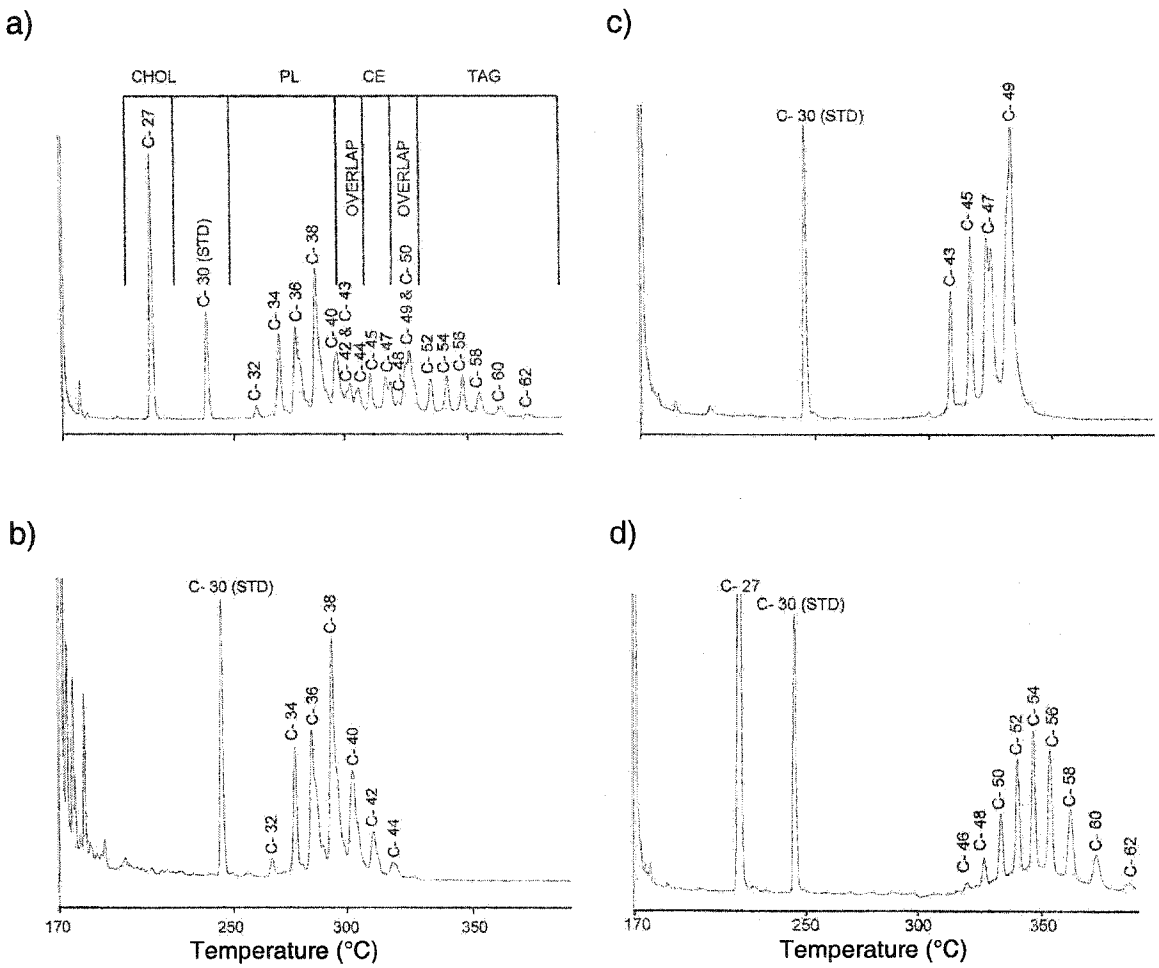

Fig 1. Gas chromatographic profiles of a) total lipid, b) phospholipids, c) cholesterol esters and d) triacylglycerols in juvenile haddock (*Melanogrammus aeglefinus*) serum analyzed by the gas chromatographic method of Kuksis *et al.*, 1978. Lipid molecular species are separated on the basis of their carbon number (excluding the glycerol moiety where appropriate). C-27, cholesterol, C-30, tridecanoin (internal standard); C-32, C-34, C-36, C-38, C-40, C-42, C-44 diacylglycerols and ceramides of phospholipids having the total number of carbons in their fatty acids equal to the indicated number; C-43, C-45, C-47, C-49 cholesterol esters containing 16, 18, 20 or 22 carbon fatty acids; C-46, C-48, C-50, C-52, C-54, C-56, C-58, C-60, C-62 triacylglycerols containing fatty acids whose total number of carbon atoms equal the designated number.

Table I. Proximate and fatty acid[1] composition of the commercial diet fed to haddock prior to blood collection.

Composition	% (dry wt.)		
Moisture	8.22	±	0.01
Protein	49.89	±	0.43
Lipid	19.07	±	0.30
Ash	13.72	±	0.26
Fatty acid	**% (total FA)**		
14:0	7.22	±	0.03
15:0	0.67	±	0.00
16:0	18.30	±	0.00
16:1n-7	9.59	±	0.05
16:2n-4	1.17	±	0.01
17:0	0.55	±	0.00
16:3n-4	1.12	±	0.01
16:4n-1	0.76	±	0.01
18:0	3.59	±	0.02
18:1n-9	9.71	±	0.03
18:1n-7	3.22	±	0.01
18:2n-6	3.64	±	0.09
18:3n-3	1.35	±	0.00
18:4n-3	2.53	±	0.01
20:1n-9	2.66	±	0.01
20:4n-6	0.77	±	0.00
20:4n-3	1.25	±	0.00
20:5n-3	9.91	±	0.03
22:1n-11	2.72	±	0.02
22:5n-3	1.75	±	0.01
22:6n-3	10.88	±	0.01
24:1n-9	0.59	±	0.02
Σ SAT	30.78	±	0.03
Σ MONO	30.31	±	0.04
Σ PUFA	37.90	±	0.02
Σ n-3	28.58	±	0.06
Σ n-6	5.31	±	0.08
DHA/EPA	1.10	±	0.00

[1]Data expressed as area percentage of FAME.

Abbreviations: FA=fatty acid, SAT=saturates, MONO=monounsaturates, PUFA=polyunsaturates, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid.

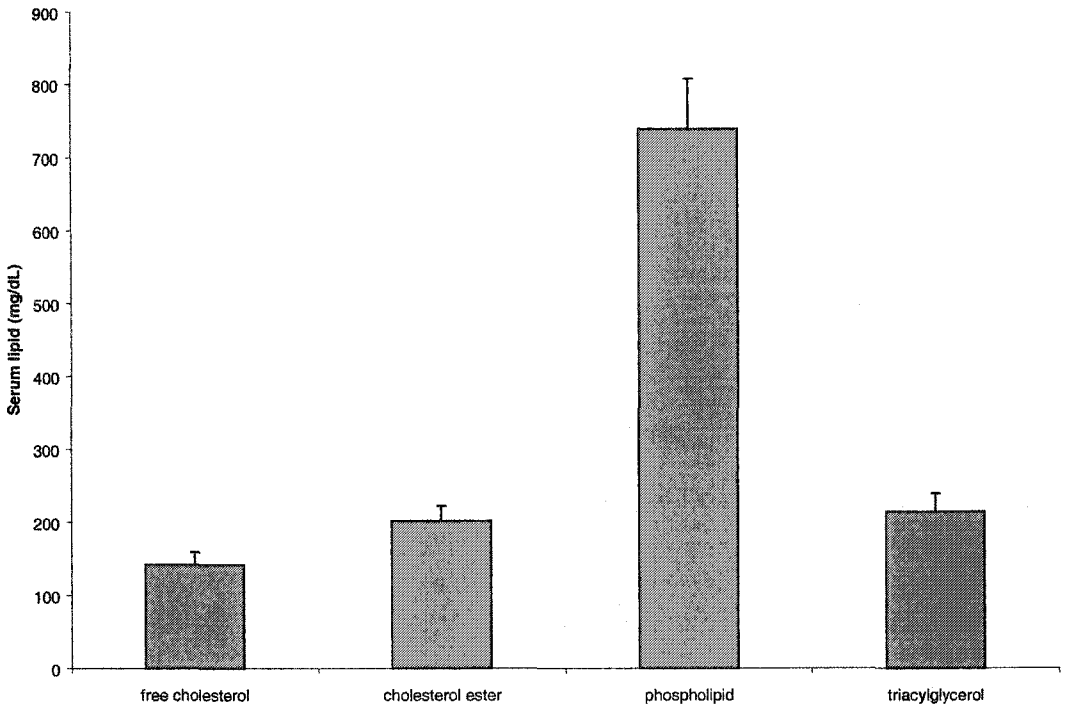

Fig 2. Lipid class composition (n=6) of whole serum in juvenile haddock (*Melanogrammus aeglefinus*).

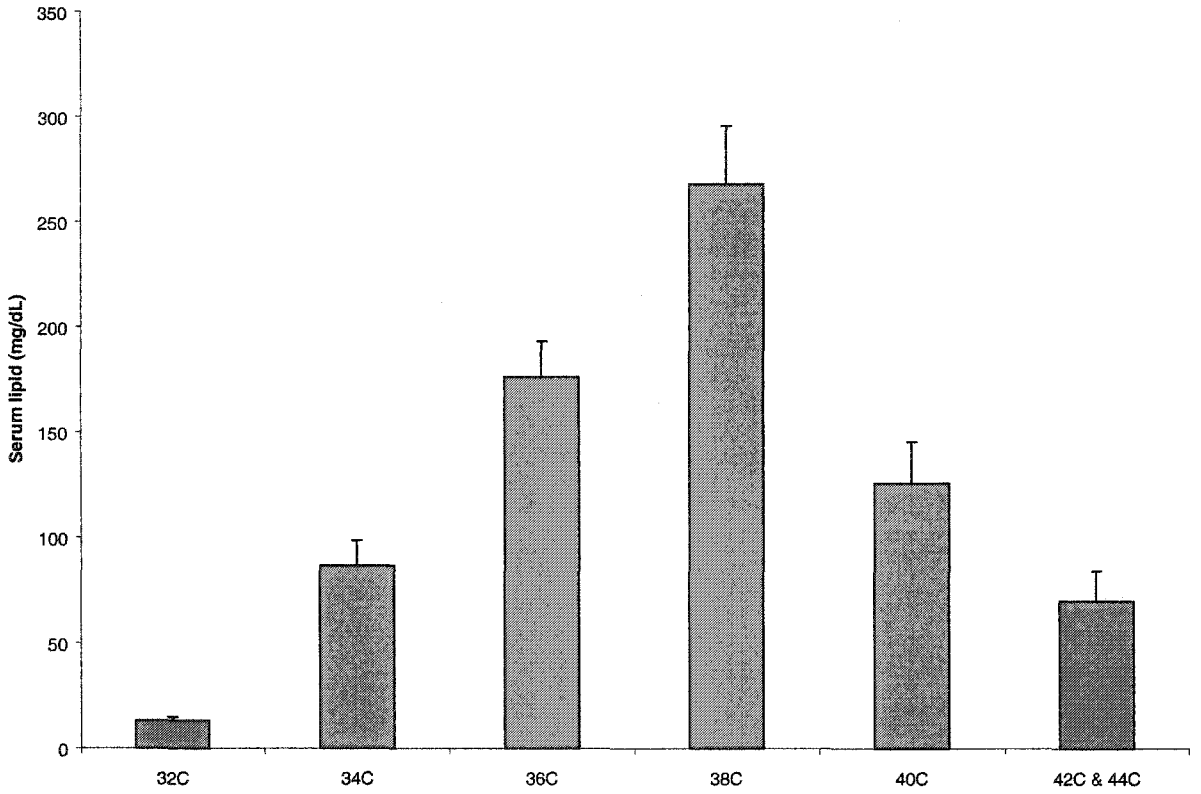

Fig 3. Phospholipid composition (n=6) of whole serum in juvenile haddock (*Melanogrammus aeglefinus*). The letter C refers to the total number of acyl carbons in the molecule.

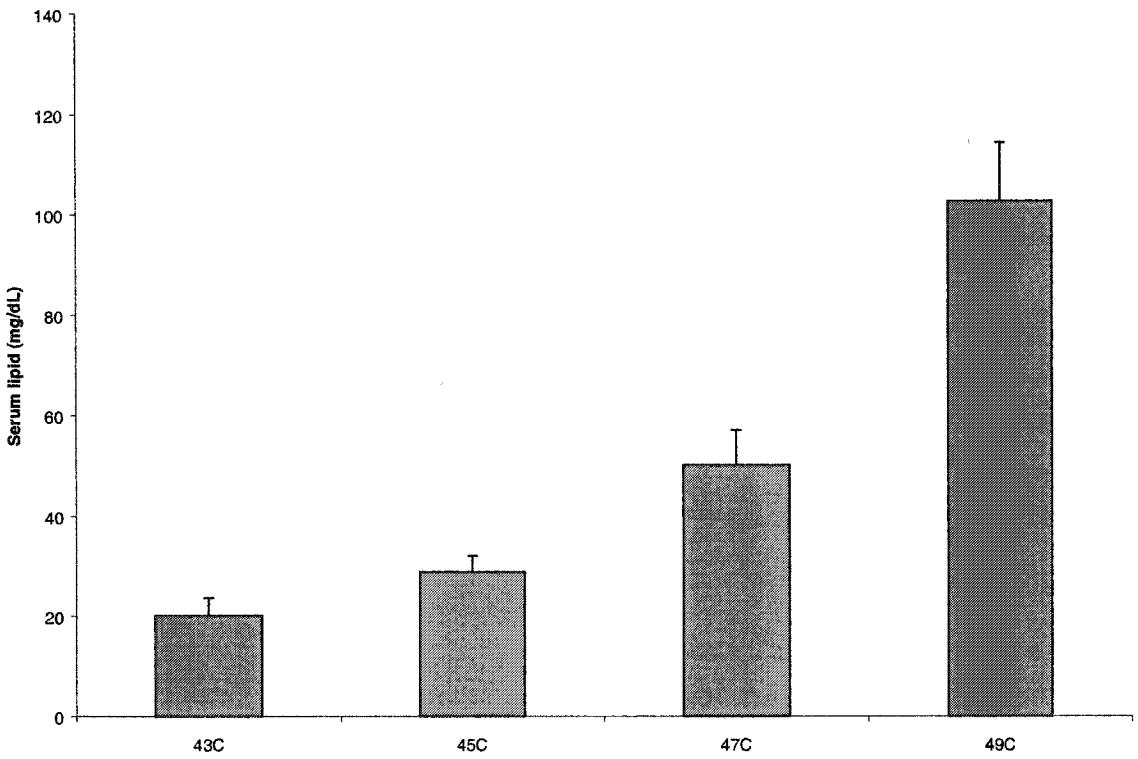

Fig 4. Cholesterol ester composition (n=6) of whole serum in juvenile haddock (*Melanogrammus aeglefinus*). The letter C refers to the total number of carbons in the molecule.

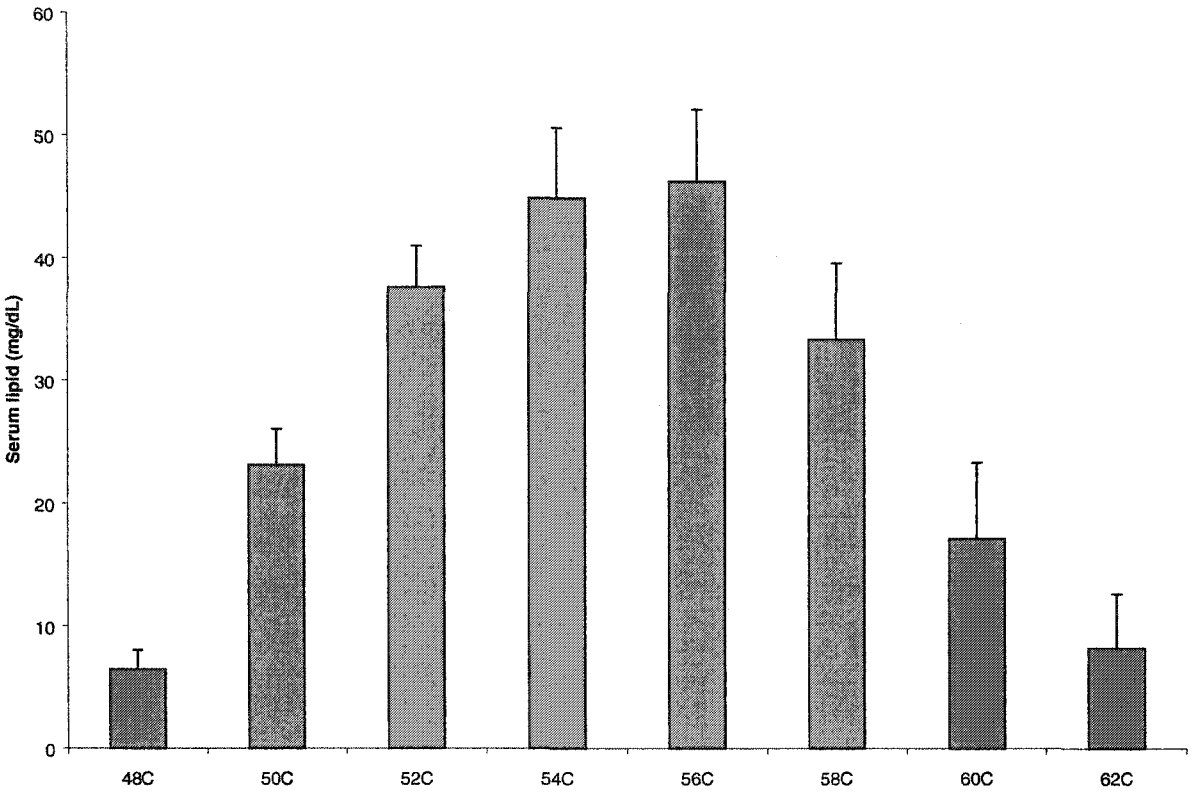

Fig 5. Triacylglycerol composition (n=6) of whole serum in juvenile haddock (*Melanogrammus aeglefinus*). The letter C refers to the total number of acyl carbons in the molecule.

Table II. Fatty acid composition[1] of total lipid (n=6), phospholipid (n=6), cholesterol ester (n=6), triacylglycerol (n=6) and free fatty acids (n=3) in juvenile haddock (*Melanogrammus aeglefinus*) serum.

Fatty acid	Total lipid	Phospholipid	Chol. ester	Triacylglycerol	Free fatty acid[2]
14:0	1.52 ± 0.22	0.87 ± 0.12	0.57 ± 0.12	2.88 ± 0.17	0.74 ± 0.13
16:0	17.91 ± 0.63	21.09 ± 0.35	9.52 ± 2.19	18.05 ± 1.31	21.92 ± 0.78
16:1n-7	3.02 ± 0.13	1.42 ± 0.07	1.76 ± 0.21	6.27 ± 0.22	2.38 ± 0.15
17:0	0.23 ± 0.11	0.26 ± 0.02	0.15 ± 0.04	0.37 ± 0.03	0.48 ± 0.06
18:0	2.58 ± 0.14	2.56 ± 0.18	1.83 ± 0.44	4.78 ± 0.92	9.08 ± 1.68
18:1n-9	13.94 ± 0.65	14.27 ± 0.86	9.82 ± 1.31	17.83 ± 0.89	15.76 ± 0.64
18:1n-7	3.96 ± 0.60	3.77 ± 0.21	2.21 ± 0.32	4.74 ± 0.49	5.56 ± 0.27
18:2n-6	3.27 ± 0.21	2.77 ± 0.15	3.81 ± 0.36	4.18 ± 0.24	3.56 ± 0.44
18:3n-3	0.45 ± 0.03	0.22 ± 0.01	0.59 ± 0.11	0.80 ± 0.05	0.36 ± 0.02
18:4n-3	0.70 ± 0.10	0.24 ± 0.04	0.62 ± 0.13	1.87 ± 0.15	1.36 ± 0.28
20:1n-9	2.97 ± 0.26	2.35 ± 0.16	2.74 ± 0.40	4.49 ± 0.37	2.98 ± 0.26
20:4n-6	1.36 ± 0.10	1.64 ± 0.14	1.29 ± 0.17	0.72 ± 0.08	1.20 ± 0.09
20:4n-3	0.78 ± 0.05	0.59 ± 0.07	1.06 ± 0.25	0.85 ± 0.06	0.90 ± 0.35
20:5n-3	11.65 ± 0.69	10.97 ± 1.15	14.71 ± 1.14	9.62 ± 0.77	8.93 ± 1.69
22:1n-11	1.45 ± 0.22	0.52 ± 0.09	2.54 ± 0.75	2.99 ± 0.46	1.40 ± 0.16
22:5n-6	0.47 ± 0.03	0.60 ± 0.03	0.59 ± 0.02	0.34 ± 0.13	0.23 ± 0.20
22:5n-3	1.72 ± 0.10	1.82 ± 0.18	1.82 ± 0.12	1.57 ± 0.19	1.44 ± 0.17
22:6n-3	25.11 ± 1.44	27.87 ± 0.68	36.45 ± 2.73	9.52 ± 1.52	12.19 ± 2.04
Σ SAT	22.62 ± 0.77	25.21 ± 0.57	12.43 ± 2.74	27.25 ± 2.19	33.02 ± 2.41
Σ MONO	27.78 ± 0.93	24.81 ± 1.13	22.39 ± 2.58	38.34 ± 1.34	31.85 ± 1.13
Σ PUFA	48.16 ± 1.53	49.75 ± 1.58	64.75 ± 3.84	32.93 ± 2.53	34.20 ± 3.20
Σ n-3	41.15 ± 1.41	42.01 ± 1.44	56.11 ± 3.73	25.40 ± 2.11	26.30 ± 2.93
Σ n-6	6.29 ± 0.24	5.94 ± 0.25	7.54 ± 0.39	5.53 ± 0.42	5.96 ± 0.88
DHA/EPA	2.16 ± 0.19	2.56 ± 0.28	2.48 ± 0.16	0.99 ± 0.09	1.37 ± 0.07

[1]Data expressed as area percentage of FAME.

[2]mg FFA/dL serum = 13.5 ± 1.9

Abbreviations: SAT=saturates, MONO=monounsaturates, PUFA=polyunsaturates, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid.

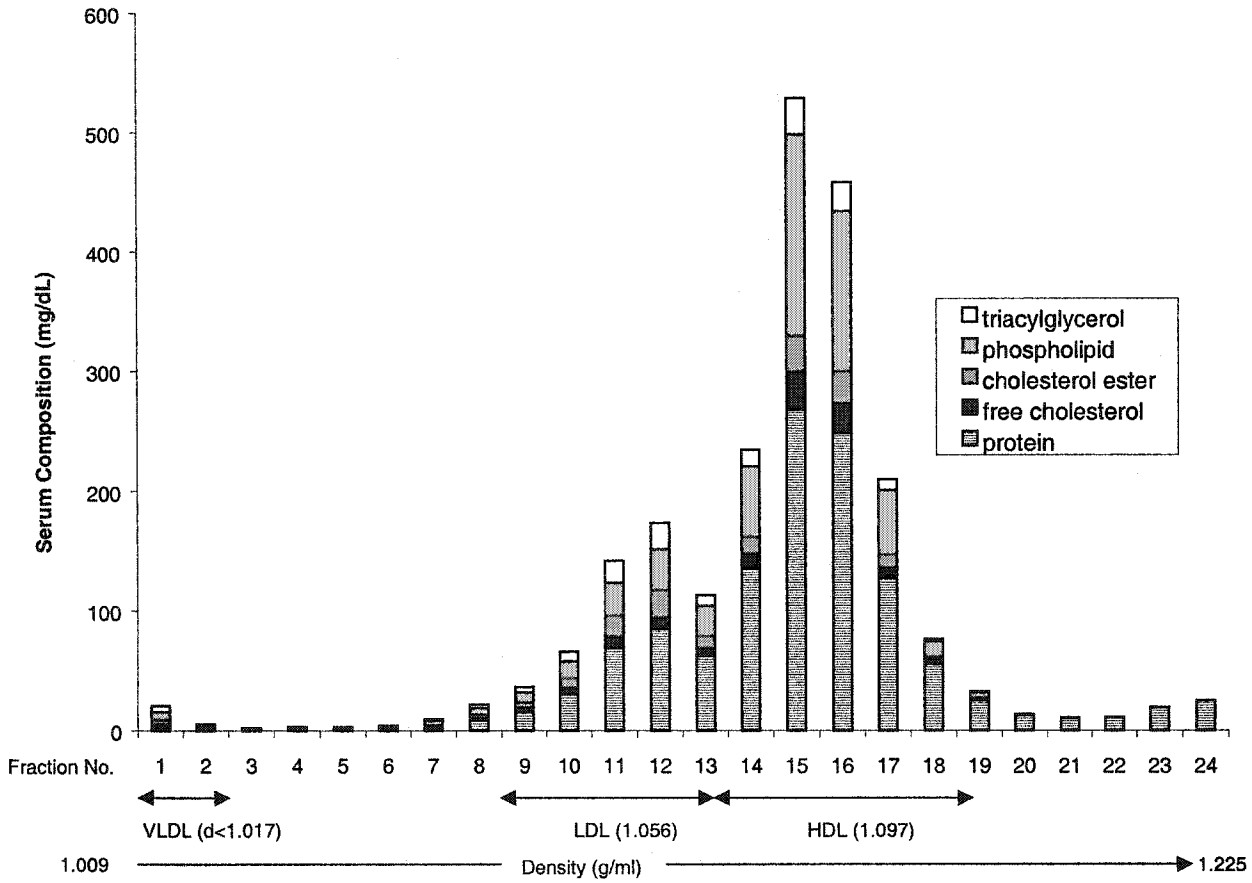

Fig. 6. Density gradient ultracentrifugation profile (n=3) of lipoproteins present in the serum of juvenile haddock (*Melanogrammus aeglefinus*). Peak density of each lipoprotein class in brackets and expressed as g/ mL. The density of the lipoprotein fractions were VLDL (<1.017g/mL), LDL (1.050-1.085 g/mL) and HDL (1.085-1.169 g/mL).

Table III. Content and composition of serum lipoproteins (n=3) in juvenile haddock (*Melanogrammus aeglefinus*). The density of the lipoprotein fractions were VLDL (<1.017g/mL), LDL (1.050-1.085 g/mL) and HDL (1.085-1.169 g/mL).

	VLDL	LDL	HDL
Lipoprotein* (mg/dL serum)	20.73 ± 12.90	467.37 ± 102.90	1516.77 ± 457.12
Cholesterol (%)	14.44 ± 2.64	5.70 ± 1.34	6.27 ± 1.27
Cholesterol ester (%)	18.59 ± 3.98	13.25 ± 1.64	5.67 ± 0.12
Phospholipid (%)	31.27 ± 5.66	20.05 ± 2.96	31.63 ± 2.74
Triacylglycerol (%)	24.56 ± 0.76	12.80 ± 2.06	5.59 ± 1.17
Apolipoprotein (%)	11.13 ± 5.64	48.20 ± 5.05	50.84 ± 3.87

* Approximation for HDL and LDL due to overlapping peak areas at fraction 14 (see Fig. 2).

Table IV. Fatty acid composition (%) of juvenile haddock (*Melanogrammus aeglefinus*) whole serum (n=6), very low density lipoprotein (VLDL; n=3) and high density lipoprotein (HDL; n=3). The density of the lipoprotein fractions was <1.017g/mL for VLDL and 1.085-1.169 g/mL for HDL.

Fatty acid	Serum	VLDL	HDL
14:0	1.52 ± 0.22	1.43 ± 0.44	1.12 ± 0.41
16:0	17.91 ± 0.63	21.31 ± 3.36	15.81 ± 5.13
16:1n-7	3.02 ± 0.13	3.84 ± 0.40	2.70 ± 0.40
18:0	2.58 ± 0.14	10.27 ± 3.40	2.38 ± 0.43
18:1n	18.26 ± 0.81	17.79 ± 2.04	19.68 ± 0.65
18:2n-6	3.27 ± 0.21	3.59 ± 0.50	3.37 ± 0.52
18:3n-3	0.45 ± 0.03	0.73 ± 0.17	0.38 ± 0.04
18:4n-3	0.70 ± 0.10	1.04 ± 0.13	0.58 ± 0.07
20:1n-9	2.97 ± 0.26	3.37 ± 0.62	2.85 ± 0.43
20:4n-6	1.36 ± 0.10	0.82 ± 0.19	1.53 ± 0.30
20:4n-3	0.78 ± 0.05	0.62 ± 0.09	0.74 ± 0.07
20:5n-3	11.65 ± 0.69	9.34 ± 1.36	12.15 ± 2.04
22:1n-11	1.45 ± 0.22	2.69 ± 0.45	1.18 ± 0.22
22:4n-6	0.45 ± 0.09	0.00 ± 0.00	0.34 ± 0.04
22:5n-6	0.47 ± 0.03	0.16 ± 0.14	0.50 ± 0.04
22:5n-3	1.72 ± 0.10	1.53 ± 0.11	1.81 ± 0.15
22:6n-3	25.11 ± 1.44	15.05 ± 1.06	27.41 ± 2.15
Σ SAT	22.62 ± 0.77	35.28 ± 6.14	19.84 ± 5.98
Σ MONO	27.78 ± 0.93	28.04 ± 3.05	28.45 ± 2.15
Σ PUFA	48.16 ± 1.53	35.74 ± 2.40	51.42 ± 4.43
Σ n-3	41.15 ± 1.41	29.18 ± 2.43	43.20 ± 3.72
Σ n-6	6.29 ± 0.24	4.85 ± 0.67	6.39 ± 0.69
DHA/EPA	2.16 ± 0.19	1.62 ± 0.12	2.29 ± 0.32

[1]Data expressed as area percentage of FAME.

Abbreviations: SAT=saturates, MONO=monounsaturates, PUFA=polyunsaturates, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid.

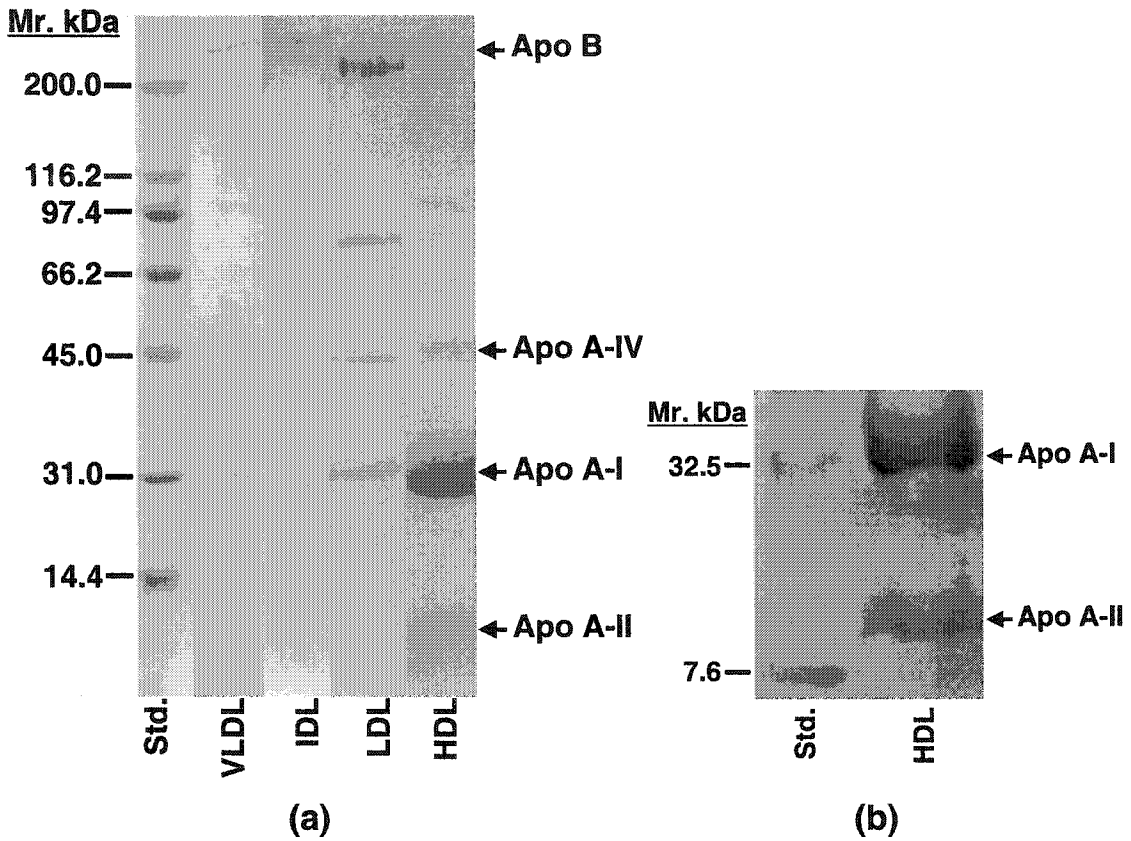

Fig 7. Sodium dodecyl sulphate polyacrylamide gel electrophoretic analysis of the apolipoproteins of the principal classes of haddock (*Melanogrammus aeglefinus*) serum lipoproteins. Gel a) was a linear gradient of 5-19% polyacrylamide and b) a linear gradient of 4-20% polyacrylamide (161-1195, Biorad, Hercules, CA). Gels were stained with Coomassie Blue R250. Standard proteins, a) 85059, Biorad and b) 85611, Biorad, of known molecular weight were run simultaneously. The density of the lipoprotein fractions were: VLDL <1.017g/mL; IDL 1.029-1.055 g/mL); LDL 1.050-1.085 g/mL; HDL 1.085-1.169 g/mL.

4. Effect of Dietary Lipid Level on Fatty Acid ß-Oxidation in Various Tissues of Haddock, *Melanogrammus aeglefinus* L.

4.1 Introduction

Fatty liver condition has been observed in cultured gadoid fish (i.e., haddock or cod) fed formulated diets. From field observations, it is obvious that somatic growth and health of fish with enlarged fatty livers is affected. Gadoids retain the majority of the incoming dietary lipid as triacylglycerol in the liver and store very little in the muscle (ca. 1% total lipid) (Nanton *et al.*, 2001; Dos Santos *et al.*, 1993). The liver lipid levels of cultured cod fed formulated diets to satiation can surpass 70% wet wt. (Shahidi & Dunajski, 1994). The hepatosomatic index (HSI) of cultured cod often exceeds 12% (Jobling, 1988; Shahidi & Dunajski, 1994), whereas in wild cod a HSI of 2-6% is considered normal (Jobling, 1988).

A low concentration of very low density lipoprotein (VLDL; <50 mg/dL) was observed in the serum of post-absorptive juvenile haddock (Nanton *et al.*, 2001). This suggests a low level of lipid transport out of the liver in haddock. Plasma VLDL is a major transporter of triacylglycerol out of the liver (Sheridan, 1988) and VLDL triacylglycerol levels in the plasma were positively correlated with muscle lipid storage in marine teleost fish species (Ando *et al.*, 1995).

A recent study on the peroxisomal and mitochondrial ß-oxidation capacities of various tissues was carried out on Atlantic salmon (Froyland *et al.*, 2000). Oxidation of palmitoyl-L-carnitine was higher in red muscle compared to the liver or white muscle in

Atlantic salmon, when expressed on a per mg protein or per g wet weight basis (Froyland *et al.*, 2000; Froyland *et al.*, 1998). The red muscle and heart of rainbow trout (Bilinski & Jonas, 1970) and mackerel (Fjermestad *et al.*, 2000) also have higher specific activity per mg protein for ß-oxidation of fatty acids than the white muscle or liver. These more active, pelagic teleost fish species tend to have a higher percentage of red fibres in their trunk musculature (Greer-Walker & Pull, 1975). These fish species also store lipid predominantly in the muscle (Fjermestad *et al.*, 2000; Polvi & Ackman, 1992). It was of interest to determine whether the lipid deposition and transport patterns, as well as the lower activity levels of the demersal haddock are reflected in their ß-oxidation enzyme activities.

In the mitochondria isolated from rainbow trout muscle, fatty acids were oxidized to carbon dioxide by mitochondria and this oxidation was greatly stimulated by coenzyme A and carnitine. This observation suggested that fatty acid oxidation in fish mitochondria occurs via the ß-oxidation pathway observed in mammals (Bilinski & Jonas, 1970). Fatty acid ß-oxidation in fish and other vertebrates also occurs in another organelle, the peroxisome (Sargent *et al.*, 1989). Peroxisomal ß-oxidation is relatively more important in the liver than the muscle of fish. The peroxisomes accounted for 30-50% (Crockett & Sidell, 1993a,b) up to 100% (Froyland *et al.*, 2000) of ß-oxidation activity in the liver of marine teleost fish.

Evaluation of the major sites of lipid catabolism may provide further insight into the cause(s) of abnormally high liver lipid deposition in cultured haddock or cod. The prevalence of fatty liver condition in these gadoid species fed formulated diets is a major

constraint for their aquaculture development. The catabolism of a radiolabelled fatty acid, [1-C^{14}] palmitoyl-CoA, through ß-oxidation (peroxisomal and mitochondrial) was evaluated in the liver, red and white muscle of juvenile haddock fed three levels of lipid (12, 18 and 24%) in the diet. The lipid and fatty acid composition of these haddock tissues was also determined and related to ß-oxidation activity. To our knowledge, this is the first attempt to measure ß-oxidation activity in the tissues of a demersal gadoid fish (i.e., haddock or cod).

4.2 Materials and Methods

Experimental conditions

Feeding trials for haddock fingerlings (initial weight 7.07 ± 0.17 g) were conducted at the NRC Aquaculture Station at Sandy Cove, Nova Scotia, where the fish were hatched and reared to attain their initial size at the start of the experiment. Fifty fish were randomly allotted into each of 6 tanks and adapted to experimental conditions for 3 weeks before the feeding trial started. Fish were hand-fed experimental diets to satiation three times daily on weekdays and twice daily on weekends during the 20 week experimental period. Filtered and UV-treated seawater (salinity, 30 ppt) was supplied to each tank at a rate of 4 L/min in a flow-through system. Water volumes in each tank were maintained at 250 L (holding capacity: 320 L) to ensure a renewal rate of approximately once every hour. Fish were held on a 12 h dark : 12 h light photoperiod with the light intensity at the water surface ranging between 40 and 60 lux. Dissolved oxygen levels and water temperature were measured every morning and averaged 9.5 mg/L (8.1-11.7 mg/L) and 11.9°C (10.5-13.5°C), respectively. Fish were bulk-weighed

and counted at the beginning and at set intervals during the experiment. Fish were fasted for 24 hours prior to weighing and counting.

Ingredients and preparation of the diets are described in Nanton *et al.* (2001). Experimental diets were formulated using practical ingredients, of which herring meal comprised the major protein source. Increasing amounts of herring oil were incorporated to provide 12, 18 and 24% lipid in isonitrogenous diets. Soybean lecithin (ADM Lecithin, Decatur, IL, USA) was included in each diet at a level of 2%.

Chemical analysis of feed and tissues

Chemical compositions of the experimental diets were evaluated following procedures described in AOAC (1990). The dry matter (DM) was determined by drying in an oven at 110°C for 24-h and crude ash by incineration in a muffle furnace at 550°C for 24 h. Crude protein (%N x 6.25) was measured using a Leco Nitrogen Determinator (Model FP-228, Leco Corporation, St. Joseph, MI)

Total lipid (Bligh & Dyer, 1959) was extracted and determined gravimetrically for the diet and haddock tissues. Fatty acid composition of the extracted lipid was estimated from the fatty acid methyl ester (FAME) derivatives of the fatty acids. The FAME were prepared using 7% boron trifluoride in methanol and heating to 100°C for 1 h (Christie, 1982). The FAME were separated by gas chromatography (Hewlett Packard 6890 GC system equipped with a flame-ionization detector) using an Omegawax 320 capillary column (Supelco, Bellefonte, PA, USA) as described in Chapter 2. FAME were identified by comparison of retention times with those of known standards (menhaden oil; Supelco, Bellefonte, PA, USA).

Homogenization of tissue for enzyme assay

Fish were transported from the NRC Aquaculture Station at Sandy Cove, Nova Scotia to the laboratory (Institute for Marine Biosciences, NRC, Halifax, Nova Scotia) alive so that fresh tissue samples could be obtained for the enzyme assay. The fish were killed with an overdose of TMS (tricaine methanesulfonate) prior to sampling.

The preparation of tissue homogenates followed the method of Froyland *et al.* (2000). The liver and parts of the white and red muscle were removed, weighed and homogenized in 20% (w/v) ice-cold sucrose solution containing 0.25 M sucrose in 10 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 (with KOH). The tissues were then centrifuged (1880 g for 10 min at 2°C). The resulting post-nuclear or E-fraction was collected and portions were used immediately to determine mitochondrial and peroxisomal ß-oxidation.

Enzyme assay

Total (mitochondrial and peroxisomal) ß-oxidation was determined in postnuclear E-fractions as acid-soluble products following incubation with radiolabelled [1-C^{14}] palmitoyl-CoA (modified method of Froyland *et al.*, 1995). The assay buffer stated below contained 12.6 mM HEPES (pH 8.1 with KOH), 15.7 mM $MgCl_2$, 82.5 mM KCl, 12.6 mM dithiothreitol, 6.3 mM ADP, 1.2 mM carnitine, 0.2 mM NAD^+ and 0.6 mM EDTA. The E-fractions from the various haddock tissues were added in 300 μL of the homogenization buffer, mixed with 250 μL of the assay buffer in microcentrifuge tubes preincubated for 2 minutes at the assay temperature of 20°C. These assay conditions

produced optimal ß-oxidation rates for these fish tissues (Stubhaug, 2002). The fatty acid substrate, [1-C^{14}] palmitoyl-coA, was then added to the tubes to give a final concentration of 27 μM. After incubation for 10 min, the oxidation was stopped by addition of 150 μL of 1.5 M KOH. Fifty μL of fatty acid free bovine serum albumin (BSA, 100 mg/mL) was added to the suspension in order to bind nonoxidized substrate and then 500 μL of 4 M $HClO_4$ was added to precipitate nonoxidized substrate bound to BSA. The total solution was then centrifuged at 1880 g for 15 min. Aliquots of 500 μL were transferred to a scintillation vial containing 8 mL of liquid scintillation cocktail (Ready Safe Scintillation Cocktail for Aqueous Samples, Beckman Instruments, Fullerton, CA, USA) and assayed for radioactivity in a liquid scintillation counter (WinSpectral 1414, Perkin Elmer Life Sciences, Wallac Oy, Turku, FIN).

The peroxisomal ß-oxidation (cyanide insensitive) was measured in the same manner as total ß-oxidation except the assay medium contained 2 mM potassium cyanide as described by Mannaerts *et al.* (1979). Mitochondrial ß-oxidation was calculated as the difference between total and peroxisomal ß-oxidation.

All assays were run in duplicate and performed under conditions where product formation was linear with respect to the amount of protein in the assay mixture. Protein was determined by the dye binding method of Bradford (1976) using a commercial kit (Bio-Rad, Hercules, CA, USA) with gamma-globulin as a standard.

The [1-C^{14}] palmitoyl-CoA, palmitoyl-CoA and other cofactors used in this enzyme assay were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analyses

Significant differences in hepatosomatic indices, lipid and fatty acid composition between diets for liver, red muscle, white muscle and heart as well as total hepatic ß-oxidation per g fish were estimated using one-way analysis of variance (ANOVA) and a post-hoc pairwise comparison test (Tukey's HSD) test at the $p<0.05$ level. Significant differences in E-fraction protein and ß-oxidation activities on a per mg protein and per g wet tissue basis were estimated using two-way ANOVA with three levels for diet (12, 18 and 24% lipid) and three levels for tissue (liver, red muscle and white muscle). Specific treatment comparisons were made using Duncan's New Multiple Range Test at the $p<0.05$ level. Percentage data were transformed using the arcsine of the square root of the proportion before ANOVA was applied. The statistical package (Systat 8.0, SPSS Inc., Chicago, IL, USA) for personal computers was used to calculate ANOVA.

4.3 Results

Diet composition

The diets were formulated using practical ingredients, of which herring meal comprised the major protein source. The lipid level was adjusted by changing the proportion of herring oil incorporated in the diets. The protein was maintained between 53-55% DM, whereas the lipid was increased from 14 to 26% DM (Table I). The fatty acid composition of the experimental diets was similar with respect to the essential fatty acids: docosahexaenoic acid (DHA) comprising ca. 8%; and eicosapentaenoic acid (EPA) comprising 9-13% of the total fatty acids. The diets had a DHA/EPA ratio of 0.6-0.8 (Table II). Soybean lecithin was added at a level of 2% to all diets to promote lipid

transport out of the liver. The proportion of soybean lecithin to total lipid was highest in the diet containing 12% lipid and decreased as the lipid content of the diet increased from 18 to 24%. This was reflected in a higher percentage of 18:2n-6 as the lipid in the diet decreased from 24 to 18 and 12%.

Growth and hepatosomatic index

Growth, feed utilization and hepatosomatic indices of juvenile haddock (initial weight of 7 g) fed experimental diets containing different lipid levels (12, 18 and 24% lipid) were measured for two time periods, from 0 to 9 and 9 to 31 weeks (Table III). The specific growth rate appeared to be higher from 0-9 compared with 9-31 weeks. Feed conversion ratios were <0.8 for juvenile haddock fed 12, 18 and 24% lipid from 0-9 and 9-31 weeks. The hepatosomatic index (HSI) increased significantly ($p<0.05$) in juvenile haddock fed an increased level of dietary lipid (12, 18 and 24%) at 31 weeks (Table III).

Lipid and fatty acid composition of tissues

The total lipid and fatty acid composition was measured in the liver (Table IV), red muscle (Table V), white muscle (Table VI) and heart (Table VII) of juvenile haddock fed graded levels of lipid for 31 weeks. The total lipid content was highest in the liver (73.8-79.3%) and increased significantly as dietary lipid was increased from 12 to 18 and 24%. There was no significant increase in the lipid content of the red muscle, white muscle or heart in juvenile haddock fed an increased level of dietary lipid. Of the tissues tested, the liver most closely mirrored the fatty acid composition of the diet. Monounsaturated fatty acids prevalent in the herring-oil based diet, in particular the long-

chain 20:1n-9 and 22:1n-11, comprised a higher percentage of the total FA in the liver compared to the red muscle, white muscle or heart. The red muscle, white muscle and heart contained a higher level of polyunsaturated fatty acids (PUFA; >55%) than the liver. In a comparison of the muscular tissues, the red muscle and heart contained a higher percentage of lipid than the white muscle (2.5 and 3.2 fold, respectively). The heart and red muscle also contained a higher level of monounsaturated fatty acids, including 20:1n-9 and 22:1n-11, than the white muscle. The 18:2n-6 composition of the diet was mirrored in the fatty acid composition of the haddock tissues. There was a significant decrease in the level of soybean lecithin-derived 18:2n-6 in the liver, heart, red muscle and white muscle of haddock as the lipid was increased (soybean lecithin: lipid ratio decreased) in the diet.

ß-oxidation activity of tissues

The rate of product formation for the total ß-oxidation enzyme assay in the liver, red and white muscle of haddock was linear for the range of E-fraction protein tested ($p<0.005$; $R^2>0.96$; Fig. 1). The amount of E-fraction protein used in the experimental ß-oxidation enzyme activity assays fell within this linear range.

The protein content of E-fraction was measured for the liver, red and white muscle of juvenile haddock fed diets containing 12, 18 and 24% lipid (Fig. 2). The E-fraction protein per g wet tissue was significantly higher in the white muscle followed by the red muscle and liver. The E-fraction protein per g wet tissue was significantly lower ($p<0.05$) in the liver and white muscle of haddock fed the highest level of dietary lipid (24%) compared to the lowest (12%).

The same general trends were observed for mitochondrial and peroxisomal ß-oxidation enzyme activities of palmitoyl-CoA expressed on a per mg protein (Fig. 3) or a per g wet tissue (Fig. 4) basis in the liver, red and white muscle of haddock. The results indicate that red muscle has significantly higher mitochondrial ß-oxidation specific activity than the white muscle. There was no mitochondrial ß-oxidation activity detected in the liver of haddock. A significantly higher amount of peroxisomal ß-oxidation was observed in the liver, followed by the red and white muscle, respectively. The peroxisomal ß-oxidation rate per g wet tissue decreased significantly in the liver of haddock fed 24% compared with 12% lipid. A significantly higher peroxisomal ß-oxidation rate per g wet tissue was observed in the red muscle of haddock fed 18% lipid compared with 12%. The white muscle (2.02 nmol/min/g) and liver (1.80 nmol/min/g) were similar in terms of their total ß-oxidation activity on a per g wet tissue basis, although it is mainly mitochondrial ß-oxidation in the muscle and entirely peroxisomal in the liver.

Hepatic ß-oxidation of palmitoyl-CoA in juvenile haddock fed 12, 18 and 24% lipid was compared on a per g of fish (wet weight) basis (Fig. 5). No significant differences ($p<0.05$) were observed despite a significant increase in the HSI ($n=5$; $p<0.05$) for the livers tested from juvenile haddock fed 12% lipid in the diet compared with 24%.

4.4 Discussion

Juvenile haddock fed experimental diets containing 12, 18 and 24% lipid grew rapidly over the first nine weeks of the study. The specific growth rate ranged from 2.63

to 2.84% and feed conversion ratios from 0.64 to 0.66 (Table III); the values were comparable to values observed for haddock cultured under similar conditions in a previous study (Nanton *et al.*, 2001). During the 9 to 31 week period, feed conversion ratios were similar (ranged from 0.66 to 0.73) to the first 9 weeks. However, a considerable decline in the specific growth rate (ranged from 0.86 to 0.88%) was observed from 9 to 31 weeks. A negative relationship between size and growth rate has been demonstrated for gadoid fish (cod; Jobling, 1988). At the end of the 31 week feeding trial, a significant increase in hepatosomatic index (HSI) was observed in haddock fed increasing levels of lipid in the diet (12, 18, and 24%). A concomitant increase of HSI with dietary lipid has been observed in previous studies (Nanton *et al.*, 2001; Lie, Lied & Lambertsen, 1988).

There was a significant increase in liver lipid along with HSI as the dietary lipid was increased from 12 to 18 and 24% (Table IV). However, there was little or no capacity for lipid storage in the muscular tissues. Increased dietary lipid did not significantly increase the lipid content of the heart, white muscle and red muscle in haddock. These observations confirm the role of the liver as the major site of lipid storage in haddock. Of the tissues tested, the liver more closely reflected the fatty acid composition of the diet. The liver contained a higher proportion of monounsaturated fatty acids (i.e., 20:1n-9 and 22:1n-11) prevalent in the diet, whereas the heart, white and red muscle contained a higher proportion of polyunsaturated fatty acids (>55%). In this manner, the liver filters lipid absorbed from the diet for transport to the muscle. This pattern of lipid and fatty acid deposition was also observed in a previous study with

juvenile haddock (Nanton *et al.*, 2001). A higher percentage of lipid was measured in the red muscle (2.5 fold) and heart (3.2 fold) compared with the white muscle of haddock. In wild cod, a similarly higher level of lipid was also observed in the red muscle (1.9%) compared with the white muscle (0.7%) (Love *et al.*, 1975). Finely dispersed intracellular lipid droplets were present in the red muscle cells but were not observed in the dorsal white muscle cells of Atlantic salmon (Zhou *et al.*, 1996). The red muscle and heart of fish (Bilinski & Jonas, 1970; Fjermestad et al., 2000; Froyland *et al.*, 2000), including the red muscle of haddock measured in this study (Fig. 3), have a greater capacity for fatty acid catabolism than the white muscle. This is reflected in the increased storage and availability of fatty acid substrate within the cells of the red muscle and heart compared to the white muscle. The increased amount of lipid deposited in the red muscle and heart compared to the white muscle is reflected in the fatty acids. The monounsaturated fatty acids (i.e., 20:1n-9 and 22:1n-11) comprised a higher proportion of the red muscle and heart fatty acids compared to the white muscle (Tables V-VII). These fatty acids are higher in the neutral (storage) lipids of haddock muscle (Nanton *et al.*, 2001) and may be preferentially catabolized in fish (Henderson *et al.*, 1982; Murata & Higashi, 1980). Thus, the preferred monounsaturated fatty acids for catabolism in fish seem to be present at higher amounts in the muscular tissues with higher ß-oxidation activities.

The lipid content and HSI of the liver in haddock fed diets containing 2% soybean lecithin (phosphatidylcholine) was relatively high in comparison with haddock fed non-lecithin supplemented diets in previous work (Nanton *et al.*, 2001). These observations suggest that the level of phosphatidylcholine (choline) was not a limiting factor for the

transport of lipid out of the liver in these haddock (Griffin *et al.*, 1994). Inclusion of soybean lecithin in the diet did have an effect on the fatty acid composition of the haddock tissues. Soybean lecithin contains a high level of 18:2n-6 (ca. 40%; Higgs & Dong, 2000) and the proportion of soybean lecithin-derived lipid in the diet increased as the total lipid decreased (Table I). Thus, the level of 18:2n-6 increased with a decrease in dietary lipid and this trend was significantly reflected in all of the tissues tested. This increase of 18:2n-6 in the muscle of haddock fed a higher proportion soybean lecithin-derived lipid may reduce potential health benefits for the human consumer (Arts *et al.*, 2001).

The protein content of the E-fraction expressed on a per gram wet tissue basis was highest in the white muscle, followed by the red muscle and liver (Fig. 2). Conversely, Atlantic salmon E-fraction protein content on a per g wet tissue basis was highest in the liver and lower in red and white muscle (Froyland *et al.*, 1998). This species difference in E-fraction protein content simply reflects differences in the major sites of tissue lipid storage. Lipid is deposited mainly in the liver of haddock (Table IV), whereas the muscle is the major lipid reserve for salmon (Polvi & Ackman, 1992). The protein content of the liver E-fraction decreased significantly in haddock fed 24 compared with 12% lipid. This may be explained by the significant increase in hepatic lipid storage of haddock fed the higher level of dietary lipid.

Peroxisomes were responsible for 100% of the hepatic ß-oxidation (Fig. 3). This contrasts with the mainly mitochondrial ß-oxidation occurring in the red and white muscle of haddock. A relatively high proportion of peroxisomal ß-oxidation was also

observed in the liver of other marine teleost fish including *Notothenia gibberifrons* (up to 30%; Crockett & Sidell, 1993a), *Myxocephalus octodecimspinosus* (at least 50%; Crockett & Sidell, 1993b) and juvenile Atlantic salmon (ca. 100%; Froyland *et al.*, 2000). In normal rat hepatocytes, peroxisomal ß-oxidation activity was estimated to contribute from 10 (Mannaerts *et al.*, 1979) to 30% (Kondrup & Lazarow, 1985) of the total cellular fatty acid oxidation of palmitate. Unlike mitochondria, peroxisomes in mammals can only chain-shorten fatty acids and cannot degrade the fatty acids fully into acetyl-CoA units (Wanders *et al.*, 2001). Acyl coA oxidases known to be present in human peroxisomes show no or hardly any activity with butyryl coA as a substrate (Vanhove *et al.*, 1993). It remains to be answered whether peroxisomes in fish are able to cycle fatty acids through ß-oxidation to acetyl-CoA. Peroxisomes were capable of oxidizing a wide range of fatty acid substrates in the liver of the marine teleost fish, *N. gibberifrons* (Crockett & Sidell, 1993a) and *M. octodecimspinosus* (Crockett & Sidell, 1993b). The physiological significance for this dominance of peroxisomal over mitochondrial ß-oxidation in the liver of haddock or Atlantic salmon juveniles is unknown. This may be an adaptation to the high amount of long-chain fatty acids present in marine lipids. In rat hepatocytes, a partially hydrogenated marine oil caused a 3-fold increase in the ß-oxidation of erucic acid (a peroxisomal substrate) compared with peanut oil (Christiansen *et al.*, 1979).

The feeding of 15% lipid compared with 5% in the diet of rats resulted in a 1.4-2.4 fold increase in total liver peroxisomal ß-oxidation (Neat *et al.*, 1980). In the liver of juvenile haddock there was no significant increase in the peroxisomal ß-oxidation activity

as dietary lipid was increased from 12, 18 to 24% on a per mg protein or per g wet tissue basis. Although the proportion of liver to total body weight (HSI) significantly increased in haddock fed a higher level of lipid, this was not reflected in an increased hepatic ß-oxidation rate per unit weight of fish. There was also no significant increase in hepatic peroxisomal or total ß-oxidation per g of fish as dietary lipid was increased from 12, 18 to 24%. Thus, juvenile haddock did not adapt to an increase in dietary lipid by increasing the hepatic ß-oxidation of palmitoyl-CoA. Post-absorptive juvenile haddock have a low concentration of serum VLDL (<50 mg/dL serum; Nanton *et al.*, 2001). These observations suggest that cultured haddock (gadoids) have little ability to transport the large amounts of deposited lipid out of the liver to the muscle for catabolism. These factors may contribute to the frequent development of fatty liver in cultured gadoids (haddock and cod) fed to satiation on a daily basis.

The red muscle of juvenile haddock had much higher ß-oxidation activity than the white muscle or liver with palmitoyl-CoA as a substrate. The red muscle of rainbow trout (Bilinski & Jonas, 1970), Atlantic salmon (Froyland *et al.*, 2000; Froyland *et al.*, 1998) and mackerel (Fjermestad *et al.*, 2000) also had a higher capacity to oxidize fatty acids than the white muscle or liver. The ß-oxidation activity in haddock red muscle was predominantly (91%) mitochondrial. The majority (80%) of red muscle ß-oxidation in Atlantic salmon also took place in the mitochondria (Froyland, 2000). The red muscle observed in a variety of fish species (pelagic and demersal) is highly vascularized, contains high levels of aerobic enzymes and abundant mitochondria (Mosse, 1979). The red muscle in haddock composes only about 1% of the total muscle in the middle section

and 8% in the tail (Fraser *et al.*, 1961), whereas Atlantic salmon have ca. 8% red muscle of the total muscle (Hamre & Lie, 1995). Fish that are more active swimmers tend to have a higher proportion of their trunk musculature as red fibres than those fish that are less active (Greer-Walker & Pull, 1975). Thus, less active, demersal fish (i.e., gadoids) would be expected to have a lower capacity for ß-oxidation of fatty acids than more active, pelagic fish due to the lower proportion of red muscle. This may be part of the reason why cultured gadoid fish (haddock and cod) fed daily to satiation often develop enlarged fatty livers.

In a less active demersal fish, the flathead (*Platycephalus bassensis*), white muscle had low vascularization, and was almost devoid of mitochondria (Mosse, 1978). The white muscle of active pelagic teleosts has higher vascularization, more abundant mitochondria and stained positively for aerobic enzymes suggesting an adaptation for a higher degree of oxidative activity (Mosse, 1979). However, this study demonstrates that the white muscle in haddock, a less active fish, has the capacity, through mitochondrial ß-oxidation, to catabolize substantial amounts of palmitoyl-CoA (2.0 nmol/min/g wet tissue) similar to that catabolized in the liver (1.8 nmol/min/g wet tissue). The fillet of a gadoid fish (3 kg wild cod) comprises over 50% of the total body weight (Watterman, 1968) and white muscle represents most of this fillet weight (Greer-Walker & Pull, 1975). These observations suggest that white muscle is the major site of palmitoyl-CoA ß-oxidation in juvenile haddock. White muscle was also the most important tissue for overall fatty acid catabolism in juvenile Atlantic salmon (Froyland *et al.*, 2000). Also, compared to liver peroxisomal ß-oxidation, fatty acids may cycle more times through

mitochondrial ß-oxidation in the muscle of juvenile haddock to produce the acetyl-CoA end product for entry into the Kreb's Cycle (Rasmussen & Wolfe, 1999).

Mammals and fish appear to regulate the mitochondrial ß-oxidation enzymes in a similar manner. Malonyl-CoA (fatty acid synthesis product) was shown to inhibit carnitine palmitoyltransferase I (CPT I; rate limiting enzyme for ß-oxidation) in the red muscle of striped bass (Rodnick & Sidell, 1994). A reduction in the activity of enzymes involved in fatty acid synthesis was observed in coho salmon (Lin *et al.*, 1977), Atlantic salmon (Arnesen *et al.*, 1993) and European seabass (Dias *et al.*, 1998) fed higher levels of dietary lipid. Thus, fish fed a higher level of lipid might increase ß-oxidation activity due to both a reduction in the level of malonyl-CoA and the increased availability of fatty acid substrate (Rasmussen & Wolfe, 1999). However, no significant differences were observed in the mitochondrial ß-oxidation activity of the red or white muscle in juvenile haddock fed 12, 18 or 24% lipid. These juvenile haddock fed the low 12% lipid diet, daily to satiation had a higher HSI and liver lipid storage than typically observed in wild gadoids (Nanton *et al.*, 2001). Thus, the supply of lipid to the muscle is probably not limited by the amount available in the liver for cultured juvenile haddock fed 12% lipid compared with 18 or 24%. Fatty acid synthesis and malonyl-CoA (mitochondrial ß-oxidation inhibitor) production was probably occurring at a similar minimal level and ß-oxidation at a maximum rate in the muscle for these haddock fed 12, 18 or 24% lipid in the diet.

The overall type and distribution of ß-oxidation activity for palmitoyl-CoA expressed on a per g wet tissue basis was similar between Atlantic salmon (Froyland *et*

al., 2000) and haddock (Fig. 4) despite differences in their major sites of lipid storage (liver in haddock vs. muscle in salmon). In the juveniles of both species, mitochondrial ß-oxidation dominated in the muscle and peroxisomal ß-oxidation in the liver. Red muscle has a much higher capacity for ß-oxidation than the white muscle in both juvenile haddock (4.3 fold) and Atlantic salmon (8.3 fold; Froyland *et al.*, 2000). Red muscle forms a higher percentage of the muscle in Atlantic salmon (Hamre & Lie, 1995) compared with haddock (Fraser *et al.*, 1961) indicating a higher capacity for overall fatty acid catabolism in the more active Atlantic salmon. The ß-oxidation rate of palmitoyl-CoA per g wet tissue was slightly lower in the liver compared to the white muscle for both juvenile haddock (89%; Fig. 4) and Atlantic salmon (75%; Froyland *et al.*, 2000). Most of the fatty acid catabolism in these fish species occurs in the muscle. However, the higher hepatosomatic index (ca. 1% in Atlantic salmon; Bjerkeng *et al.*, 1999) and lower proportion of red muscle observed in haddock compared with salmon suggests the increased importance of the liver for overall fatty acid catabolism in juvenile haddock.

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Table I. Chemical composition[1] of experimental diets.

	Dietary lipid level (%)		
Chemical analysis (%DM)	12	18	24
Moisture	12.2	9.9	6.9
Protein	55.1	54.5	53.2
Lipid	14.1	20.3	26.2
Ash	8.4	8.3	8.2

[1] Average of two replicates.

Table II. Fatty acid composition[1] of experimental diets.

	Dietary lipid level (%)		
Fatty acid	12	18	24
14:0	5.3	6.2	6.6
16:0	16.2	16.5	16.6
16:1 n-7	5.5	6.6	7.1
18:0	2.6	2.8	2.9
18:1 n-9	11.6	11.2	11.0
18:1 n-7	2.5	2.6	2.6
18:2 n-6	11.8	7.7	5.6
18:3 n-3	1.4	1.1	0.9
18:4 n-3	1.3	1.7	1.9
20:1n-9	5.4	4.2	3.6
20:4 n-6	0.7	0.8	0.9
20:4n-3	0.4	0.5	0.5
20:5 n-3	9.3	12.1	13.4
22:1n-11	8.4	6.4	5.5
22:5 n-3	1.0	1.3	1.5
22:6 n-3	7.5	8.0	8.2
Σ SAT	25.0	26.4	27.0
Σ MONO	36.5	33.6	32.4
Σ PUFA	38.1	39.7	40.2
Σ n-3	21.4	25.3	27.1
Σ n-6	13.1	9.3	7.5
DHA/EPA	0.8	0.7	0.6

[1]Data expressed as area % of FAME, n=2.
Abbreviations: DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, MONO=monounsaturates, PUFA=polyunsaturates, SAT=saturates.

Table III. Growth[1], feed utilization[1] and hepatosomatic index[6] of juvenile haddock fed graded levels of dietary lipid[2].

Parameter	Dietary lipid level (%) 12 (14)	18 (20)	24 (26)
		0-9 weeks	
Wt. gain[3]	35.23	33.80	30.35
SGR[4]	2.84	2.79	2.63
FCR[5]	0.66	0.63	0.64
		9-31 weeks	
Wt. gain[3]	111.75	108.61	104.02
SGR[4]	0.86	0.86	0.88
FCR[5]	0.66	0.73	0.73
HSI[6]	10.02 ± 1.31^{a}	12.55 ± 1.31^{b}	15.19 ± 1.61^{c}

[1]Values represent 2 tanks/ diet.

[2]Values within brackets are % lipid in diet on dry matter basis.

[3]Weight gain (g wet wt./ fish). Average initial weight was 7.07 g.

[4]Specific growth rate (%) = 100 x (ln(final wt.)-ln(initial wt.))/duration (d).

[5]Feed conversion ratio = feed intake (expressed as dry matter)/wet wt. gain.

[6]Hepatosomatic index (%) = 100 x wet liver wt./body wt.; HSI of initial fish was 5.38%. Values (means ± SD of 16 fish/ diet) containing different superscripts were significantly different ($p<0.05$).

Table IV. Total lipid[1] and fatty acid[2] composition of the liver in juvenile haddock fed 12, 18 and 24% lipid.

	Dietary lipid level (%)		
Fatty acid	12	18	24
Lipid (%)	73.84 ± 1.90[a]	77.86 ± 1.19[b]	79.29 ± 1.85[b]
14:0	3.7 ± 0.2[a]	4.0 ± 0.2[b]	4.0 ± 0.1[b]
16:0	16.5 ± 0.4[a]	15.3 ± 0.3[b]	14.6 ± 0.3[c]
16:1 n-7	5.6 ± 0.3[a]	6.4 ± 0.2[b]	6.7 ± 0.1[b]
18:0	3.9 ± 0.4[a]	3.5 ± 0.2[a]	3.5 ± 0.2[a]
18:1 n-9	19.7 ± 0.7[a]	16.9 ± 0.8[b]	16.4 ± 0.7[b]
18:1 n-7	3.8 ± 0.1[a]	3.9 ± 0.0[a]	4.0 ± 0.2[a]
18:2 n-6	11.0 ± 0.2[a]	7.6 ± 0.2[b]	5.8 ± 0.1[c]
18:3 n-3	1.4 ± 0.0[a]	1.1 ± 0.0[b]	0.9 ± 0.0[c]
18:4 n-3	1.2 ± 0.0[a]	1.7 ± 0.0[b]	1.8 ± 0.0[c]
20:1 n-9	5.7 ± 0.3[a]	4.8 ± 0.1[b]	4.2 ± 0.1[c]
20:4 n-6	0.6 ± 0.0[a]	0.7 ± 0.0[b]	0.8 ± 0.0[c]
20:4 n-3	0.4 ± 0.0[a]	0.6 ± 0.0[b]	0.7 ± 0.0[c]
20:5 n-3	8.1 ± 0.2[a]	12.0 ± 0.3[b]	13.7 ± 0.3[c]
22:1n-11	4.9 ± 0.2[a]	4.2 ± 0.1[b]	3.5 ± 0.1[c]
22:5 n-3	1.1 ± 0.1[a]	1.5 ± 0.1[b]	1.7 ± 0.1[c]
22:6 n-3	6.0 ± 0.1[a]	7.7 ± 0.1[b]	8.4 ± 0.2[c]
Σ SAT	24.8 ± 0.5[a]	23.7 ± 0.2[b]	23.0 ± 0.3[c]
Σ MONO	41.5 ± 0.3[a]	37.9 ± 0.7[b]	36.6 ± 0.6[c]
Σ PUFA	33.6 ± 0.4[a]	38.4 ± 0.8[b]	40.4 ± 0.5[c]
Σ n-3	18.5 ± 0.3[a]	25.0 ± 0.5[b]	27.9 ± 0.5[c]
Σ n-6	12.3 ± 0.2[a]	9.2 ± 0.3[b]	7.6 ± 0.1[c]
DHA/EPA	0.7 ± 0.0[a]	0.6 ± 0.0[b]	0.6 ± 0.0[c]

[1] Percent total lipid of wet tissue, n=4.
[2] Data expressed as area % of FAME, n=4.
[1,2] Values in the same row containing different superscripts were significantly different ($p<0.05$).
Abbreviations: DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, MONO=monounsaturates, PUFA=polyunsaturates, SAT=saturates.

Table V. Total lipid[1] and fatty acid[2] composition of the red muscle in juvenile haddock fed 12, 18 and 24% lipid.

	Dietary lipid level (%)		
Fatty acid	12	18	24
Lipid (%)	1.86 ± 0.06[a]	2.00 ± 0.15[a]	1.95 ± 0.15[a]
14:0	0.8 ± 0.1[a]	0.9 ± 0.2[ab]	1.2 ± 0.2[b]
16:0	15.7 ± 0.3[a]	15.9 ± 0.4[a]	16.3 ± 0.2[a]
16:1 n-7	1.4 ± 0.1[a]	1.7 ± 0.2[a]	2.1 ± 0.2[b]
18:0	4.2 ± 0.3[a]	4.3 ± 0.4[a]	4.2 ± 0.3[a]
18:1 n-9	11.6 ± 0.6[a]	10.9 ± 0.3[a]	10.6 ± 0.7[a]
18:1 n-7	3.3 ± 0.2[a]	3.7 ± 0.2[b]	3.9 ± 0.1[b]
18:2 n-6	8.5 ± 0.6[a]	5.5 ± 0.5[b]	4.3 ± 0.4[c]
18:3 n-3	0.6 ± 0.1[a]	0.5 ± 0.0[b]	0.4 ± 0.1[b]
18:4 n-3	0.3 ± 0.0[a]	0.3 ± 0.0[a]	0.4 ± 0.1[b]
20:1 n-9	2.7 ± 0.3[a]	2.4 ± 0.3[a]	2.1 ± 0.3[a]
20:4 n-6	2.4 ± 0.1[a]	2.6 ± 0.2[a]	2.8 ± 0.2[a]
20:4 n-3	0.4 ± 0.0[a]	0.4 ± 0.1[a]	0.5 ± 0.0[b]
20:5 n-3	12.9 ± 0.5[a]	13.5 ± 2.0[a]	14.6 ± 1.1[a]
22:1n-11	1.3 ± 0.2[a]	1.3 ± 0.2[a]	1.4 ± 0.2[a]
22:5 n-3	2.1 ± 0.1[a]	2.5 ± 0.2[b]	3.0 ± 0.3[c]
22:6 n-3	25.8 ± 2.1[a]	27.2 ± 2.5[a]	26.1 ± 1.8[a]
Σ SAT	22.0 ± 0.5[a]	22.3 ± 0.5[a]	22.7 ± 0.3[a]
Σ MONO	21.5 ± 1.2[a]	21.2 ± 1.0[a]	21.1 ± 0.5[a]
Σ PUFA	55.4 ± 0.7[a]	55.4 ± 1.6[a]	55.1 ± 0.5[a]
Σ n-3	42.5 ± 1.4[a]	45.1 ± 1.6[ab]	45.7 ± 0.8[b]
Σ n-6	12.2 ± 0.7[a]	9.4 ± 0.7[ab]	8.2 ± 0.6[b]
DHA/EPA	2.0 ± 0.2[a]	2.1 ± 0.4[a]	1.8 ± 0.3[a]

[1] Percent total lipid of wet tissue, n=5.
[2] Data expressed as area % of FAME, n=4.
[1,2] Values in the same row containing different superscripts were significantly different ($p<0.05$).
Abbreviations: DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, MONO=monounsaturates, PUFA=polyunsaturates, SAT=saturates.

Table VI. Total lipid[1] and fatty acid[2] composition of the white muscle in juvenile haddock fed 12, 18 and 24% lipid.

	Dietary lipid level (%)		
Fatty acid	12	18	24
Lipid (%)	0.77 ± 0.04[a]	0.79 ± 0.03[a]	0.82 ± 0.02[a]
14:0	1.0 ± 0.3[a]	0.9 ± 0.2[a]	1.2 ± 0.2[a]
16:0	19.2 ± 0.3[a]	19.9 ± 0.4[a]	19.6 ± 0.7[a]
16:1 n-7	1.6 ± 0.3[a]	1.4 ± 0.2[a]	1.7 ± 0.3[a]
18:0	3.8 ± 0.2[a]	3.9 ± 0.4[a]	4.0 ± 0.2[a]
18:1 n-9	10.4 ± 1.3[a]	8.6 ± 0.5[b]	7.9 ± 0.9[b]
18:1 n-7	2.6 ± 0.2[a]	2.6 ± 0.1[a]	2.8 ± 0.2[a]
18:2 n-6	9.7 ± 0.8[a]	5.7 ± 0.3[b]	4.1 ± 0.5[c]
18:3 n-3	0.7 ± 0.1[a]	0.4 ± 0.0[b]	0.4 ± 0.1[b]
18:4 n-3	0.4 ± 0.1[a]	0.4 ± 0.1[a]	0.4 ± 0.0[a]
20:1 n-9	1.8 ± 0.2[a]	1.6 ± 0.2[a]	1.5 ± 0.2[a]
20:4 n-6	1.8 ± 0.2[a]	2.0 ± 0.1[b]	2.2 ± 0.1[b]
20:4 n-3	0.5 ± 0.1[a]	0.5 ± 0.0[a]	0.5 ± 0.1[a]
20:5 n-3	17.0 ± 0.7[a]	20.2 ± 0.9[b]	21.4 ± 1.2[b]
22:1n-11	0.6 ± 0.1[a]	0.6 ± 0.1[a]	0.6 ± 0.1[a]
22:5 n-3	2.0 ± 0.1[a]	2.4 ± 0.1[b]	2.7 ± 0.3[c]
22:6 n-3	23.1 ± 2.6[a]	24.7 ± 1.1[a]	25.0 ± 2.5[a]
Σ SAT	24.9 ± 0.3[a]	26.1 ± 0.4[b]	26.1 ± 0.6[b]
Σ MONO	17.6 ± 2.2[a]	15.4 ± 0.7[a]	15.2 ± 1.6[a]
Σ PUFA	57.2 ± 2.0[a]	58.4 ± 0.8[a]	58.7 ± 1.3[a]
Σ n-3	44.1 ± 2.9[a]	49.0 ± 0.8[b]	50.7 ± 1.8[b]
Σ n-6	12.4 ± 0.7[a]	8.6 ± 0.3[b]	7.1 ± 0.4[c]
DHA/EPA	1.4 ± 0.1[a]	1.2 ± 0.1[a]	1.2 ± 0.2[a]

[1] Percent total lipid of wet tissue, n=5.
[2] Data expressed as area % of FAME, n=5.
[1,2] Values in the same row containing different superscripts were significantly different ($p<0.05$).
Abbreviations: DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, MONO=monounsaturates, PUFA=polyunsaturates, SAT=saturates.

Table VII. Total lipid[1] and fatty acid[2] composition of the heart in juvenile haddock fed 12, 18 and 24% lipid.

	Dietary lipid level (%)		
Fatty acid	12	18	24
Lipid (%)	2.49 ± 0.12[a]	2.51 ± 0.32[a]	2.59 ± 0.34[a]
14:0	1.0 ± 0.1[a]	1.1 ± 0.2[a]	1.5 ± 0.2[b]
16:0	15.5 ± 0.4[a]	15.4 ± 0.3[a]	15.7 ± 0.4[a]
16:1 n-7	1.5 ± 0.1[a]	1.8 ± 0.2[a]	2.3 ± 0.2[b]
18:0	4.6 ± 0.2[a]	5.2 ± 0.2[b]	4.8 ± 0.4[a]
18:1 n-9	12.0 ± 0.4[a]	11.4 ± 0.5[ab]	11.2 ± 0.5[b]
18:1 n-7	3.3 ± 0.0[a]	3.4 ± 0.1[b]	3.6 ± 0.1[c]
18:2 n-6	8.4 ± 0.7[a]	5.2 ± 0.4[b]	4.2 ± 0.3[c]
18:3 n-3	0.7 ± 0.1[a]	0.5 ± 0.1[b]	0.5 ± 0.1[b]
18:4 n-3	0.3 ± 0.0[a]	0.3 ± 0.1[b]	0.4 ± 0.0[b]
20:1 n-9	2.7 ± 0.2[a]	2.4 ± 0.2[b]	2.4 ± 0.1[b]
20:4 n-6	3.1 ± 0.2[a]	3.6 ± 0.3[ab]	3.6 ± 0.3[b]
20:4 n-3	0.4 ± 0.1[a]	0.5 ± 0.3[a]	0.4 ± 0.1[a]
20:5 n-3	10.9 ± 0.4[a]	11.2 ± 0.7[a]	12.4 ± 0.8[b]
22:1n-11	1.1 ± 0.1[a]	1.0 ± 0.2[a]	1.2 ± 0.2[a]
22:5 n-3	1.8 ± 0.4[a]	2.1 ± 0.4[a]	2.5 ± 0.6[a]
22:6 n-3	25.9 ± 2.1[a]	27.5 ± 1.6[a]	26.1 ± 2.1[a]
Σ SAT	22.1 ± 0.5[a]	22.9 ± 0.2[b]	23.2 ± 0.4[b]
Σ MONO	21.5 ± 1.0[a]	21.1 ± 1.3[a]	21.5 ± 0.9[a]
Σ PUFA	56.1 ± 0.8[a]	55.6 ± 1.6[a]	54.9 ± 0.9[a]
Σ n-3	41.7 ± 1.5	44.0 ± 1.3[b]	44.1 ± 1.0[b]
Σ n-6	12.9 ± 0.9[a]	9.9 ± 0.6[b]	8.8 ± 0.5[b]
DHA/EPA	2.4 ± 0.3[a]	2.5 ± 0.3[a]	2.1 ± 0.3[a]

[1] Percent total lipid of wet tissue, n=5.
[2] Data expressed as area % of FAME, n=5.
[1,2] Values in the same row containing different superscripts were significantly different ($p<0.05$).
Abbreviations: DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, MONO=monounsaturates, PUFA=polyunsaturates, SAT=saturates.

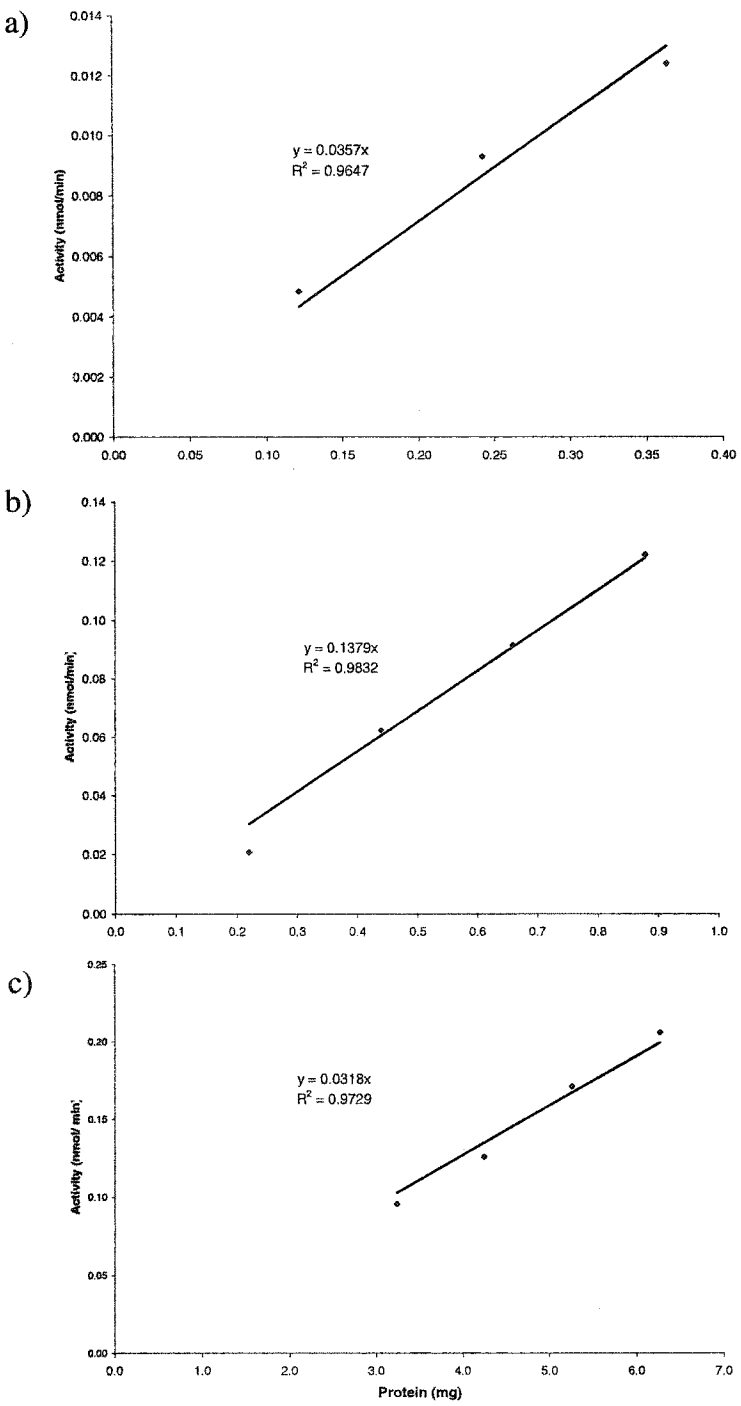

Fig. 1. ß-oxidation activity of palmitoyl-CoA at 20°C for the linear ($p<0.005$) range of haddock liver (a), red muscle (b) and white muscle (c) E-fraction protein used in the experimental assays.

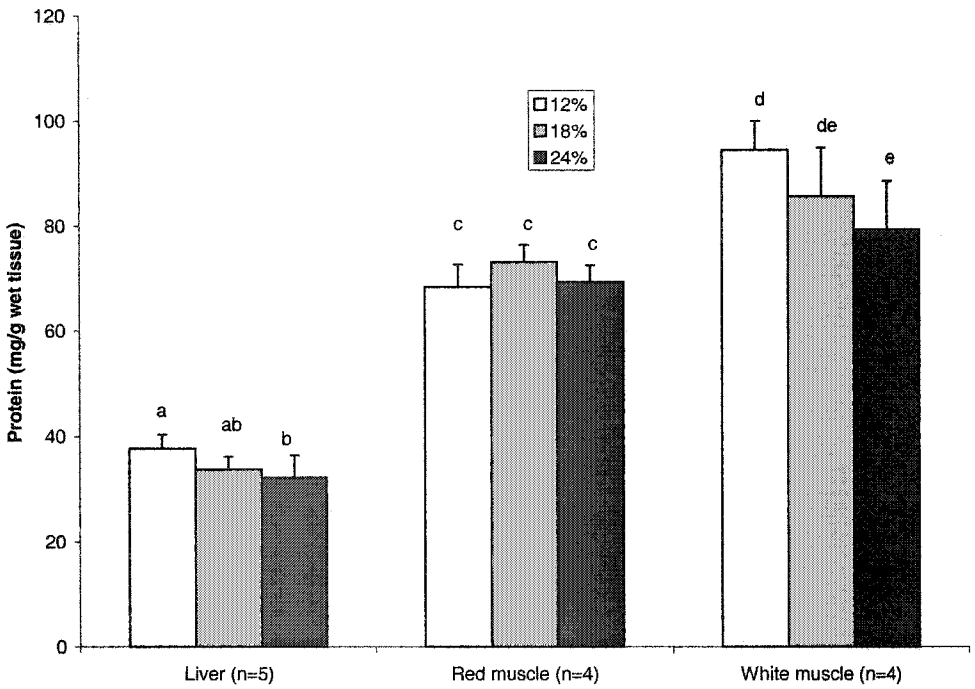

Fig. 2. Protein content of E-fraction in juvenile haddock fed graded levels of dietary lipid (12, 18 and 24%). Different lettering represents significant differences ($p<0.05$).

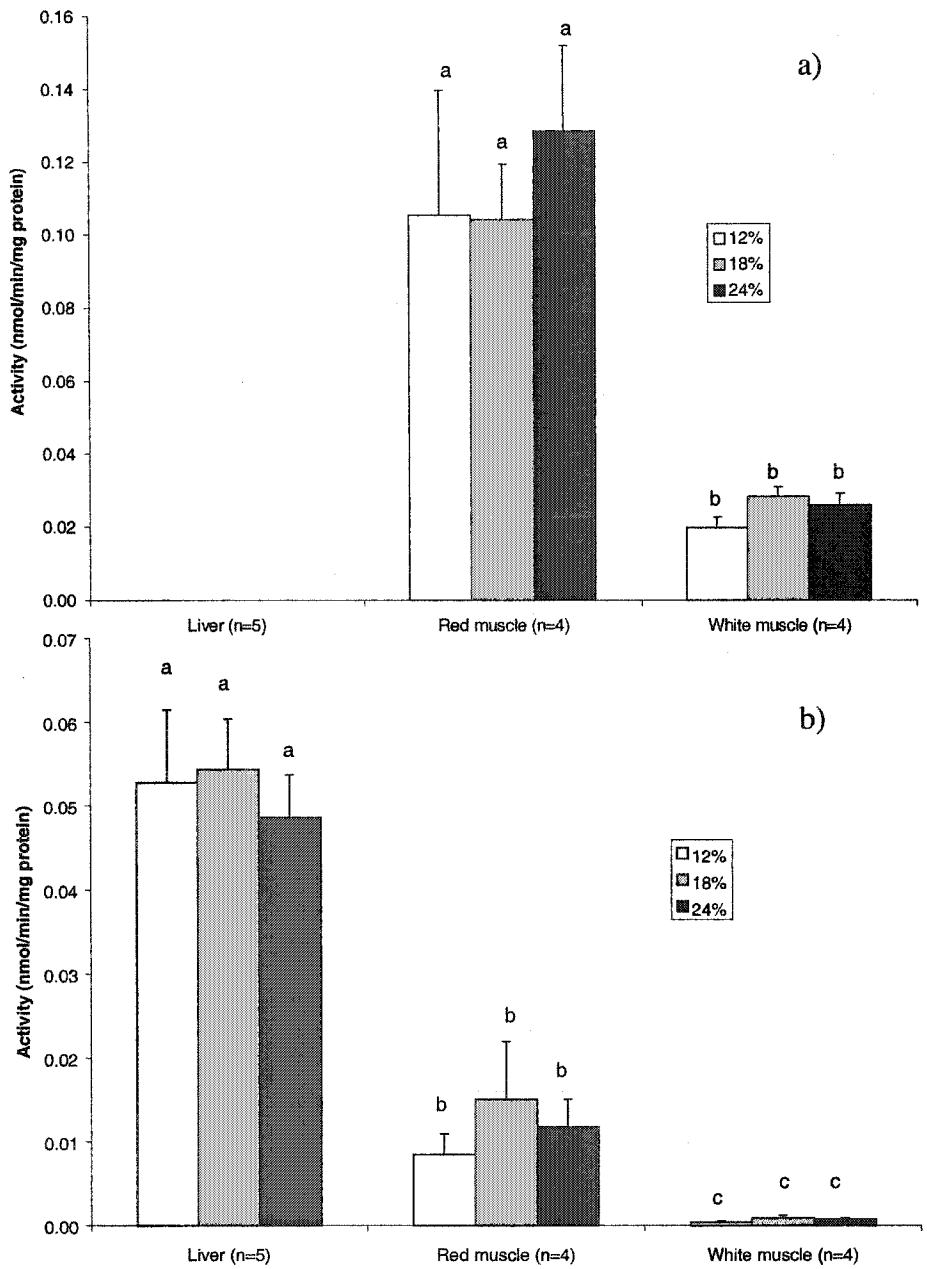

Fig. 3. Mitochondrial (a) and peroxisomal (b) ß-oxidation of palmitoyl-CoA at 20°C in juvenile haddock fed graded levels of dietary lipid (12, 18 and 24%). Results (means ± SD) are expressed as nmol/min/mg protein. Different lettering represents significant differences ($p<0.05$).

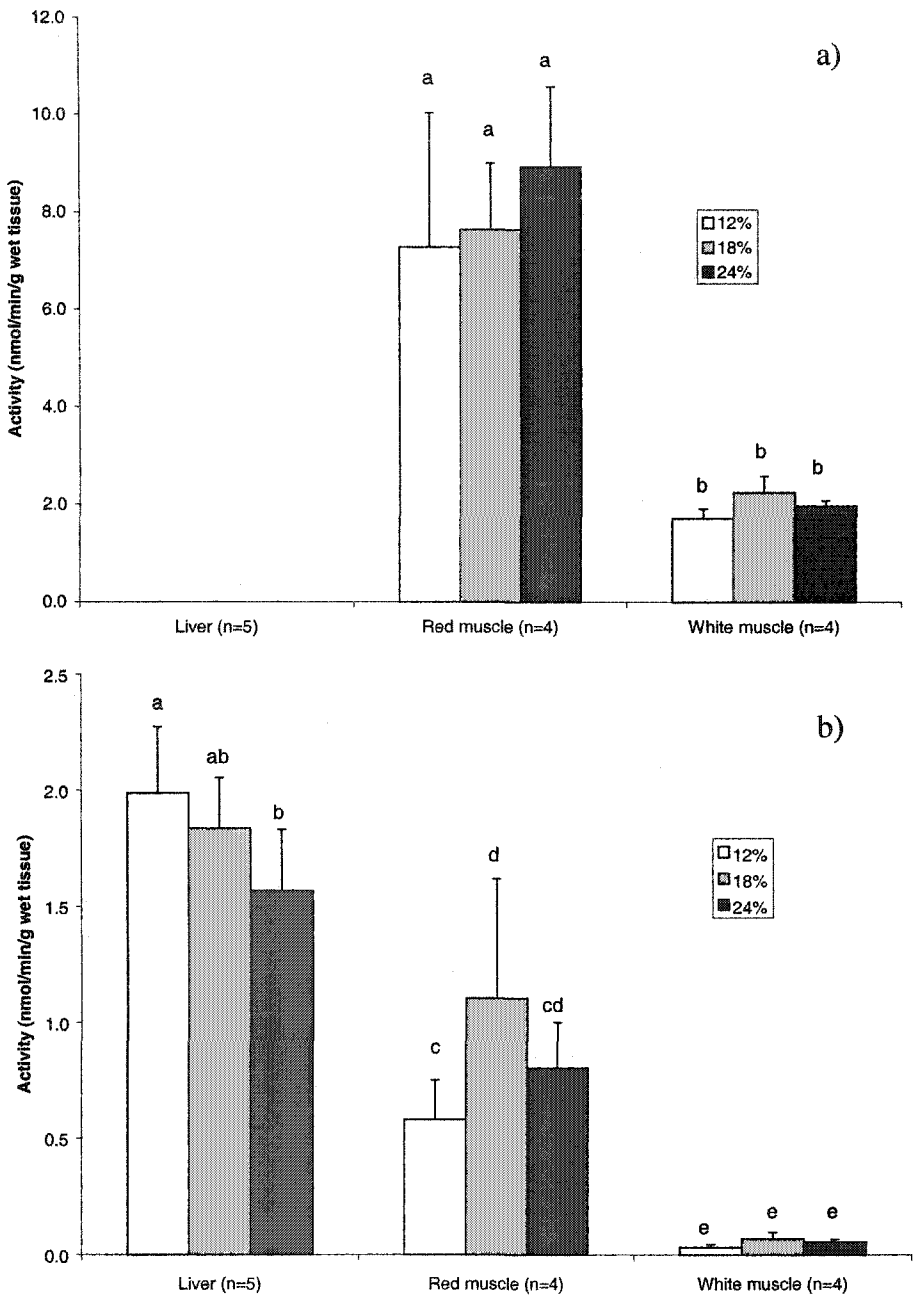

Fig. 4. Mitochondrial (a) and peroxisomal (b) ß-oxidation of palmitoyl-CoA at 20°C in juvenile haddock fed graded levels of dietary lipid (12, 18 and 24%). Results (means ± SD) are expressed as nmol/min/g wet tissue. Different lettering represents significant differences ($p<0.05$).

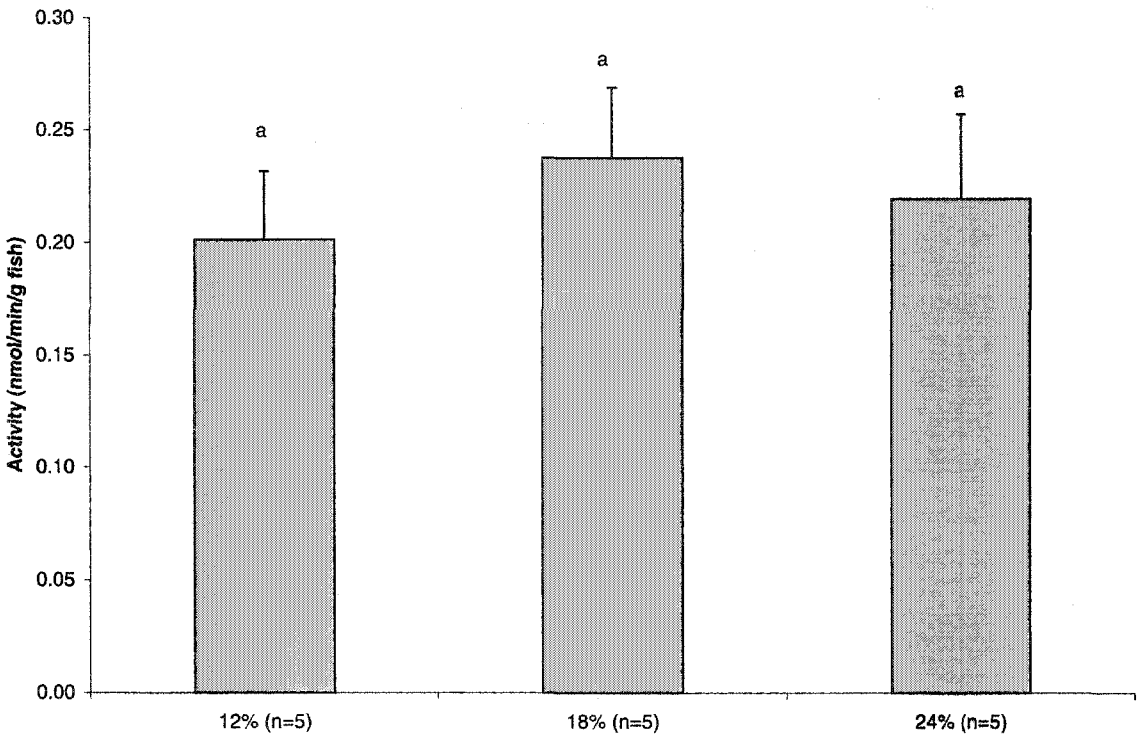

Fig. 5. Hepatic ß-oxidation of palmitoyl-CoA at 20°C in juvenile haddock fed graded levels of dietary lipid (12, 18 and 24%). Results (means ± SD) are expressed as nmol/min/g fish. Different lettering represents significant differences ($p<0.05$).

5. Effect of Hepatosomatic Index on Liver Function Parameters in the Plasma of Juvenile Haddock.

5.1 Introduction

Fatty liver frequently occurs in cultured haddock fed formulated diets. Gadoids (i.e., cod or haddock) fed formulated diets generally deposit more lipid in the liver and have higher hepatosomatic indices (HSI) compared with wild stocks (Nanton *et al.*, 2001; Shahidi and Dunajski, 1994; Jobling *et al.*, 1991). To assess the implications of this fatty liver condition on the health of cultured gadoids, it is important to determine if liver function is affected.

Because of the many functions of the liver, a battery of tests rather than one test alone is routinely performed on blood samples to provide a more complete indication of liver health in mammals. These liver function tests can suggest if cell integrity, cholestasis or metabolic function of the liver is affected (Hawker, 1993). Increases in the plasma levels of liver-specific enzymes such as alanine aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase indicate a pathologic condition in which the liver cells are leaking cellular enzymes into blood. Increases in plasma alkaline phosphatase and gamma-glutamyl transferase may indicate the liver is synthesizing these enzymes and releasing them in a situation of cholestasis (Kaplan, 1993). The liver synthesizes most serum proteins. If hepatic function is significantly reduced it can be detected by reductions in serum protein, particularly albumin levels (Hawker, 1993). An animal with reduced hepatic function will have inflated post meal bile acid serum levels.

This is a fairly sensitive test based on the idea that reduced hepatic function significantly interferes with the normal enterohepatic circulation of bile acids. Impaired liver function may also be reflected in the reduced hepatic clearance of bilirubin (derived from the catabolism of heme) (Kaplan, 1993).

The purpose of this study was to compare liver function parameters in cultured haddock with different hepatosomatic indices (HSI). These tests may suggest potential cause(s) of haddock mortality associated with fatty liver.

5.2 Materials and Methods

Experimental conditions

The liver function trial was conducted at the NRC Aquaculture Station at Sandy Cove, Halifax, Nova Scotia, where the haddock were hatched and reared to attain their initial size (ca. 98g; ca. 1.5 years post hatch from 1999 year class) at the start of the experiment. Fifty fish were randomly allotted into each of 2 tanks and adapted to experimental conditions for 2 weeks before feeding the experimental diets. Fish were hand-fed to satiation three times daily on weekdays and twice daily on weekends during the 92 d experimental period. Filtered and UV-treated seawater (salinity, 30 ppt) was supplied to each tank in a flow-through system. Water volumes in each tank were maintained at 3000 L. Fish were held on a 12 h dark : 12 h light photoperiod. Dissolved oxygen levels and water temperature were measured every morning and averaged 10 mg/L and 12.5°C, respectively. Fish were bulk-weighed and counted at the beginning and end of the experiment. Fish were fasted for 72 hours prior to weighing and counting.

Originally, the results of the liver function tests were to have been compared

between the 1999 year class haddock fed 12 and 22% lipid in the diet. However, there was no significant difference in the HSI of these two groups. Instead these tests were performed on haddock sampled randomly from the 1998 year class (ca. 2.5 years post hatch) fed a commercial formulation (19% lipid of dry wt.) and the 1999 year class (ca. 1.5 years post hatch) fed the experimental 12% lipid diet. These haddock were then divided into three significantly different groups (low, medium and high) according to their HSI. Haddock from the 1998 year class were cultured under similar conditions to those described above for the 1999 year class haddock fed the experimental lipid diets.

Diet preparation

Herring oil was incorporated to provide levels of 12 and 22% lipid in two isonitrogenous diets. Fish meal was ground and passed through a fine screen using a Fitz mill (Fitzpatrick Co., Ill., USA) before mixing with other ingredients. All dry ingredients, except the choline chloride, starch and fish oil, were weighed and mixed in a large Hobart mixer (Model H600T, Rapids Machinery Co., Iowa, USA) for 15 minutes. The choline chloride was then added and the ingredients were mixed for 15 more minutes before adding the fish oil and continuing to mix for an additional 15 minutes. Diets were steam pelleted into 2.4 mm pellets using a laboratory pellet mill (California Pellet Mills, San Francisco), dried in an air-convectioned drier at 30°C and screened prior to feeding. The commercial haddock diet used in this feeding trial was supplied by Ziegler Bros. Inc. (Gardners, PA, USA). The diets were stored in a -20°C freezer until needed.

Sampling and analytical methods

At the beginning of the feeding trial, 5 fish were sampled and sacrificed with an overdose of TMS (tricane methanesulfonate). These fish were individually weighed and dissected to remove the liver. At the end of the experiment, 10 fish from each tank were sampled and killed in a similar manner. Chemical compositions of the experimental diets were evaluated following procedures described in AOAC (1990). The dry matter was determined by drying in an oven at 110°C for 24 h and crude ash by incineration in a muffle furnace at 550°C for 24 h. Crude protein (%N x 6.25) was measured by the Dumas method (Ebling, 1968) using a Leco Nitrogen Determinator (Model FP-228, Leco Corporation, St. Joseph, MI). Total lipid was extracted (Bligh and Dyer, 1959) and determined gravimetrically for each diet. Fatty acid compositions of the diets were estimated from their fatty acid methyl ester (FAME) derivatives. The FAME were prepared using 7% boron trifluoride in methanol and heating to 100°C for 1 h (Christie, 1982). The FAME were separated by gas chromatography (Hewlett Packard 6890 GC system equipped with a flame-ionization detector) using an Omegawax 320 capillary column (Supelco, Bellefonte, PA) as described in Chapter 2. The FAME were identified by comparison of retention times with those of known standards (menhaden oil, Supelco).

Liver Function Tests

At the end of the feeding trial, 72 h fasted, juvenile haddock were sampled randomly from the 1998 year class (ca. 2.5 years post hatch) fed a standard formulation (19% lipid of dry wt.) and the 1999 year class (ca. 1.5 years post hatch) fed the experimental 12% lipid diet. Fish were killed with an overdose of anaesthetic (MS-222)

and blood was collected from the caudal vein in heparinized tubes. The blood was immediately subsampled in duplicate for hematocrit (Houston, 1990). The remaining blood was left at 0°C for 30 min. The plasma was obtained from the blood by low speed centrifugation. The plasma was transferred to 1 mL centrifuge tubes, placed on dry ice and stored at –80°C. These fish were weighed and dissected, the livers weighed and the plasma samples grouped according to their HSI (low, medium or high; n=5). Liver function tests were performed using an automated Boehringer Manheim/ Hitachi 917 Analyzer. The liver function parameters were determined using the following clinical kits and reagents (cat. no. and supplier in brackets): alanine aminotransferase (1876805, Roche Diagnostics, Laval, PQ); albumin (1970909, Roche); alkaline phosphatase (1972596; Roche); aspartate aminotransferase (1876848, Roche); gamma-glutamyl transferase (1551906, Roche); sorbitol dehydrogenase (50-UV, Sigma Chemical, St. Louis, MO.); total bilirubin (1552414, Roche); total protein (1929917, Roche).

For bile acid analyses, the fish were separated into low, medium and high HSI groups as described for the liver function panel. These fish were tagged to keep track of individual bile acid levels pre- and post-meal. These tagged fish were then fasted for 72 h. Blood was collected in non-heparinized tubes and the serum separated, as described above for the liver function panel. Approximately two weeks later, blood was again taken to estimate post-feeding bile acid levels. The bile acid measurements of the 1998 year class (8.4°C) were performed later in the year and at colder water temperatures than those for the 1999 year class (11.1°C). Therefore the blood was taken 4.0 h post-meal for the 1998 year class and 2.5 h post-meal for the 1999 year class. The HSI of these fish

were determined as described for the liver function panel. The bile acids were measured using a clinical kit (BI-1689, Randox Labs, Crumlin, Ireland) and expressed as umol/L serum.

Statistical analysis

Analyses of variance (ANOVA) with pairwise comparisons (Tukey's HSD) were performed at the $p<0.05$ level to determine significant differences between liver function tests for the low, medium and high HSI groups. Percentage values were square root, arcsine transformed prior to analysis. Pearson correlations and the corresponding probability matrix were used to determine significant correlations ($p<0.05$) between ungrouped liver function parameters. Statistical analyses were performed using the Systat 5.1 software package.

5.3 Results

Experimental diets were formulated using practical ingredients, of which herring meal comprised the major protein source (Table I). The lipid level was adjusted by changing the proportion of herring oil incorporated in the experimental diets. The chemical composition of the diets are presented in Table II. The protein was maintained at a constant level of ca. 54% of dry wt. whereas the lipid level in the experimental diets was increased from 13.3 to 22.4% of dry wt. The fatty acid composition of the experimental diets was similar with respect to the essential fatty acids: docosahexaenoic acid (DHA) composing ca. 10%; and eicosapentaenoic acid (EPA) comprising 13-15% of the total fatty acids. The diets had a DHA/EPA ratio of 0.65-0.77 (Table III). The

formulation (Table I), chemical (Table II) and fatty acid composition (Table III) of the commercial diet (19% lipid of dry wt.) used to feed 1998 year class fish (ca. 2.5 years post hatch) is presented along with the experimental diets.

The mean growth in terms of both weight gain per individual fish and specific growth rate was slightly higher in the tank fed the lower lipid level (Table IV). The feed: gain ratio appeared to be lower for the haddock fed the lower lipid level. This was not a growth trial and only one tank was used for each diet. Statistical analyses were therefore not performed on the growth measurements. The HSI for individual haddock fed 12 versus 22% lipid were not significantly different at the end of the feeding trial. The hematocrit (% red blood cell volume) was significantly higher ($p<0.05$) in the fish fed 22% lipid in the diet.

Originally, the results of a panel of liver function tests were to have been compared between the haddock fed 12 and 22% lipid in the diet. However, there was no significant difference in the HSI of these two groups. Instead these tests were performed on haddock sampled randomly from the 1998 year class (ca. 2.5 years post hatch) fed a commercial formulation (19% lipid of dry wt.) and the 1999 year class (ca. 1.5 years post hatch) fed the experimental 12% lipid diet. These haddock were then divided into three significantly different groups (low, medium and high) according to their HSI (Table V). No significant differences ($p<0.05$) were observed between the low, medium and high HSI groups for any of the liver function parameters tested. This may have been partly due to the large variability, particularly in the plasma enzyme activity values. Total protein in the plasma averaged 32.7 g/l of which albumin composed 32.5%.

Correlations of individual HSI and the results of corresponding liver function tests were then evaluated (Table VI). Only one significantly positive correlation (+0.601; $p<0.05$) between individual HSI and total bilirubin was observed. Other liver function parameters which were significantly correlated ($p<0.05$) were total protein with albumin (+0.647) and aspartate aminotransferase (+0.530), as well as aspartate aminotransferase with alanine aminotransferase (+0.540).

The measurement of bile acid in the serum of the haddock divided into low, medium and high HSI groups was also performed. The variation was large for bile acid measurements in the fasting fish (mean=11 μmol/L, coefficient of variation=175%) and the post meal bile acid levels did not always increase for individual fish even though food was observed in the gut for all fish tested. Therefore, the results from this test were not presented and this assay would not be recommended as a measure of liver function in fish.

5.4 Discussion

The mean HSI was not significantly higher in haddock fed 22% compared to those fed 12% lipid in the diet. As seen in our previous study with haddock (Nanton *et al.*, 2001), it was expected that an increase in dietary lipid would increase the HSI. However, the low tank stocking density may have reduced the normal aggressive feeding behaviour and feed intake. The specific growth rate (0.76%) for these fish was lower than expected. Interestingly, the hematocrit level was significantly higher in the fish fed 22% lipid in the diet. This may have been due to the lower ingestion and total amount of highly unsaturated fatty acids in the diet of fish fed 12% lipid (Fujii *et al.*, 1976). These

fatty acids are present in high concentrations in the erythrocyte membranes of gadoids (Lie *et al.*, 1989).

No significant differences were observed between the liver function test results of the low, medium and high HSI groups. This suggests that liver function was not affected significantly in fish with a high mean HSI of 17.3%. However, there was a large within group variation for some of the liver function parameters, particularly the plasma enzyme activity assays, which could potentially mask some aspects of hepatic dysfunction.

There was a weak (+0.601) but significant positive correlation between individual HSI and total bilirubin. Total bilirubin levels in the blood may indicate impairment in its uptake by the hepatocytes (Kaplan, 1993). However, the total bilirubin concentration in the plasma of haddock was in the "normal" range for teleost marine fish (Casillas, 1983) and certainly not high enough to suggest hepatic dysfunction. Bilirubin was not elevated in the plasma of English sole (*Paraphrys vetulus*) after experimentally induced toxic liver damage. Thus, plasma bilirubin levels were not reliable indicators for acute liver toxicity in this marine species (Casillas, 1983).

Aminotransferase activity in the plasma has been used as a measure of liver function in other fish species. There was no detectable activity of alanine or aspartate aminotransferases in the plasma of the marine fish, red drum (*Sciaenops ocellatus*), fed diets containing 0 to 21% lipid (Craig *et al.*, 1999). The activity of aspartate aminotransferase averaged 281 U/L in sturgeon (*Acipenser transmontanus*; Fynn-Aikins *et al.*, 1993) and 196 U/L in rainbow trout (*Salmo gairdneri*; Racicot *et al.*, 1975) plasma. Alanine aminotransferase activities were much lower averaging 49 U/L in sturgeon

(Fynn-Aikins *et al.*, 1993) and 27 U/L in rainbow trout (Racicot *et al.*, 1975) plasma. In haddock the aspartate and alanine aminotransferase activities were much lower than those reported for sturgeon and rainbow trout, the haddock averaging 22 and 3 U/L respectively. All of these fish species have a high ratio of aspartate to alanine aminotransferase activity in their plasma when detected. The enzymatic activities of aspartate aminotransferase and alkaline phosphatase were significantly higher in the plasma of sea-bass (*Dicentrarchus labrax*) fed a diet without polyunsaturated fatty acids. However there was no significant difference in alanine aminotransferase activity. Necrosis of liver hepatocytes was also observed in these fish fed a polyunsaturated fatty acid deficient diet. This demonstrates that plasma aspartate aminotransferase and alkaline phosphatase can act as indicators of liver necrosis in fish (Lemaire *et al.*, 1991). Neither aspartate, alanine aminotransferase nor alkaline phosphatase were correlated with increasing HSI in the plasma of these haddock which indicates the absence of liver necrosis.

Elevated levels of plasma sorbitol dehydrogenase provided a reliable indicator of sublethal hepatotoxicity in rainbow trout. Elevated sorbitol dehydrogenase levels were also correlated with decreased serum protein levels in fish exposed to hepatotoxic chemicals. Plasma sorbitol dehydrogenase activity in healthy, non-fasted rainbow trout averaged 11 U/L (Dixon *et al.*, 1987). Sorbitol dehydrogenase activity averaged 21 U/L in haddock plasma and was not correlated with HSI or plasma protein.

In the plasma of 24 h fasted Atlantic salmon (*Salmo salar*) protein averaged 49 g/L and albumin 20 g/L. The albumin/total protein ratio averaged 41% (Sandnes *et al.*,

1988). The protein, albumin and albumin/total protein ratio was comparatively lower in the 72-h fasted haddock averaging 33 g/L, 11 g/L and 33%, respectively. The time interval between the last feeding and blood sampling will affect these values and should be considered before performing this liver function test. Plasma albumin was measured because it serves as a more specific liver function test than total protein. Albumin is synthesized only in the liver, whereas plasma proteins can come from other sources. The albumin and protein concentrations were significantly correlated (+0.647). However, the plasma protein and albumin concentrations in haddock were not negatively correlated with HSI in this study.

Other than total bilirubin, there were no significant correlations between HSI and the liver function parameters tested for HSI from 10.6 to 19.6% suggesting normal liver function for HSI in this range. However, it should be stated that these tests are only indicators of liver dysfunction. It is possible to obtain normal liver function test results in human patients with serious liver disorders such as cirrhosis or hepatocellular carcinoma (Kaplan, 1993).

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Table I. Formulations of diets used for haddock liver function trial.

Ingredient (%DM)	12% lipid	22% lipid	Commercial[1]
Herring meal[2]	49.5	49.5	39.4
Soybean meal	0.0	0.0	8.0
Blood meal	0.0	0.0	4.0
Crab meal[3]	4.0	4.0	12.6
Corn gluten meal[4]	13.0	14.0	8.0
Ground wheat	0.0	0.0	13.2
Krill hydrolysate[5]	0.2	0.2	0.2
Binder (DAKA)	0.0	0.0	2.0
Herring oil[6]	6.0	16.0	8.0
Soybean lecithin	0.0	0.0	2.0
Whey dried[7]	7.0	7.0	0.0
Wheat middlings[4]	9.2	5.2	0.0
Gelatin[8]	1.5	1.5	0.0
Celufil[8]	7.0	0.0	0.0
Choline chloride[8]	0.6	0.6	0.6
Vitamin mixture[9]	1.0	1.0	1.0
Mineral mixture[10]	1.0	1.0	1.0
Total	100.0	100.0	100.0
CP (%)	49.0	49.0	49.9
DP (%)	46.0	46.0	46.2
Lipid (%)	12.0	21.7	15.8
CHO (%)	16.2	14.4	16.5
Fiber (%)	1.7	1.4	2.5
Ash (%)	9.5	9.3	10.3
DE (cal/g)	3855.3	4699.6	4117.8
g DP/MJ DE	28.5	23.4	26.8

[1]Zeigler Bros. Inc., Gardner, PA
[2]Sea Life Fisheries Inc., Canada
[3]St. Laurent Gulf Products Ltd., Caraquet, NB
[4]Dover Mills Ltd., Canada
[5]Special Marine Products Ltd., West Vancouver, Canada
[6]Stabilized with 0.06% ethoxyquin, Commeau Seafood, Saulnierville, NS
[7]Farmers, Truro, Canada
[8]US Biochemical, Cleveland, OH
[9]Vitamin added to supply the following (per kg diet): vitamin A, 8000 IU; vitamin D_3, 4500 IU; vitamin E, 300 IU; vitamin K_3, 40 mg; thiamine HCl, 50 mg; riboflavin, 70 mg; d-Ca pantothenate, 200 mg; biotin, 1.5 mg; folic acid, 20 mg; vitamin B_{12}, 0.15 mg; niacin, 300mg; pyridoxine HCl, 20 mg; ascorbic acid, 300 mg; inositol, 400 mg; BHT, 15 mg; BHA, 15 mg
[10]Mineral added to supply the following (per kg diet): manganous sulfate (32.5% Mn), 61.5 mg; ferrous sulfate (20.1% Fe), 62.3 mg; copper sulfate (25.4% Cu), 19.8 mg; zinc sulfate (22.7% Zn), 165.2 mg; cobalt chloride (24.8% Co), 20.2 mg; sodium selenite (45.6% Se), 1.1 mg; sodium fluoride (45.2% F), 9.4 mg

Table II. Proximate composition of commercial and experimental diets used for haddock liver function trial (dry matter basis).

Composition (g/100g)	Commercial[a,b]	12% lipid[c]	22% lipid[c]
Moisture	8.22	9.26	9.15
Protein	49.89	53.81	54.17
Lipid	19.07	13.32	22.38
Ash	13.72	8.53	8.41

[a]Produced by Zeigler Bros. Inc., Gardners, PA, USA.

[b]Average of three replicates.

[c]Average of two replicates.

Table III. Fatty acid composition of commercial and experimental diets used for haddock liver function trial.

Fatty Acid	Commercial[a,b]	12% lipid[c]	22% lipid[c]
14:0	7.22	5.52	6.34
16:0	18.30	16.52	16.57
16:1n-7	9.59	6.49	7.27
18:0	3.59	2.59	2.84
18:1n-9	9.71	11.17	10.99
18:1n-7	3.22	2.77	2.72
18:2n-6	3.64	4.87	2.97
18:3n-3	1.35	0.81	0.70
18:4n-3	2.53	2.77	2.56
20:1n-9	2.66	4.80	3.62
20:4n-6	0.77	0.78	0.91
20:4n-3	1.25	0.54	0.60
20:5n-3	9.91	13.33	15.00
22:1n-11	2.72	5.78	4.38
22:5n-3	1.75	1.31	1.65
22:6n-3	10.88	10.30	9.74
Σ SAT	30.78	25.68	26.88
Σ MONO	30.31	33.43	31.02
Σ PUFA	37.90	40.89	42.09
Σ n-3	28.58	29.66	30.96
Σ n-6	5.31	6.21	5.01
DHA/EPA	1.10	0.77	0.65

[a]Produced by Zeigler Bros. Inc., Gardners, PA, USA.

[b]Data expressed as area % of FAME, n=3.

[c]Data expressed as area % of FAME, n=2.

Table IV. Growth, hepatosomatic index (HSI) and hematocrit of haddock fed 12 and 22% lipid experimental diets.

Diet	Wt. gain[1] (g/ fish)	SGR[2]	Feed: gain[3] Ratio	HSI[ns]	Hematocrit*
12% lipid	108.7	0.79	0.90	11.9 ± 1.6	28.8 ± 2.3
22% lipid	93.8	0.73	0.99	11.4 ± 1.7	33.0 ± 2.9

[1] Initial weight of fish was 98.5g; average for two tanks.

[2] Specific growth rate (%) = 100 x (ln(final wt.)-ln(initial wt.))/duration (d).

[3] Feed intake (expressed as dry matter)/wet wt. gain

[ns] HSI (%) = wet liver wt./body wt. x 100.
HSI were not significantly different ($p>0.05$, mean±SD, n=10). HSI of initial fish was 11.87±2.12% (n=5).

* Hematocrit values (% red blood cell volume) were significantly different ($p<0.05$, mean±SD, n=20).

Table V. Liver function test results for haddock[1] with low, medium and high hepatosomatic indices (HSI).

	Low HSI	Medium HSI	High HSI
HIS	11.1 ± 0.6[a]	13.0 ± 0.5[b]	17.3 ± 1.7[c]
Liver function test			
TBili[ns]	0.20 ± 0.45	0.60 ± 0.55	1.00 ± 0.71
AlkPh[ns]	3.40 ± 1.82	3.80 ± 1.30	3.20 ± 2.17
ALT[ns]	2.80 ± 1.64	2.20 ± 1.79	2.80 ± 1.30
GGT[ns]	1.60 ± 2.07	1.80 ± 2.68	2.20 ± 4.92
TProt[ns]	35.60 ± 10.26	30.20 ± 2.68	32.20 ± 7.09
Alb[ns]	10.80 ± 0.84	10.20 ± 0.84	10.80 ± 1.92
SDH[ns]	13.60 ± 4.77	27.20 ± 11.37	22.40 ± 7.83
AST[ns]	40.20 ± 39.54	19.80 ± 13.70	5.20 ± 7.40

[1]Grouped from haddock fed 12% experimental or commercial diet (Ziegler Bros. Inc., Gardners, PA, USA).

Abbreviations and units:
Alb (albumin, g/L); AlkPh (alkaline phosphatase, units/L); ALT (alanine aminotransferase, units/L); AST (aspartate aminotransferase, units/L); GGT (gamma-glutamyltransferase, units/L); HSI (hepatosomatic index, %); SDH (sorbitol dehydrogenase, units/L); TBili (total bilirubin, umol/L), TProt (total protein, g/L).

Values (means ± SD; n=5) in the same row containing different superscripts were significantly different ($p<0.05$).

[ns]Not significantly different ($p>0.05$).

Table VI. Pearson correlation matrix and corresponding probability matrix of liver function parameters of haddock[a]. Correlations in bold type are significant ($p<0.05$).

a) Pearson correlation matrix

	HSI	TBILI	ALKPH	ALT	GGT	TPROT	ALB	SDH	AST
HSI	1.000								
TBILI	**0.601**	1.000							
ALKPH	-0.298	-0.282	1.000						
ALT	0.116	0.496	-0.316	1.000					
GGT	0.064	0.183	-0.398	0.047	1.000				
TPROT	-0.121	-0.126	-0.145	0.277	-0.284	1.000			
ALB	-0.053	-0.036	0.198	-0.015	-0.319	**0.647**	1.000		
SDH	0.166	0.457	0.233	0.129	-0.306	0.143	0.138	1.000	
AST	-0.490	-0.027	-0.014	**0.540**	0.033	**0.530**	0.228	-0.007	1.000

b) Matrix of probabilities

	HSI	TBILI	ALKPH	ALT	GGT	TPROT	ALB	SDH	AST
HSI	0.000								
TBILI	0.018	0.000							
ALKPH	0.281	0.309	0.000						
ALT	0.681	0.060	0.251	0.000					
GGT	0.821	0.513	0.142	0.867	0.000				
TPROT	0.668	0.656	0.605	0.317	0.305	0.000			
ALB	0.850	0.898	0.479	0.957	0.246	0.009	0.000		
SDH	0.555	0.087	0.403	0.647	0.267	0.611	0.624	0.000	
AST	0.063	0.923	0.960	0.038	0.906	0.042	0.415	0.981	0.000

[a]From haddock fed 12% experimental or commercial diet (Ziegler Bros. Inc., Gardners, PA, USA).

Abbreviations:
Alb=albumin; AlkPh=alkaline phosphatase; ALT=alanine aminotransferase; AST=aspartate aminotransferase; GGT=gamma-glutamyltransferase; HSI=hepatosomatic index; SDH=sorbitol dehydrogenase; TBili=total bilirubin; TProt=total protein.

6. Conclusions

Experimental and field studies show that hepatosomatic index (HSI; liver to body weight ratio) of haddock may exceed 20% when the diets contain high amount of lipid (>15%). These highly fragile, friable livers can be physically disrupted resulting in the breaking of capillaries and bacterial infections (C. Frantsi, Heritage Salmon Ltd., Blacks Harbour, NB; personal communication). Also, cultured haddock with fatty livers convert dietary energy into flesh less efficiently. Thus, the occurrence fatty liver in cultured haddock is a major constraint for the commercial culture of haddock juveniles to market size. Studies were designed to investigate the lipid utilization and metabolism in haddock as well as to provide insight into the cause(s) or mechanism behind the frequent occurrence of fatty liver in cultured gadoids. To achieve this, the patterns of lipid deposition and catabolism were determined in the tissues of cultured juvenile haddock fed graded levels of dietary lipid. The transport of lipids in the blood of haddock was also evaluated by measuring lipoprotein composition in the serum. Liver function tests were carried out on the plasma of cultured haddock to determine if the metabolism of nutrients and health were affected in fish with high HSI.

In the study on lipid deposition, juvenile haddock were fed graded levels of herring oil to supply 14, 16, 19 and 22% lipid (dry wt.) in a herring meal-based, isonitrogenous diets. The growth and feed efficiency of juvenile haddock were not significantly ($p<0.05$) affected by increasing the lipid content of the diet. The liver was the major lipid storage organ in haddock. A significant increase in hepatosomatic index (9.8-12.1%) and total liver lipid (63.2-69.0%) were observed in haddock fed 14% versus

22% lipid (dry wt.). Muscle lipid levels remained low (1.0%) and did not increase significantly with dietary lipid. A dietary lipid level of 14% (dry wt.) or less is recommended for juvenile haddock. Growth did not decrease significantly, whereas the hepatosomatic index and liver lipid were comparatively lower in haddock fed 14% lipid (dry wt.).

Studies conducted on tissue fatty acid composition and lipid transport mechanisms of haddock showed that lipid composition of the serum resembled that of the muscle in haddock. Phospholipid (57%) and polyunsaturates (48%) were the major lipid and fatty acid classes, respectively. The low level of VLDL (<50mg/dL) circulating in the serum suggests a low level of lipid transport out of the liver to the muscle in haddock.

Lipid metabolism studies did not show a significant increase in the ß-oxidation activity of liver and muscle as the dietary lipid level increased from 12 to 24%. Peroxisomal ß-oxidation (100%) dominated in the liver whereas mitochondrial ß-oxidation dominated in the red (91%) and white muscle (97%) of juvenile haddock. Of the tissues tested, red muscle possessed the highest capacity for ß-oxidation expressed as per mg protein or per g wet weight. There is a lower proportion of red muscle in less active, demersal fish such as haddock (Greer-Walker & Pull, 1975) suggesting a lower capacity for overall fatty acid catabolism. The white muscle appears to be the most important tissue in juvenile haddock for overall ß-oxidation of fatty acids.

A series of liver function tests were performed on the plasma of cultured haddock grouped on the basis of their hepatosomatic indices (HSI). There was no clear evidence

or trend of hepatic dysfunction in haddock with individual HSI ranging from 10.6 to 19.6%, or differences between groups with an average HSI of 11.1, 13.0 and 17.3%.

Unlike wild fish, intensively cultured fish are generally fed to satiation on a daily basis to optimize growth. The lipid contained in the diet is highly digestible (NRC, 1993) and either incorporated within the fish or catabolized. This study demonstrated that the incoming dietary lipid was deposited almost exclusively in the liver with negligible amounts of lipid accumulation in the muscle. However, the muscle was the most important site for overall fatty acid catabolism. VLDL, a major transporter of lipid out of the liver to muscle, was comparatively low (<50 mg/dL) in the blood of haddock. Thus, it appears that the transport of lipid from the predominant storage (liver) to the catabolic (muscle) site is low in haddock. Also, haddock did not increase fatty acid catabolism in the liver with an increase in dietary lipid. Of the tissues tested, the red muscle was the most active for catabolizing fatty acids in haddock. Haddock is a demersal, comparatively inactive fish species with a low red to total muscle ratio (Greer-Walker and Pull, 1975). A lower red to total muscle ratio may indicate a lower overall capacity for lipid catabolism. These observations suggest the underlying cause behind the frequent occurrence of fatty liver in cultured gadoid fish.

Although an increase in the dietary protein content and the reduction in lipid level may be the simplest way to reduce fatty liver conditions in cultured haddock (Kim *et al.*, 2001), this will increase the feed cost as well as nitrogen excretion. Protein is the single most expensive ingredient in fish feeds. The use of high protein diets also increases indigestible protein and ammonia excretions which increases the nitrogen load from fish

farms. Carnitine has been used in the diet, with mixed results, to reduce fat deposition in fish by increasing the rate of mitochondrial ß-oxidation (Gaylord and Gatlin, 2000). However, it appears that peroxisomal ß-oxidation predominates in the liver of juvenile haddock and transport of lipid out of the liver to the muscle for mitochondrial ß-oxidation may be low. Phosphatidylcholine (lecithin) has also been used in fish to reduce liver lipid deposition by increasing synthesis of VLDL (transport of lipid out of the liver) for which it is a major component (Griffin *et al.*, 1994). However, lecithin was incorporated at 2% of the diet in the ß-oxidation study and the HSI of the haddock fed 24% lipid averaged over 15%.

One way to proceed in this research on the fatty liver problem in gadoids is to examine ways of increasing fatty acid catabolism in the liver. To achieve this goal, the ß-oxidation activity of haddock liver could be measured for various types of radiolabelled fatty acids (see Crockett et al, 1993a,b) including medium or short-chain fatty acids (Nordrum *et al.*, 2000). A diet could then be tailored using oils containing a high percentage of those fatty acids which induced a higher rate of ß-oxidation in the haddock liver. Dietary conjugated linoleic acid (CLA) reduced the accumulation of adipose tissue in mammals (Belury, 2002). The effects of dietary CLA on the catabolism of lipid in the liver of haddock could also be evaluated.

A salt-resistant triacylglycerol lipase enzyme in the liver of cod was ca. five times more active than the same enzyme in rainbow trout liver (Black *et al.*, 1983). The more active salt-resistant triacylglycerol lipase may be used by the cod to mobilize its comparatively larger liver lipid stores (Sargent *et al.*, 1989). Longer-chain free fatty acids

(>10C) released into the plasma may be transported on albumin-like carrier proteins in teleost fish (Sheridan, 1988). Measurement of salt-resistant triacylglycerol lipase activity in the liver of haddock fed different amounts or types of lipid might also prove beneficial.

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7. APPENDIX A

Table I. Common and scientific names of fish species discussed in this thesis.

Common name	Species name
Amberjack	*Seriola dumerili*
Arctic char	*Salvelinus alpinus*
Atlantic salmon	*Salmo salar*
Ayu	*Plecoglossus altivelis*
Black cod	*Erilepis zonifer*
Capelin	*Mallotus villosus*
Channel catfish	*Ictalurus punctatus*
Cod (Atlantic)	*Gadus morhua*
Coho salmon	*Oncorhychus kisutch*
Carp (common)	*Cyprinus carpio*
Eel (Japanese)	*Anguilla japonica*
English sole	*Parophrys vetulus*
Flathead	*Platycephalus bassensis*
Flounder (Japanese)	*Paralichthys olivaceus*
Gilthead seabream	*Sparus auratus*
Goldfish	*Carassius auratus*
Greenland halibut	*Rheinhardtius hippoglossoides*
Haddock	*Melanogrammus aeglefinus*
Herring	*Clupea harengus*
Hybrid striped bass	*Morone saxatilis* X *Morone chrysops*
Lake trout	*Salvelinus namaycush*
Mackerel	*Scomber scombrus*
Nile tilapia	*Oreochromis nilotica*
Pacific cod	*Gadus macrocephalus*
Plaice (American)	*Hippoglossoides platessoides*
Rainbow trout	*Salmo gairdnerii*
Red drum	*Sciaenops ocellatus*
Red seabream	*Pagrus major*
Sandeel	*Ammodytes spp.*
Seabass (European)	*Dicentrarchus librax*
Steelhead trout	*Salmo gairdnerii*
Striped bass	*Morone saxatilis*
Striped jack	*Caranx delicatissimus*
Sturgeon	*Acipenser spp.*
Tilapia (Zilli's)	*Tilapia zilli*
Turbot	*Scophthalmus maximus*
Yellow perch	*Perca flavescens*