THE EFFECT OF DIETARY ARACHIDONIC ACID

CONCENTRATION ON ATLANTIC HALIBUT

(*HIPPOGLOSSUS HIPPOGLOSSUS*) BROODSTOCK

PERFORMANCE. ASSESSMENT OF EGG, MILT AND

LARVAL QUALITY

THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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BY

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GLOSSARY

Asian sea bass	*Lates calcarifer*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Baltic herring	*Clupea harengus m.*
Capelin	*Mallotus villosus*
Cod	*Gadus morhua*
Coho salmon	*Oncorhynchus kisutch*
Cultured sturgeon	*Acipenser fulvescens*
European seabass	*Dicentrarchus labrax*
Flounder	*Platichthys flesus*
Goldfish	*Carassius auratus*
Gilthead seabream	*Sparus aurata*
Japanese flounder	*Paralichthys olivaceus*
Murray cod	*Maccullochella. peelii peelii*
Pike	*Esox luxius*
Rainbow trout	*Onchorynchus mykiss*
Senegal sole	*Solea senegalensis*
Striped bass	*Morones saxatilis*
Trout cod	*Maccullochella macquariensis*
Turbot	*Scophthalmus maximus*
Walleye	*Stizostedion vitreum*
White sea bream	*Diplodus sargus*

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ABSTRACT

In order to evaluate the impact of four different diets on halibut broodstock, eight tanks were set up. Each tank contained 15 females and 5 males. The four broodstock diet formulations contained 0.4% arachidonic acid (20:4n-6; ARA), (0.4g ARA/100g dry feed), 0.6% ARA, a control feed not supplemented with ARA and a control feed in which a proportion of the fish meal was replaced with squid meal, to improve palatability. The experimental diets were fed for a period of 3 years. The spawning period was regulated by photoperiod. Four tanks spawned in May and the four others spawned in July. A pit tag was used to identify each fish. This allowed individual length and weight data to be collected every 2 months. During the spawning season, milt, eggs, at different developmental stages, and yolk sac larvae were sampled for subsequent biochemical analyses (lipids, fatty acids and prostaglandins). Fish fed the ARA enriched diets showed significantly better growth compared to the other two treatments. During the second spawning season, fish fed the 0.4% ARA diet showed significantly improved egg production while fish fed on the 0.6% ARA diet showed a delay in their spawning season and poor reproductive performance. Fatty acid analyses on eggs, yolk sac larvae and milt showed significant ARA uptake and deposition in relation to the diet. Broodstock females fed the 0.4% ARA diet produced early life stages with what could be regarded as an optimal DHA: EPA: ARA ratio. Furthermore, males fed the 0.4% ARA diet produced the best quality milt over an extended period.

Acknowledgements

Chapter 1 General Introduction

The Atlantic halibut (*Hippoglossus hippoglossus*) is emerging as a promising species for marine cold-water aquaculture in the countries of the North Atlantic. The potential for farming Atlantic halibut has been studied in Norway, the UK, Iceland and Canada. Fishing by long-lining and non-specific trawling has largely depleted Atlantic stocks of halibut. Catches of wild fish are therefore sporadic and those caught are of variable size and quality (Arthur, 1999). With demand high due to the premium consumer image of the fish, market values around £15 per Kg (fillet) are common.

FARMED ATLANTIC HALIBUT PRODUCTION IN EUROPE
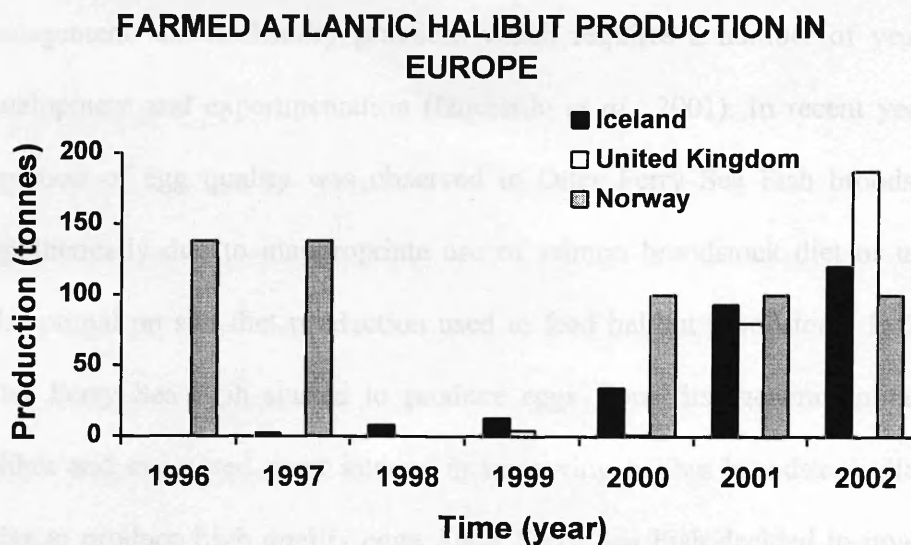
Figure 1. Atlantic halibut production in Europe, data from FAO (2003)

As a consequence of this market opportunity, there has been over 15 years of heavy investment into technology to enable farming of Atlantic halibut to progress (Arthur, 1999). The first halibut broodstock was established in 1982 in Norway (Lønning *et al.,* 1982). Little is known about how broodstock feeding affects egg and larval viability in halibut. Halibut research has been hampered by the low availability of broodstock fish. Ideally, broodstock fish should be maintained under controlled conditions, which as far as possible match or improve upon those to which the fish will have been exposed in the wild. In practice, however, it may not be possible to manage all of the rearing conditions. Water quality, feeding regime and diet, stocking density, exposure to pathogens and handling stress parameters may be optimised by appropriate management and husbandry practices which requires a number of years of development and experimentation (Izquierdo *et al.*, 2001). In recent years, a depletion of egg quality was observed in Otter Ferry Sea Fish broodstock, hypothetically due to inappropriate use of salmon broodstock diet or use of sub-optimal on site diet production used to feed halibut broodstock. In 2000, Otter Ferry Sea Fish started to produce eggs from first generation farmed halibut and expressed great interest in improving halibut broodstock diets in order to produce high quality eggs. Otter Ferry Sea Fish decided to invest in halibut broodstock and tanks to perform this scientific project.

1 Atlantic Halibut

1.1 Halibut species

The Atlantic halibut *Hippoglossus hippoglossus* (L.) is the largest flatfish in the

North Atlantic. Within the order Pleuronectiformes the genus Hippoglossus

belong to the family Pleuronectidae, sub-family Pleuronectinae (Hensley &

Ahlstrom 1984). Studies using genetically determined electrophoretically

detectable protein variants to test for genetic differences have revealed a

genetic difference between Atlantic and Pacific halibut of a magnitude that

confirms the treatment of the two taxa as separate species (Grant *et al.* 1984).

Separate stocks, as indicated by both tagging experiments and the examination

of various population parameters, have also been suggested for halibut in

Faeroese and Canadian Atlantic waters (Haug & Fevolden, 1986).

1.2 Atlantic halibut geographical distribution

The halibut is an arctoboreal species, distributed in parts of the Arctic Ocean

and in the northern part of the Atlantic Ocean. Occasionally found as far south

as the Bay of Biscay and New York on the eastern and western side of the

Atlantic, respectively. It is commonly found in the southwestern parts of the

Barents Sea as far north as Bear Island, and occasionally on the west coast of

Spitzbergen. It is particularly numerous along the Norwegian coast, off the

Faeroes and Iceland, and off southern Greenland, and it may also be

encountered in the North Sea and western part of the Baltic Sea (Haug, 1990).

Atlantic halibut have been shown to return to the same spawning site in

successive years, suggesting that distinct breeding populations could exist. Genetic screening of 43 loci in Faeroes halibut revealed only four polymorphic loci. Comparing these four loci, including stocks from the Faeroes, the Norwegian coast, and Greenland gave evidence of general genetic homogeneity over the sampling range. This lack of genetic differentiation is taken as strong evidence of substantial gene flow between geographical areas (Fevolden & Haug, 1988). While feeding, mature halibut are dispersed widely on offshore banks and in shallow inshore waters. Prior to and during spawning, fish congregate on the spawning grounds, which are certain soft clay, or mud covered deep Norwegian fjords or ocean-bank pools (Haug & Tjemsland, 1986).

1.3 Atlantic halibut; Description of the genus *Hippoglossus*

Halibut are laterally compressed, asymmetrical with pigmentation occurring only on the right side (Stickney *et al.*, 1991). The body is elongated, covered with small cycloid scales and smaller supplementary scales. The lateral line has a steep bend above the pectoral fin. Both eyes are on the right side of the head. Jaws are large, symmetrical with large pointed teeth directed posteriorly. Teeth, on the upper jaw, are in two rows and on the lower jaw, are in one row. The vomer is toothless. Infrapharyngeal teeth are sharp, in two rows. The inner series has enlarged teeth. An anal spine overgrown by skin is present in adult specimens. Pectoral fins are better developed on the eye side. The caudal fin is weakly emarginated. They have between 49 and 53 vertebrae. The colour of

the ocular or dorsal side is dark brown, darker in adult specimens and lighter in juveniles. The blind side is usually white, although on very rare occasions coloration of various amounts is present. In extreme cases, an incomplete rotation of the eye and hooked dorsal fin has been observed. In some very rare cases, the general rule of dextral pigmentation/eye localization is broken and reversed and unusual specimens may occur. Although some statistically significant differences have been confirmed by unvariate and multivariate analysis of morphometric variables, no visual morphological differences between the sexes is evident (Haug, 1990).

1.4 Atlantic halibut biology

The species thrives at low temperatures and produces a highly regarded, firm, and white-fleshed meat (Cordero *et al.*, 1994). The Icelandic stock of the Atlantic halibut spawns annually from early March to late May (Foss *et al.*, 1998). A peak in halibut spawning activity in late January and early February is indicated in deep waters on the continental slope southwest of the Faroe Islands (Jákupsstovu & Haug, 1987) and in northern Norway (Kjørsvik *et al.*, 1987). Each individual female spawns several batches of eggs over a period of about four weeks. The adult fish have a seasonal migration in the North Atlantic Ocean from shallow coastal waters in summer to oceanic depths in winter. Tagging experiments in Norwegian waters have revealed that mature halibut show a remarkable homing response, returning to the same area for repeated spawning (Foss *et al.*, 1998). Spawning takes place in relatively warm (5-7°C)

saline (34.5-35‰) bottom waters at depths ranging from 350 to over 1000m (Haug *et al.*, 1982; Haug *et al.*, 1984). In wild fish there is a clear sexual difference in growth rates from the age of 6-7 years, when the males mature. There is a considerable variation in age at first maturation in halibut, and maturity seems to be more a function of growth rate and size than age (Jákupsstovu & Haug, 1988).
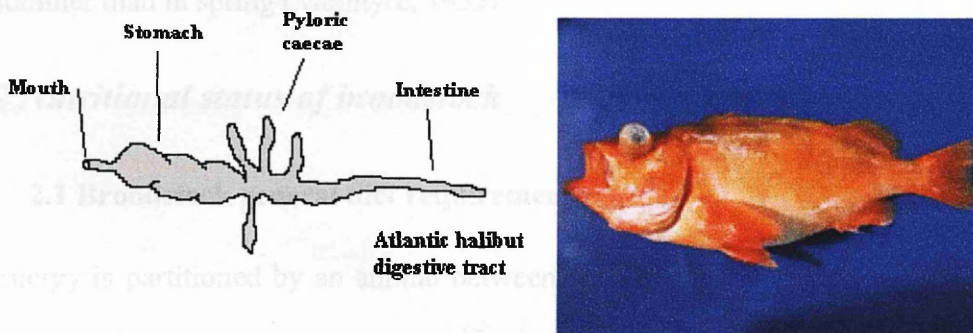
Picture 1. Atlantic halibut digestive tract and *Sebastes marinus* (www.redfish.de/biology/classification.html)

Atlantic halibut has a digestive tract which is relatively short and is divided into regions of stomach, pyloric caecae and intestine (Picture 1). This fish stomach is well-defined and muscular accounting for 26% of the total gut length as opposed to 50% for the intestine (Glass *et al.*, 1989). Halibut is a large predatory fish. In general, the number of organisms which serve as food for halibut is limited. A change in the diet can be observed with increasing size of the fish. Fish up to about 30 cm contained invertebrate food almost

exclusively, mostly annelids and crustaceans. Those from 30 to 80 cm contained either invertebrates or fish or both (Kohler, 1967). The large halibut found off Iceland were feeding on only 11 species of organisms, and a single species of fish, *Sebastes marinus* (Picture 1), constituted over 75% of the food volume. The adult halibut demand food organisms large in size or rich in abundance to make their capture easy. In view of this diet preference, halibut must leave the bottom in pursuit of prey. The feeding intensity is greater in summer than in spring (McIntyre, 1952).

2 Nutritional status of broodstock

2.1 Broodstock general diet requirements

Energy is partitioned by an animal between each of the various physiological processes involved in maintenance, growth and reproduction. The end result of this partitioning is that it is not possible for energy to be expended for more than one purpose. Energy used for growth cannot also be used for reproduction. In general, the maintenance requirements of an animal are met first and then additional energy is divided between growth and reproduction. The relative partitioning of energy between growth and reproduction varies both between species and between strains of an individual species, and further generalizations are difficult to make (Izquierdo *et al.,* 2001). The total amount of energy available for utilization in various physiological processes has been found to affect size, quality and number of eggs produced. Reduced fecundity,

reported in several marine fish species, could be caused either by the influence

of a nutrient imbalance on the brain-pituitary-gonad endocrine system or by

restriction in availability of a specific biochemical, component, or components,

required for optimal egg formation (Izquierdo *et al.*, 2001).

2.2 Carbohydrate metabolism in fish

The utilisation of carbohydrates by marine species is still controversial. In the

natural environment fish have limited access to carbohydrate sources and are

not well adapted, at the digestive and metabolic levels, to deal with high

amounts of dietary carbohydrates. Glucose injection seemed to enhance

catabolism of body stores in seabream and to stimulate anabolism in seabass

(Peres *et al.*, 1999). Carbohydrate in fish diets also has to be carefully

controlled since the excess deposited as glycogen is subsequently less readily

available to the fish for use as energy than other chemical forms. The plasma

glucose level in resting fish reflects the dietary level of carbohydrate. The

plasma glucose response is significantly more affected by stress in

carbohydrate-consuming cod than in cod on a carbohydrate-free diet (Hemre *et

al.*, 1991). Cod fed diets with either 10% or 21% carbohydrate and then fasted

for 4 weeks showed significantly higher weight loss than cod fed a diet without

carbohydrates (Hemre *et al.*, 1993). Moreover, reduction of dietary protein

levels from 51% to 34% together with an increase in dietary carbohydrate level

from 10% to 32% reportedly reduced egg viability in seabass (Cerdá *et al.*,

1994). Diets of varying carbohydrate levels have no strong influence on live

weight or condition factor in halibut. The lower average plasma glucose

concentration may indicate an ability to adapt to starch levels higher than those

found in the natural diet of halibut. It is generally accepted that in flatfish, the

main energy reserves are deposited in the liver and muscle tissues, and these

organs are the primary targets for insulin in fish. The higher concentration of

labelled glucose found in halibut heart, relative to kidney, gills and

gastrointestinal wall, indicate a higher utilisation of glucose for energy in the

heart than in the other organs (Garcia-Riera & Hemre, 1996).

2.3 Essential minerals

Table 1: The trace mineral requirement ranges for fish (Watanabe *et al.*, 1997)

Mineral	Requirement (mg mineral kg^{-1} dry diet)
Iron	30-170
Copper	1-5
Manganese	2-20
Zinc	15-40
Cobalt	0.05-1.0
Selenium	0.15-0.5
Iodine	1-4

Minerals are required for the normal life processes, and animals, including fish,

need these inorganic elements. Fish may derive these minerals from their diets

and also from ambient water. Characteristic concentrations and functional

forms of the minerals need to be maintained within narrow ranges for ordinary

metabolic activities in cells and tissues. The minerals are responsible for

skeletal formation, maintenance of colloidal systems, regulation of acid-base

equilibrium and for biologically important compounds such as hormones and

enzymes. If excess amounts of the elements are ingested and assimilated, toxicity may develop. Therefore, the animal maintains a delicate balance of body levels of trace metals by integrating the various parameters of uptake, storage and excretion.

2.3.1 Selenium

Selenium is essential for animals, including fish. It is an integral component of glutathione peroxidase. The activity of this enzyme in liver or plasma is indicative of selenium supply to the organism. Glutathione peroxidase protects cells and membranes from oxidative damage by destroying hydrogen peroxide and lipid hydroperoxides employing reducing equivalents from glutathione. Selenium in conjunction with vitamin E is essential for avoidance of nutritional muscular dystrophy. Selenium compounds are also capable of protecting against toxicity of heavy metals such as cadmium and mercury (Watanabe *et al.*, 1997). Mackerel, herring and flatfish species contain 0.262-0.498 µg Se/g wet weight (Önning, 2000).

2.3.2 Copper

Copper is involved in the activity of enzymes such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase and tyrosinase. In addition, copper-proteins and chelates also have metabolic roles. Copper levels are high in the eyes where it is found along with melanins, bound to protein.

Organs such as liver, brain and heart also contain comparatively large amounts

of copper (Watanabe *et al.*, 1997).

2.4 Essential vitamins and pigments

Vitamins are organic molecules that act as cofactors or substrates in some

metabolic reactions. They are generally required in relatively small amounts in

the diet and, if present in inadequate amounts, may result in nutrition-related

disease, poor growth or increased susceptibility to infections. The addition of a

vitamin supplement to the whole frozen herring diet of juvenile halibut

produced a beneficial effect on growth (Goff & Lall, 1989). Inclusion of

vitamins in diets is complicated further since most of them are highly labile

molecules and are readily destroyed during processing. Individual vitamins or

premixes of vitamins prepared for specific purposes are commercially available

(Izquierdo *et al.*, 2001).

2.4.1 Vitamin D_3

Livers from marine fish contain large amounts of vitamin D_3. Fish species

living in a marine environment have large calcium supply and use a great deal

of energy to excrete this from the body. Freshwater species have the opposite

problem, needing a calcium transport mechanism from the water to the animal.

The vitamin D metabolites regulate the calcium balance. The $24,25(OH)_2D_3$

has a hypocalcaemic function and is the quantitatively predominant metabolite

in salmon, cod and halibut. This may be explained by the requirement of the

species living in a calcium-rich environment to decrease, rather than increase, their calcium absorption (Graff *et al.*, 1999).

2.4.2 Vitamin A

Vitamin A is considered important for embryo and larval development due to its role in bone development, retina formation and differentiation of immune cells. Furuita *et al.* (2001) indicate that feeding Japanese flounder broodstock a higher level of vitamin A increases the vitamin A content in eggs but does not affect egg quality because excess dietary vitamin A was stored mainly in the broodstock liver.

2.4.3 Vitamin K

Vitamin K is required for carboxylation of glutamate residues to γ-carboxyglutamate residues in many proteins. The γ-carboxyglutamate residues are necessary for normal blood coagulation as they specifically bind calcium ions. The intestinal production of vitamin K can be affected by composition of diets and antibiotics. In salmonids, the vitamin K requirement for growth is suggested to be 10 mg kg^{-1} dry diet. Increased blood clotting time, anaemia and haemorrhages in gills, eyes and vascular tissues have been reported in salmonids fed diets low in vitamin K, or diets supplemented with antibiotics. However cod seems to have a low vitamin K requirement, below 0.2 mg kg^{-1} (Grahl-Madsen & Lie, 1997)

2.4.4 Vitamin E

Vitamin E, also known as α-tocopherol, is an antioxidant, particularly protecting polyunsaturated fatty acids. It acts as an inter- and intra-cellular antioxidant to maintain homeostasis of labile metabolites in the cell and tissue plasma. Farmed halibut can suffer from dorsal sub-dermal fat deposits, nominally termed fat cell necrosis syndrome (FCNS). The disease does not appear to have an infectious or malignant aetiology, but may be related to an imbalance between dietary oxidants and antioxidants combined with an exposure to sunlight. It is possible that increasing the levels of antioxidants, such as α-tocopherol, in the diet may induce resistance to the syndrome (Bricknell et al., 1996). In terms of reproduction effect, an increase in the level of dietary α-tocopherol significantly reduced the percentage of abnormal gilthead seabream eggs, and resulted in an improvement in the percentage of normal eggs (Fernández-Palacios et al., 1998).

2.4.5 Vitamin C, Ascorbic acid

Ascorbic acid is a highly labile vitamin easily destroyed by cooking and lengthy, or improper, storage of food. It is usually added to feeds at five to ten fold the requirement to allow degradation during storage and to provide extended shelf life of the feed. The first recognized function of ascorbic acid is its role in hydroxylating proline to hydroxyproline for use in cartilage synthesis. It also acts as a strong reducing agent in a number of other reactions and is involved in carnitine synthesis and in detoxification of pesticides and

other toxicants in processes involving cytochrome P450. In fish reproduction,
ascorbic acid is a co-factor in the biosynthesis of steroid hormones and
neurohormones. Herring, eel, Atlantic salmon, cod, mackerel, halibut and
turbot do not have the ability to synthesize ascorbic acid; dietary
supplementation is required in the culture of teleost fish (Mæland & Waagbø,
1998). A vitamin C dose of 2000 mg kg^{-1} feed, delivered every second day to
seabass and gilthead seabream broodstock, increases collagen synthesis in
embryos and fasting larvae in comparison with a diet containing vitamin C at
the recommended concentration for growth (Terova *et al*, 1998 a). The
ascorbic acid requirements for broodstock rainbow trout (Blom & Dabrowski,
1995) and broodstock seabass and seabream (Terova *et al*, 1998 a) might be
higher than for the growth of immature fish. The research of Cecchini *et al*.
(2000) seems to demonstrate a positive correlation between L-ascorbic acid
supplementation in seabass broodstock diet and lysozyme activity in some
stages of embryos and larvae, probably reflecting better activation of the non-
specific immune system. The egg oil globule significantly increased in turbot
fed with a vitamin C supplemented diet but was not correlated with any of the
hatching or fertilisation characteristics (Lavens *et al*, 1999).

2.4.6 Astaxanthin

All animals, including fishes are unable to biosynthesise carotenoids *de novo*,
and depend on dietary supply for these pigments (Bjerkeng & Berge, 2000).
Astaxanthin, along with other carotenoids, constitutes one of the most

important pigment classes in fish, with a wide variety of functions including protection from damaging light conditions, a provitamin A source, chemotaxis of spermatozoa and antioxidant functions including singlet oxygen quenching. However, Christiansen & Torrisen (1997) suggest that astaxanthin is not essential for fertilization and egg survival in Atlantic salmon. The higher apparent digestibility coefficients (ADC) of astaxanthin in halibut than in salmon may be caused by the lower feed intake and possibly longer gastrointestinal passage time in the former (Bjerkeng & Berge, 2000).

2.5 Amino acid and protein requirements

All studies on finfish to date have shown that they need the same essential amino acids as most other animals. Fish age is an important factor in determining the level of dietary protein and hence amino acid requirements. Generally, protein requirements diminish as the fish grows older. However, evidence of possible maternal compensating in Nile tilapia female has been observed. The females attempt to channel essential nutrients to developing oocytes even when their nutrition is sub-optimal. Broodstock fish seem to ensure that all the important amino acids are incorporated into oocytes from female body stores regardless of dietary quality, this warrants further investigation (Gunasekera et al., 1997). Dietary essential amino acids (EAA) control seabream fecundity, egg and larval quality mainly via the synthesis and selective uptake of yolk constituents (Harel et al., 1995). The pelagic eggs of marine teleosts have a high content of free amino acids (FAA) and the FAA

pool is mainly found in the yolk (Rønnestad, 1992). The protein content of halibut developing eggs appears to be a function of the greater size. Neither protein nor FAA content varied significantly between similar weight and size eggs (Finn *et al.,* 1991). Exact knowledge of the amount and specificity of each enzyme present in a particular digestive system and the conditions under which hydrolysis of proteins takes place would allow us to predict the digestibility of new feeds with greater accuracy before progressing to feeding trials. All marine species would appear to rely on the trypsin / chymotrypsin combination for random cleavage of proteins, with initial hydrolysis being carried out by pepsin in the case of the adult flatfish, halibut, sole and turbot. Flatfish digestive tract shows alkaline protease, possibly elastase, activity (Glass *et al.,* 1989). For Atlantic halibut, growth and feed efficiency ratios were linearly correlated with protein digestibility (Aksnes & Mundheim, 1997). Halibut seem to have a higher protein demand than plaice and salmonids. When decreasing the level of carbohydrates and increasing the level of protein in the diet, a significant increase in growth was observed. No difference in growth or feed efficiency was found with increasing dietary lipid level from 12.7% to 32.5% (Aksnes *et al.,* 1996). Halibut have a high requirement for protein in accordance with other marine carnivorous fish however for good growth of 140g and 1 kg Atlantic halibut it is not necessary to include more than 51% and 48% protein respectively. (Helland & Grisdale-Helland, 1997; Grisdale-Helland *et al.,* 1998).

2.6 Fish dietary lipid requirements

Lipids have a fundamental importance in animal growth, namely their roles as sources of metabolic energy and sources of essential highly unsaturated fatty acids (HUFA) for the formation of cell membranes. This is because lipid rather than protein or carbohydrates is the favoured source of metabolic energy in most fish species. Seasonal variations in the levels of lipids in fish are related, fundamentally, to the reproductive cycle. It is the norm in the natural environment that fish accumulate large lipid reserves during the period spring-late summer when food is plentiful prior to developing gonads during late winter-early spring. The majority of marine fish spawn in spring, and so fertilised eggs hatch when there is a plentiful supply of food available from the plankton blooms of spring-early summer. Consequently, marine fish generally contain their highest and lowest levels of lipid in early winter and early spring respectively. Normally, food availability during winter is low, so that some of the lipid accumulated during spring-summer will be used for metabolic energy for maintenance purposes when fish over-winter. However, such maintenance costs, whether they be associated with short-term swimming activity, osmoregulation or tissue turnover, are likely to be low compared with the metabolic cost of growth (Sargent, 1995). Lipids are organic molecules containing many carbon atoms in a variety of chain or ring conformations. They are substances of biological origin and their properties result in them being soluble in organic solvents, but only slightly soluble, if at all, in water.

There are five major classes of lipids: Triacylglycerols, sterols, sphingolipids, phospholipids, and fatty acids (King, 2001).

2.6.1 Triacylglycerols

Fatty acids are stored for future use as triacylglycerols in all cells, but primarily in adipocytes of adipose tissue. Triacylglycerols constitute molecules of glycerol to which three fatty acids have been esterified. The fatty acids present in triacylglycerols are predominantly saturated or monounsaturated but, in fish, also contain polyunsaturated fatty acids. The major building block for the synthesis of triacylglycerols, in tissues other than adipose tissue, is glycerol. Fatty acids rarely occur free in nature, but more generally occur in esterified form, as triacylglycerols, so named since they are triesters of glycerol. In these three fatty acids are bonded to the glycerol molecule. Triacylglycerols are an efficient form in which to store metabolic energy mainly because they are less oxidized than carbohydrates or proteins and hence yield significantly more energy on oxidation (King, 2001).

2.6.2 Sterols

The presence of four fused carbon rings identifies sterols. A variety of side chains attached to carbon rings at c3 and c17 give them their individual characteristics. The most frequent sterol is cholesterol, being a major component of animal cell membranes and a precursor of steroid hormones and bile acids (King, 2001).

2.6.3 Sphingolipids

Sphingolipid is a derivative of sphingosine, a long-chain unsaturated amino alcohol $C_{(18)}H_{(37)}O_{(2)}N$ found especially in nervous tissue and cell membranes. It has a similar structure to a glycerol-based phospholipid, having a polar head group as well as two hydrophobic hydrocarbon chains (one is the sphingosine and the other is a fatty acid chain). Sphingolipids are one principal group of lipids in the cell membranes. They play roles in cell signalling, development, differentiation, host-pathogen interactions and definition of physical state of membranes and lipoproteins. In addition to those functions, current research has proposed sphingolipids as intracellular Ca^{2+} mediators. Important cellular membrane constituents of mammalian cells, sphingolipids have been shown to be essential for the survival of eukaryotes from yeast to man. Besides acting as signalling molecules, sphingolipids are bulk structural components of the membrane that can segregate laterally from glycerophospholipids into domains with cholesterol. These domains recruit specific membrane proteins, notably those anchored to the outside of the plasma membrane by a glycolipid (GPI), and are important in health and disease (King, 2001).

2.6.4 Phospholipids

Table 2. Major phospholipids classifications (King, 2001)
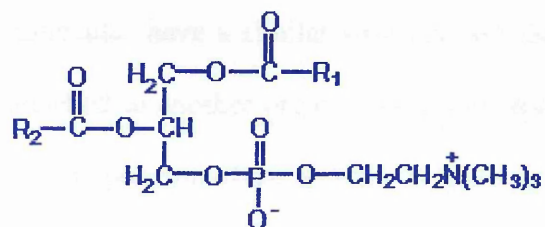
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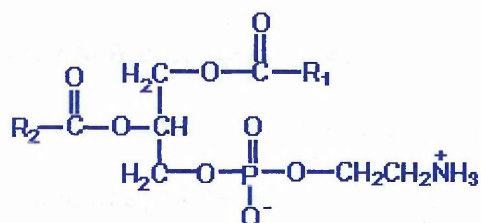
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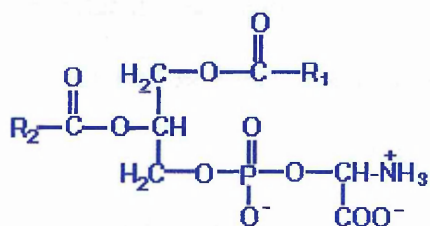
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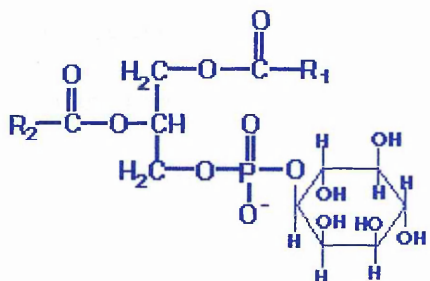
Phosphatidylinositol (PI)
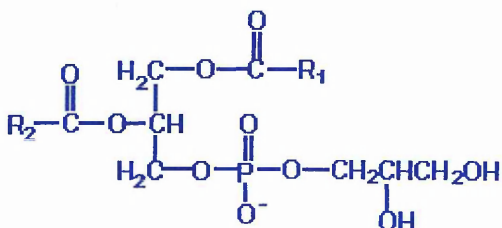
Phosphatidylglycerol (PG)

20

Another class of lipids is the glycerophospholipids, also called phospholipids. They are the major lipid components of biological membranes. These molecules have a similar structure to triacylglycerols except that a phosphate attached to another organic molecule replaces one of the fatty acid chains on the *sn*3 position of the glycerol backbone. Phospholipids are the primary fuel in fish embryonic and larval development. After hatching and up to the stage prior to first-feeding, catabolism of PL, particularly PC, has been demonstrated in larvae of halibut, plaice and cod, but not in turbot, whereas PE tended to be synthesized in all four species (Rainuzzo *et al.,* 1997). The consumption of PC was related to the reduction in DHA observed in these species. It is likely that PC was used as a source of metabolic energy at this developmental stage. High levels of triacylglycerol are linked with the poorer quality eggs from seabass fed low EFA diets during the period of vitellogenesis. The phospholipid, phosphotidylethanolamine (PE), is the most important phospholipid in neural cell membranes. In herring, *Clupea harengus*, 40% of the brain dry matter was found to be lipid and 10% of that lipid was PE (Sargent *et al.,* 1995). This phospholipid class is rich in long chain PUFA's, particularly DHA (Sargent *et al.,* 2001).

2.7 Fish dietary fatty acid requirements

In broodstock seabream, dietary fatty acid composition is reflected in body composition, especially in the ovaries (Almansa *et al.*, 2001). Czesny *et al.* (2000) demonstrated that sturgeons' environment, and thus their diet along with species-specific characteristic life history (i.e., freshwater or marine origin) play an important role and markedly influence egg fatty acid composition. Thus, egg fatty acid profile can be a viable tool in discrimination of different sturgeon populations with respect to caviar source and can ultimately be used to protect endangered wild populations of sturgeon.

Fatty acids are the simplest lipids, made from a hydrophobic carbon chain and a carboxylic acid group. The carbon chain can be either saturated (no double bonds) or unsaturated (one or more double bonds). All vertebrate species have a requirement for essential fatty acids (EFA) which they are unable to synthesise *de novo*, and must be supplied by the diet. All animals species are believed to have a requirement for both (n-3) and (n-6) PUFA, though the exact ratios and specific fatty acids within the series will vary. Terrestrial animals have a greater requirement for long chain PUFA of the (n-6) series than teleost fish while marine fish have a relatively higher requirement for long chain PUFA of the (n-3) series (Sargent *et al.* 1995; Sargent *et al.*, 1999 a).

2.7.1 Loss of metabolic pathways

Long-chain highly unsaturated fatty acid (HUFA) compositions are determined both by dietary input and by tissue desaturase and elongase enzymes. The exact cause of the differences in essential fatty acid requirements between species is not fully understood, although it is speculated that the level of piscivory is more important than environmental salinity in determining the EFA requirements of fish species. This is because longer chain fatty acids are selectively retained by piscivores in the food chain (Sargent *et al.* 1995). Generally cold water, piscivorous, marine fish have a higher requirement for the longer chain (*n-3*) EFA, eicosapentaenoic acid, (EPA=20:*5n-3*) and docosahexaenoic acid (DHA=22:*6n-3*) due to low activity of the metabolic pathways for their conversion from linolenic acid, 18:3 (*n-3*) (Sargent *et al.*, 1995, Sargent *et al.*, 2001). It is postulated that this requirement for long chain (*n-3*) EFA, EPA and DHA, has developed in marine organisms because of the high levels of fatty acids of the (*n-3*) HUFA readily available from marine phytoplankton. Freshwater phytoplankton, on the other hand, generally have lower levels of (*n-3*) fatty acids and higher levels of (*n-6*) fatty acids. Therefore, top-level predators that rely on food chains that are based on marine phytoplankton appear to have lost the ability to convert linolenic acid to longer chain fatty acids, EPA and DHA, and linoleic acid to arachidonic acid (ARA=20:*4n-6*), because they are supplied preformed in their natural diet. The enzymes, $\Delta 5$ desaturase and/or C_{18} to C_{20} elongase enzymes, are responsible

for desaturating long chain fatty acids and the necessary step in their elongation (see Figure 2). In many animals the above enzymatic steps, by which the precursors linoleic acid and α-linolenic acid can act as nutritionally the functionally essential fatty acids, are missing. That also applies for the marine fish studied to date (Sargent *et al.* 1999 a). The situation has become less clear with the discovery that mature pike, *Esox lucius*, a freshwater obligate piscivore, lacks the ability to convert linolenic to the longer chain fatty acids of the (*n-3*) series, probably due to the piscivorous nature of their diet (Buzzi *et al.*, 1997). However, Atlantic halibut, a cold-water marine piscivore, is considered to have an absolute requirement for preformed EPA, DHA and ARA (Sargent *et al.*, 1995).

Long chain PUFA's have been shown to be important in maintaining cell integrity. They are found largely in the phospholipids that make up cell membranes, and erythrocyte fragility has been used as a measure of EFA deficiency (Farndale *et al.*, 1999; Fokkema *et al.*, 2002). In terrestrial animals, ARA is the dominant HUFA in most tissues. In fish it appears that high levels of EPA and DHA in membrane phospholipids fulfils the role of maintaining cell integrity (Sargent, 1995). Long chain fatty acids with a greater degree of desaturation generally have a lower melting point than more saturated fatty acids with the same carbon chain lengths. Increased numbers of double bonds in the carbon chain give rise to an irregular physical structure. It is therefore

suggested that there may be a higher requirement for them in cold-water species, such as Atlantic halibut, because they may assist in the maintenance of membrane fluidity (Sargent *et al.*, 1999 a). Dietary supplementation of both n-3 and n-6 PUFA is essential to improve gonadal maturation, breeding performance and spawn recovery in catla female broodstock (Nandi *et al.*, 2001). A lower egg production in seabass broodstock fed an n-3 HUFA deficient diet is extended to spawning quality such as lower percentages of fertilised and hatched eggs (Rodriguez *et al.*, 1998). Moreover, the fatty acid composition of seabass eggs is affected by the fatty acid composition of the diets but not by the total quantity of lipid administered to the broodstock. (Thrush *et al.*, 1993; Navas *et al.*, 2001)

Figure 2. Pathway from linoleic acid to arachidonic acid (King, 1996).

2.7.2 EPA: Eicosapentaenoic acid or 20:5(n-3)

$$CH_3CH_2(CH=CHCH_2)_5(CH_2)_2COOH$$

EPA has an important physiological function in modulating eicosanoid action. EPA competitively inhibits the formation of eicosanoids from ARA (Horrobin, 1983, Bell *et al.* 1996). The functions of DHA and EPA during early developmental stages of marine fish larvae are apparently different. High amounts of EPA in relation to DHA may create an imbalance in the structural composition of phospholipids, which could affect normal growth and the quality of the larvae (Rodriguez *et al.*, 1998).

2.7.3 DHA: Docosahexaenoic acid or 22:6(n-3)

$$CH_3CH_2(CH=CHCH_2)_6CH_2COOH$$

Larval herring, another marine piscivore, fed a diet low in DHA were less active predators, particularly at low light intensities, than those fed on a diet higher in DHA (Bell *et al.*, 1995). It could therefore be surmised that the normal development of larval brain and retinal tissue is dependent on adequate levels of DHA (Sargent *et al.*, 1995). The eyes and other organs of halibut larvae are fully functional around 150° days post hatching, approximately 60° days before first feeding in commercial hatcheries (Kvenseth *et al.*, 1996). Although further development of halibut eyes does occur after first feeding, it could be assumed that if high levels of DHA are required for normal neural and retinal development, adequate amounts of DHA must be incorporated into eggs before ovulation (Shields *et al.*, 1999 a). Therefore, low levels of DHA in the fertilised egg may affect development of the important brain and retinal tissue; this could have its greatest effect in the important first feeding stage, when larval halibut must capture prey in conditions of low light intensity. In juvenile turbot, dietary DHA is required for proper development of neural tissues (Mourente *et al.*, 1991)

2.7.4 ARA: Arachidonic acid or 20:4(n-6)

$$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$$

Arachidonic acid is an essential fatty acid in marine organisms lacking $\Delta 5$ fatty acyl desaturase and/or C_{18}-C_{20} elongase. Castell *et al.* (1994) demonstrated a

significant increase in growth rate in juvenile turbot when their diet was supplemented with increased levels of ARA. The addition of arachidonic acid to the food for fish fry leads to an appreciable increase in the yield of full-grown fish (Castell *et al.,* 1994) or prawns (Xu *et al.,* 1994) A moderately increased ARA concentration in seabass diet is rapidly and efficiently incorporated into cellular lipid (Farndale *et al.,* 1999).

Arachidonic acid is also a precursor in the biosynthesis of prostaglandins, thromboxanes, and leukotrienes. It is the main precursor for the production of the more biologically active eicosanoids, the 2-series prostanoids and thromboxanes and the 4-series leukotrienes. In humans and rats EPA has been shown to competitively inhibit this process by promoting the production of the less biologically active eicosanoids, the 3-series prostanoids and thromboxanes and the 5-series leukotrienes, by competing for the same enzymatic pathways (Higgins & Braunwald, 1972; Olson 1998). Consequently the ratio of EPA to ARA will affect the production of biologically active eicosanoids; higher ratios will inhibit the production of the more active eicosanoids. The process is believed to be the same in marine teleosts (Sargent *et al.,* 1995; Farndale *et al.,* 1999). Thromboxanes TXB_2 and TXB_3 derived from ARA and EPA, respectively, were measured in seabass plasma. In all dietary treatments more TXB_2 than TXB_3 was produced with the highest values in seabass fed an increased ARA diet. The percentages of 20:4n-6 varied in accordance with dietary input with the highest levels in leucocytes from seabass fed the

increased ARA diet (two-fold compared to 2 other diets) (Farndale *et al.*, 1999). There are three main metabolic pathways for ARA conveniently identified by the class of enzymes instrumental in the initial determination of product. The lipoxygenase lead to formation of hydroxyeicosatetranoic acids (HETEs) and leukotrienes, cycloxygenase to thromboxanes, prostaglandins (PGs) and prostacyclin (PGI2) and cytochrome P450 epoxygenases to epoxyeicosatrienoic acids (EETs plus 20-HETE) (Olson, 1998).

2.8 Eicosanoids

2.8.1 Eicosanoid metabolism

The major eicosanoids consist of the prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). The PGs and TXs are collectively identified as prostanoids. Prostaglandins were originally shown to be synthesized in the prostate gland, thromboxanes from platelets (thrombocytes) and leukotrienes from leukocytes, hence the derivation of their names (Higgins & Braunwald, 1972).

Table 3: Structures of clinically relevant eicosanoids (King, 1996)
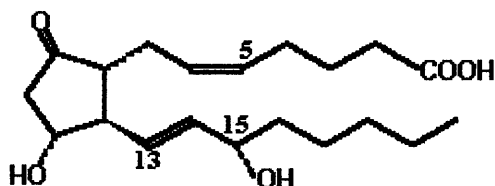
PGE$_2$
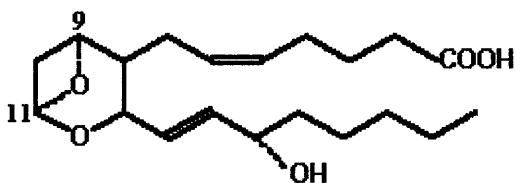
TXA$_2$
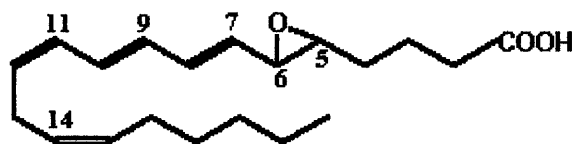
LTA$_4$

The eicosanoids produce a wide range of biological effects on inflammatory responses (predominantly those of the joints, skin and eyes), on the intensity and duration of pain and fever, and on reproductive function (including the induction of labour) (Higgins & Braunwald, 1972). They also play important roles in inhibiting gastric acid secretion, regulating blood pressure through vasodilation or constriction, and inhibiting or activating platelet aggregation and thrombosis (Kelley, 2001). The principal eicosanoids of biological significance are a group of molecules derived from the C_{20} fatty acid, arachidonic acid. Minor eicosanoids are derived from dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid. Series-2 eicosanoids are extremely potent,

able to cause profound physiological effects at very low concentrations. All eicosanoids function locally at the site of synthesis, through receptor-mediated G-protein linked signaling pathways leading to an increase in cAMP levels. Two main pathways are involved in the biosynthesis of eicosanoids. The cyclic pathway synthesizes the prostaglandins and thromboxanes. The linear pathway synthesizes the leukotrienes
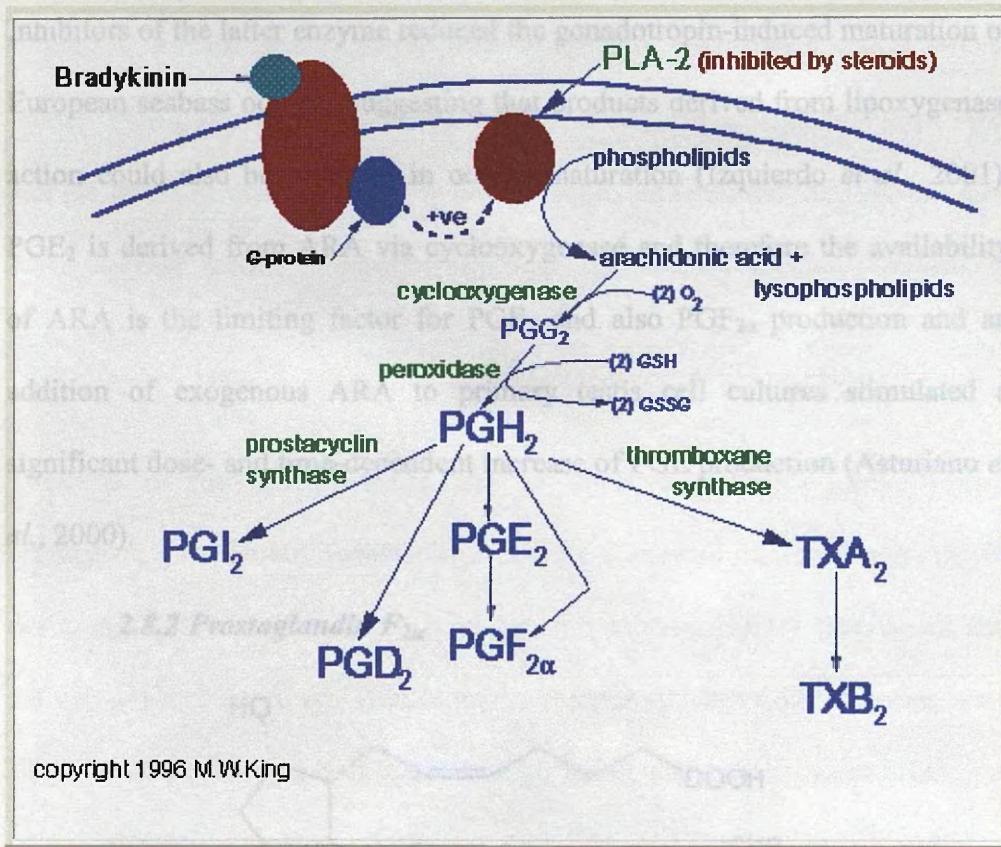
Figure 3. Synthesis of the clinically relevant prostaglandins and thromboxanes from arachidonic acid (King, 1996).

Numerous stimuli (e.g. epinephrine, thrombin and bradykinin) activate phospholipase A_2, which hydrolyzes arachidonic acid from membrane phospholipids.

Trout ovaries have a high capacity to generate eicosanoids, among them prostaglandin E (PGE) derived from cycloxygenase action and leukotrienes LTB_4 and LTB_5 derived from lipoxygenase action (Knight *et al.*, 1995). Inhibitors of the latter enzyme reduced the gonadotropin-induced maturation of European seabass oocytes suggesting that products derived from lipoxygenase action could also be involved in oocyte maturation (Izquierdo *et al.*, 2001). PGE_2 is derived from ARA via cyclooxygenase and therefore the availability of ARA is the limiting factor for PGE_2 and also $PGF_{2\alpha}$ production and an addition of exogenous ARA to primary testis cell cultures stimulated a significant dose- and time-dependent increase of PGE production (Asturiano *et al.*, 2000).

2.8.2 Prostaglandin $F_{2\alpha}$
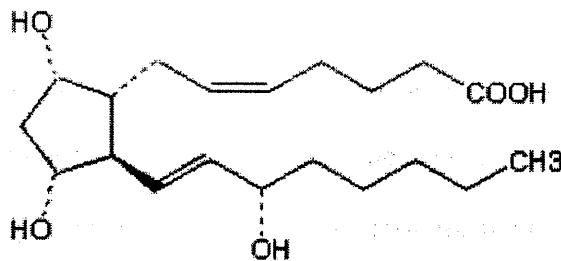
Prostaglandin F $_{2\alpha}$ (PGF$_{2\alpha}$) is one of the five primary prostaglandins derived

enzymatically directly from the endoperoxide PGH$_2$. Matsumoto *et al.* (1989)

were the first to describe prostaglandin synthesis in marine fish thrombocytes.

PGF$_{2\alpha}$ was initially discovered in seminal fluid, and to date the majority of the

functional roles ascribed to it relate to fertility, pregnancy, and parturition

(Higgins & Braunwald, 1972). PGF$_{2\alpha}$ is a potent luteolytic agent and is used to

induce ovulation in domestic livestock. It is also a potent uterine stimulant and

is part of the cascade of myometrial stimulants which induce and sustain

labour. PGF$_{2\alpha}$ is rapidly metabolised to 13,14-dihydro-15-keto PGF$_{2\alpha}$ *in vivo*,

and the levels of this metabolite in both plasma and amniotic fluid are elevated

during active labour in several different mammalian species (Higgins &

Braunwald, 1972).

The aim of the present study was to improve overall Atlantic halibut

broodstock production quality. The project focussed on the feasibility of

designing a high quality commercial diet for Atlantic halibut broodstock and

the use of arachidonic acid diet as a reproduction enhancer. Other issues were

also studied including a milt production period extender, the standardisation of

milt and egg collection protocols and the standardisation of artificial

fertilisation and egg incubation protocols. To monitor the effect of arachidonic

acid on eggs, yolk sac larvae and milt quality, biochemical analyses such as

lipid class analyses and fatty acid profile have been performed. For a better

understanding of physiological effects of increased arachidonic acid concentration, $PGF_{2\alpha}$.was analysed in milt

3 Overall Aims

> Standardisation of Atlantic halibut broodstock husbandry, feeding, stripping frequency and process

> Standardisation of Atlantic halibut egg artificial fertilisation, incubation and maintenance protocols

> Design of a high quality commercial Atlantic halibut broodstock diet

> Analysis of the effect of an arachidonic acid enriched diet on halibut broodstock growth and reproductive performance

> Assessment of egg, milt and larvae quality through lipid and fatty acid analyses

> Physiological impact of arachidonic acid on $PGF_{2\alpha}$ concentration in milt

Chapter 2 General Materials and Methods

1 Halibut broodstock husbandry

1.1 Otter Ferry Sea fish Ltd.

The halibut broodstock nutrition trial was set up on a fish farm on the west coast of Scotland: Otter Ferry Sea Fish Ltd. Otter Ferry Sea fish Ltd. is situated 130 km west of Glasgow on the east coast of Loch Fyne. This unit changed from Atlantic salmon production to culture Atlantic halibut in 1995 and is the only juvenile halibut supplier in Scotland (Table 4) with production on two separate sites.

Table 4: Otter Ferry Ltd hatchery production 1997-2002

Year	Number of females stripped	Number of eggs incubated (10^3)	FR (%)	HR (%)	Yolksac larvae survival (%)	First feeding survival (%)	Weaning survival (%)	Overall survival (%)
1995	22	3100	N/A	33	20	4	57.5	0.14
1997	35	4000	N/A	56	29	8	42	0.55
1998	53	4240	N/A	47	35	24	88	3.47
1999	53	5880	74	61	38	13	56	1.69
2000	78	7270	55.9	45	35	9	71	1.01
2001	298	7992	46	33	34	21	72	1.70
2002	340	14780	61	41	32	12	39	0.67

1.2 Fish farm hatchery structure
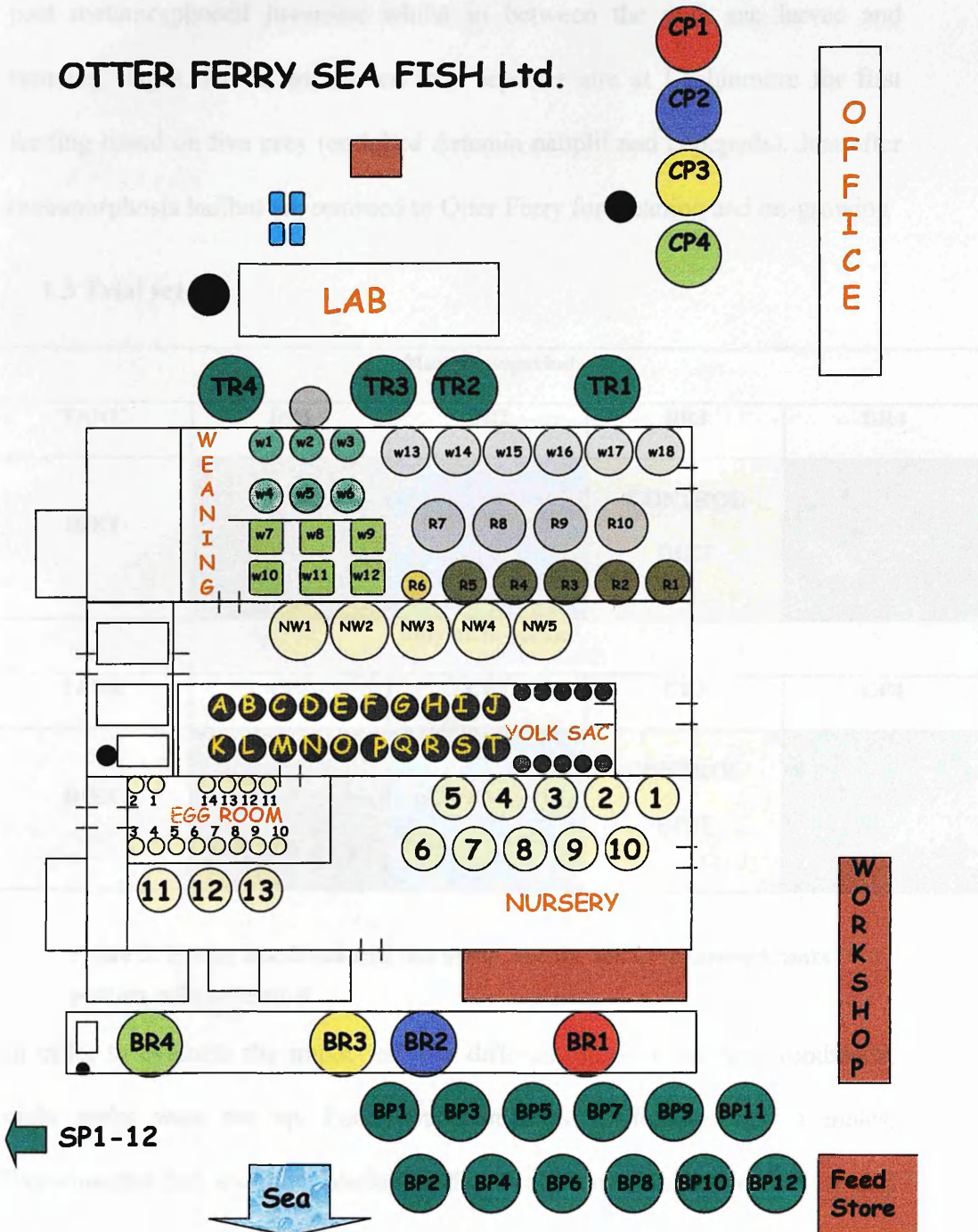
Figure 4. Otter Ferry Seafish Ltd hatchery site. BR, CP and SP are broodstock tanks. W and R are weaning tanks. NW and BP are ongrowing tanks.

The hatchery (Figure 4) contains halibut broodstock, eggs, yolk sac larvae and post metamorphosed juveniles whilst in between the yolk sac larvae and weaning stages, larvae are moved to a separate site at Lephinmore for first feeding based on live prey (enriched Artemia nauplii and copepods). Just after metamorphosis halibut are returned to Otter Ferry for weaning and on-growing

1.3 Trial set up

May photoperiod				
TANK	BR1	BR2	BR3	BR4
DIET	HIGH Arachidonic Acid Diet (0.6%)	SQUID DIET	CONTROL DIET	LOW Arachidonic Acid Diet (0.4%)
July photoperiod				
TANK	CP1	CP2	CP3	CP4
DIET	HIGH Arachidonic Acid Diet (0.6%)	SQUID DIET	CONTROL DIET	LOW Arachidonic Acid Diet (0.4%)

Figure 5: Halibut broodstock trial diet set up. For BR and CP broodstock tanks position, refer to Figure 4.

In order to evaluate the impact of four different diets on halibut broodstock, eight tanks were set up. Each tank contained 15 females and 5 males. Experimental fish are under photoperiod control. Four tanks spawn in May and the four others spawn in July. The four broodstock diet formulations contained 0.4% ARA, (0.4g ARA/100g dry feed), 0.6% ARA, (0.6g ARA/100g dry feed),

a control feed not supplemented with ARA and a control feed in which 10% of

the fish meal was replaced with squid meal.

1.4 Trial tanks
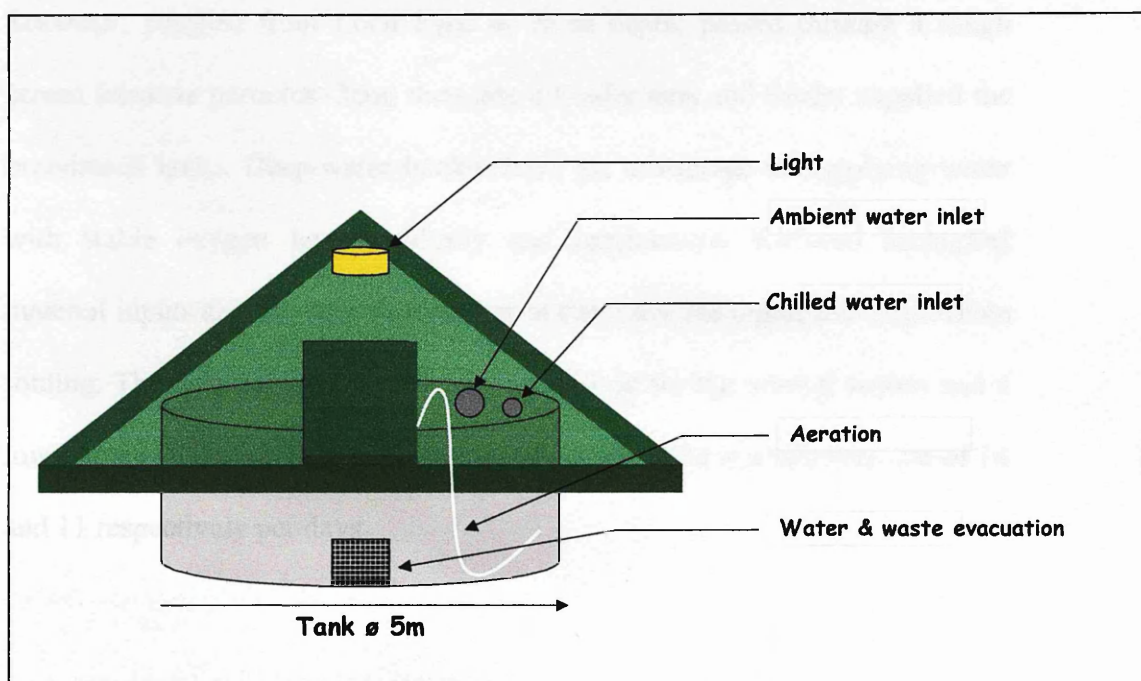
Figure 6. Halibut broodstock nutrition experimental tank

Halibut broodstock for the project were kept in land-based 5m diameter

concrete tanks with 80 cm of water depth (60 cm during spawning season). The

tanks were covered with a wood and tar roof to facilitate blackout for

photoperiod control. The inside of the tank was covered with antifouling blue

paint. Healthy halibut spend 90-95% of the time on the bottom immobile and

prefer a coarse floor to a smooth surface, which can induces scarring and

wounding (Rønnestad & Rødseth, 1989). Each tank also had continuous aeration and an airstone linked to an emergency oxygen bottle in case of pump malfunction.

1.5 Environmental parameters

Seawater, pumped from Loch Fyne at 25 m depth, passed through a rough screen (remove particles>3cm) then into a header tank and finally supplied the broodstock tanks. Deep-water intakes have the advantage of supplying water with stable oxygen levels, salinity and temperature. Reduced biological material inputs are also less likely either to carry disease organisms or promote fouling. The flow rate was maintained at 2.5l/sec for the normal season and a lower rate of 1.5l/sec during the spawning season. This is a turnover rate of 14 and 11 respectively per days.

Temperature is one of the most important environmental variables in fish farming. Optimal temperature for halibut growth decreases with increasing fish size, being approximately 14°C for 10-60g fish, 11.4°C for 100-500 g fish and 9.7°C for 3-5 kg fish. The dome-shaped relationships of growth rate and growth efficiency versus temperature became flatter with increased size of fish, suggesting that thermal sensitivity close to the optimum decreases as fish size increases (Björnsson & Tryggvadottir, 1996; Jonassen *et al.,* 1999). Temperatures were measured every day and salinity once a week during

spawning season. Apart from the spawning season, temperature was not modified from natural pumped seawater temperature and over the three-year trial varied from 4°C to 14°C according to season. Salinity was 33‰±1‰. Daily oxygen level monitoring was performed using a Handy Alpha Oxyguard ® with galvanic probe. Percentage oxygen saturation was read and recorded every morning at the water output drain after feeding the fish. To avoid any contamination of water by organic waste (faeces, excess feed) or ammonia and sulphur accumulation, tanks were flushed twice a week.

1.6 Fish feeding frequency

Fish were fed 5 days a week during the normal season and 2 days a week during spawning season with trial diet under moist sausages form made on site. Numbers and weight of sausages distributed were recorded. Fish were fed to excess and unconsumed 'waste' sausages were removed and recorded daily.

1.7 Halibut broodstock handling

1.7.1 Halibut tagging

At the beginning of the experiment, every fish was injected with a microchip included in a transponder in order to collect individual fish data. The microchip was a computer chip that contained a unique identification number assigned to the transponder and measured approximately 1 mm square. The chip used by Otter Ferry Sea Fish was the ID-100 protocol made by Trovan. The unique ID is guaranteed tamperproof. A transponder is an electronic device encapsulated

in biocompatible glass and used to store a unique identification number. Transponder size is 2.12 mm x 11.5 mm, they are inert, once implanted they are unlikely to be lost or altered and will not wear out. Scanners in fish farm environments have to be used carefully because they are not waterproof and exposure to seawater often causes failure to read the microchip.

1.7.2 Halibut anaesthesia

When needed for measurement, hormone injection or tagging, broodstock fish were moved in a plain crowder (60x150x100 cm), and then anaesthetised with 250 ml of phenoxyethanol in 900 L of seawater. Anaesthetics are frequently used in aquaculture to minimise fish stress response and to prevent a negative impact on performance (Pickering, 1998). In many cases their effectiveness depends on the procedure used, because severe anaesthesia may itself induce a stress response in fish. Fish are anaesthetised by adding anaesthetic to the water. A safe anaesthetic requires both short exposure time and relatively short recovery time (dependent on the anaesthesia mean) (Gerwick *et al.*, 1999). Fish should be ready for handling within 5 min of exposure to an anaesthetic and should recover within 10 min. It is important to take into account individual variations and to control reflexes and pigmentation of the fish (Malmstrøm *et al.,* 1993). The behavioural response to an anaesthetic is also important. Generally, a calm induction and recovery from anaesthesia is required. It is recommended to avoid contact between the anaesthetic solution and eggs or milt ((Ross & Ross, 1984; Ortuño *et al.*, 2002).

1.8 Halibut broodstock trial

Fish for the experiment were been randomly selected from the first generation of farmed halibut broodstock on the fish farm. The first generation of farmed halibut was spawned in 1995 and selected according growth performance. The original parent stock were wild Atlantic halibut caught in Icelandic waters. Halibut were maintained under controlled photoperiod. Under natural photoperiod, halibut females spawn from January to March. In order to extend experimental spawning season, to fullfill fishfarm production needs two different delayed spawning seasons were set up using photoperiod control. The first one planned to start spawning in May and the second one planned to start spawning in July.

1.8.1 May spawners

The May spawner group had a longer adaptation time than the July spawners. They had been placed a year before the start of the experiment in trial tanks (5m). However at first, fish were not eating and showed some wounding so for management reasons they were put back in 13m tanks six months prior to beginning the experiment. Finally, in November 2000, 60 females and 20 males were placed in the trial tanks (BR tanks,Figure 4).

1.8.2 July spawners

60 females and 20 males were moved under anaesthesia from the 13m tanks into the trial tanks (CP tanks, Figure 4) in January 2001.

2 Analytical techniques

Over the three years of the trials, eggs, milt, yolk sac larvae and diet were sampled for different biochemical analyses.

2.1 Lowry protein measurement

250µl of 1M NaOH 0.25% sodium dodecyl sulphate (SDS) were added to 200µl defrosted sample (previously taken from homogenised tissue in HBSS) in Eppendorf tubes. Standard concentrations of 0-100µg protein were prepared in 6 tubes with respectively 0, 20, 40, 60, 80, 100 µl of bovine serum albumine (BSA) (1mg/ml) and volume made up to 250µl with 1M NaOH 0.25% SDS. Samples and standards were heated at 60°C for 45 minutes in a water bath. 15µl of sample was transferred to 235µl of 1M NaOH 0.25%SDS to obtain the same volume as the standard. One ml of a solution of 1% (w/v) $CuSO_4$ and 1 ml of 2% (w/v) of sodium potassium tartrate was made up to 100 ml with 2% (w/v) $NaCO_3$. 2.5ml of this solution was added to standards and samples. After 15 minutes 250µl of folin-ciocaulteau phenol reagent diluted 1:1 with water was added to standard and samples. Standard and sample tubes were read on a spectrophotometer (Jenway 6405 UV/Vis) at 660nm between 30 and 60 minutes later. A standard curve was drawn and protein concentration was calculated in mg/ml (Lowry *et al.*, 1951)

2.2 Kjeldahl protein using the Tecator Kjeltec system

Kjedahl method (Helrich, 1990) was carried out for the determination of experimental diet total proteins. Approximately 200mg samples (weighed to 4dp) were placed into digestion tubes in triplicate. 3 standards with 50mg of urea and 3 blank tubes were prepared. Two mercury kjeltabs and 5 ml of concentrated sulphuric acid were added to each tube. Tubes were placed in a digestion block for 1 hour at 400°C under a fume extractor. After digestion and cooling 20ml of de-ionised water and 5 ml of sodium thiosulfate solution were added to each tube. Samples, standard and blank were then distilled in the Kjeltec unit to obtain their titration.

2.3 Lipid extraction

Total lipid was extracted from tissues and diets by the method of Folch *et al.* (1957). After homogenisation in at least 10 volumes of ice-cold chloroform: methanol (2:1, v/v), 0.2 volume of 8.8g KCl/L was added and the solutions were mixed on a vortex mixer. The extracts were redissolved in chloroform: methanol (2:1, v/v) at a final concentration of 10mg lipid/ml and stored at − 20°C. All solvents contained 50 mg of butylated hydroxytoluene per litre as an antioxidant.

2.4 High-performance thin layer chromatography

Quantification of lipid classes was performed using double-development method of Olsen & Henderson (1989) employing high-performance thin layer chromatography (HPTLC) and scanning densitometry. 10 µg of total lipid were applied to a 10 x 10-cm HPTLC plate that had been pre-run in first solvent: propan-2-ol: chloroform: methanol: 0.25% (w/v) aqueous KCl (25:25:25:10:9 by volume) and activated at 110°C for 30 min. Plates were developed to 5-cm in first solvent to separate polar lipid classes with neutral lipids running at the solvent front. After drying in a vacuum dessicator, the plates were developed fully in second solvent: hexane: diethyl ether: acetic acid (85:15:1 v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualised by charring at 160°C for 15min after spraying with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid (Fewster *et al.*, 1969) and identified in comparison with commercially available standards. Scanning densitometry was conducted using CAMAG TLC scanner 3 and winCATS Planar Chromatography Manager software to quantify lipid classes. The slit dimensions were 8.00 x 0.2 mm with a scanning speed of 20mm/s and a wavelength of 370nm.

2.5 Fatty acid methylesters and gas-liquid chromatography

Fatty acid methyl esters (FAME) of total lipid were prepared by acid-catalysed transesterification of 0.5 mg of total lipid overnight at 50°C in 2-ml of 2%

sulphuric acid in methanol plus 1 ml toluene as described by Christie (1982).

An internal standard of 17:0 free fatty acid was added prior to transmethylation

to quantify fatty acids. The methyl esters were extracted, after neutralisation

with 2 ml of 6% $KHCO_3$, in 5 ml of isohexane: diethyl ether (1:1 v:v) samples

were then centrifuged and the upper organic layer transferred to a new tube

then re-extracted with 5 ml of isohexane: diethyl ether added to the original

tube. The solvent was evaporated under nitrogen and the residue dissolved in

100μl of isohexane. 100μl were loaded on TLC plates to purify methyl esters.

Plates were run in isohexane: diethyl ether: acetic acid (90:10:1 v:v:v) then

sprayed with 1% iodine in $CHCl_3$ after drying to visualise the FAME. The

FAME chromatograph has a doublet band around halfway up the TLC plate.

Saturated and mono unsaturated fatty acids formed the upper band,

polyunsaturated the lower band. Both bands were scraped into a test tube and

eluted with isohexane: diethyl ether 1:1 (v:v) to separate silica gel and FAME.

Solvent was evaporated under nitrogen and the sample transferred to a 2ml

glass vial in 150 μl isohexane+BHT and stored under nitrogen in a freezer until

GLC analysis. FAMEs were quantified by gas-liquid chromatography using a

Fisons GC 8000 gas chromatograph fitted with an on-column injector and

fused silica capillary column (CP wax 52CB, 30m x 0.32mm id, 0.25-μm film

thickness, Chrompack, UK) using hydrogen as carrier gas (Ghioni *et al.,* 1999).

Temperature at injection was 50°C; the thermal gradient was 40°C/min till

150°C then 2°C/min to 225°C (Navarro *et al.,* 1992). Data were analysed using Chrom-Card for windows software.

2.6 Eicosanoids

2.6.1 Extraction and purification of eicosanoids from tissues

Tissues were homogenised in 4 volumes of Hanks balanced salt solution (HBSS) containing 15% (v/v) ethanol and 50μl/ml of 0.2M formic acid. Sampled were centrifuged to remove debris and the supernatant was loaded onto a C18 "Sep-Pak" cartridge which had been washed first with 5 ml methanol followed by 10ml distilled water. The sample was washed with 10ml distilled water followed by 5ml of 15% ethanol (v:v) followed by 5ml of hexane: chloroform 65:35 (v:v). Eicosanoids were then eluted with 10ml of ethyl acetate (Powell, 1982). The extract was dried under nitrogen, dissolved in 0.1ml methanol and stored in freezer before analysing by immunoassay.

2.6.2 $PGF_{2\alpha}$ immunoassay

$PGF_{2\alpha}$ was measured by competitive enzyme immunoassay using Cayman Chemical Company kit. This assay is based on the competition between $PGF_{2\alpha}$ and a $PGF_{2\alpha}$-acetylcholinesterase (AChE) conjugate ($PGF_{2\alpha}$ tracer) for a limited number of $PGF_{2\alpha}$-specific rabbit antiserum binding sites (Granström & Kindahl, 1982). The plate was washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) was added to

the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of PGF traces bound to the well, which is inversely proportional to the amount of free PGF present in the well during the incubation.

Chapter 3 Halibut broodstock nutrition

1 Introduction

Broodstock nutrition is, without any doubt, one of the most poorly understood areas of finfish nutrition. The lack of research in this area has been mainly due to the necessity of suitable indoor or outdoor culture facilities for maintaining large groups of adult fish and the consequent higher cost of running and conducting extended broodstock-feeding trials. In human and livestock nutrition, the dietary nutrient requirements of broodstock are different from those of rapidly growing juvenile animals (Bromage & Roberts, 1995). Moreover, as in other animals, it has already been demonstrated that many of the deficiencies and problems encountered during the early rearing phases of newly hatched finfish larvae are directly related to the feeding regime, including nutrient level, of the broodstock (Izquierdo *et al.,* 2001).

Winter flounder inhabiting inshore waters off Newfoundland have a prolonged winter fast and will not initiate or maintain the following year's spawn if they have poor nutritional status in the critical period which occurs for females close to the normal spawning season (Burton, 1995). The basic data necessary for feed formulation are the nutrient requirements of the species being cultivated and its feeding behaviour. Local availability and cost of nutrients of the prepared diet are also important. Furthermore, for feed formulation to be

performed correctly, knowledge of the digestibility of each nutrient included in the diet is essential although estimating these parameters can be tedious and time-consuming (Robaina *et al.,* 1999). In order to minimize the leaching of dry matter, the optimal inclusion level of binder and water in the diet has also to be determined (Hillestad *et al.,* 1999).

1.1 Fish feed processing

Halibut broodstock used to be fed on frozen capelin (*Mallotus villosus*) and herring (*Clupea harengus*) (Björnsson, 1995). However, diets based on fresh fish products (as commonly used for most farmed marine broodstock) have been implicated as a possible route for accidental introduction of pathogens such as nodavirus and viral haemorrhagic septicaemia (VHS) to the culture system (Dannevig *et al.,* 2000). Industrial processing of commercial extruded diets reduce this risk. Moreover, compujnded diets allow better control biochemical composition of the feed (Asturiano *et al.,* 2001).

Some physical parameters have also to be studied to obtain a diet adapted to the specific feeding behaviour of the fish species. For example, the degree of floating of dry pellets is negatively correlated to halibut growth rates, especially for small and one-eyed fish (Nortvedt & Tuene, 1995). Sinking velocity should therefore be introduced as a new target variable. It is important to note that large pellets resulted in the highest feed conversion ratio, especially

in bigger fish (Nortvedt & Tuene, 1995).. To summarize, the sinking / dry / large pellet showed the highest growth and conversion of feed into flesh in halibut. Feeds should consequently maximize these parameters within reasonable biological limits to achieve the best utilisation of the feed (Nortvedt & Tuene, 1995).

The "Marine Broodstock Mix" from Skretting Aquaculture is a complete dry diet in meal form and is designed to be mixed on-farm with fish oil and water in order to produce a moist paste. This paste must then be extruded on-farm into conventional sausage skins and either fed fresh to marine broodstock or kept frozen for later use. The inclusion of wet fish in this mix is neither nutritionally necessary nor desirable from a hygiene point-of-view. All ingredients used have been screened for their high quality and digestibility.

1.2 Binders

The use of binders is important in the manufacturing of moist feeds and experimental diets in aquaculture in order to improve water stability, thus increasing feed efficiency, reducing wastage and fouling of water systems (Ross & Ross, 1984). Natural binders commonly used in fish diets include alginates, starches, gums, gelatine and agar. They generally represent fibres or filler material and it is assumed that they have no nutritional value (Fagbenro & Jauncey, 1994). Alginate can be used as a thickening or gelling agent

depending on the presence or absence of calcium ions (Montero & Pérez-Mateos, 2001). The effect of the calcium cation on a gel made with alginate is an increase in elasticity and lightness. Extruded wheat gluten has good pelleting properties and does not gel during mixing of feedstuffs before pelleting (Fagbenro & Jauncey, 1994). In plants, there is only one well-documented elastomeric protein system, the alcohol-soluble seed storage proteins (gluten) of wheat. The elastic properties of these proteins have no known biological role, the proteins acting as a store for the germinating seed (Tatham et *al.*, 2001).

1.3 Fish meal

1.3.1 Protein origin: Raw material

Fishmeal is produced almost exclusively from small, bony species of pelagic fish, for which there is little or no demand for human consumption. Virtually all fishmeal in the UK is supplied by South American (Peru, Chile) and European (UK, Denmark, Norway, Iceland) fisheries. The fish used to produce fishmeal are commonly referred to as "industrial or feed grade fish", since the majority of the catch is processed into meal and oil. Seven key species are used to produce fishmeal and fish oil in Europe. These can be divided into three groups:

➢ Not suitable for human consumption (inedible feed grade fish – sand eel, capelin, Norway pout)

➢ Potential use for human consumption but mainly used for fishmeal because of limited outlets for human consumption (blue whiting, European sprat)

➢ Primary use is human consumption but any surplus within the Total Allowable Catch (TAC) or process waste may be used for fishmeal (Atlantic herring, horse mackerel, and Atlantic mackerel).

Fishmeal quality depends on both the freshness of the raw material used and the fishmeal processing conditions. Fishmeals provide high contents of essential amino and fatty acids, are low in carbohydrates, are usually well digested and, provided they are fresh, contain few anti-nutritional factors (Taija-Riitta & Esmark, 2003). Marine based ingredients, especially fishmeals, are highly sought after as the protein source of choice for many formulated aquaculture diets. Recent developments in fishmeal manufacturing technology have made it possible production of low-ash fishmeal from fish processing waste. The protein quality of these fishmeals is higher than conventional fishmeals, due to removal of poorly digested connective tissue during the process. Not only does this allow the formulation of low-phosphorus, environmentally friendly fishmeal-based feeds, but it will also reduce

environmental issues associated with disposal of fish processing waste in ocean or land dumping (Hardy, 1999).

1.3.2 Squid meal

Some workers suggest that squid meal contains components which are beneficial for successful spawning in marine species (Zohar, 1995). Previous studies in gilthead seabream suggest a positive effect of the fat-insoluble fraction of squid meal on the total number of eggs, egg viability and the number of larvae produced daily per kilogram of seabream female. Squid meal was shown to be a good protein and lipid source in the diet for gilthead seabream broodstock (Fernández-Palacios *et al.*, 1997)

1.4 Mackerel oil

Fish is composed of three major fractions: solids (fat-free dry matter), oil and water. The purpose of fishmeal and oil processing is to separate these parts from each other as much as possible. This is followed by a pressing step separating solids from liquids. The oil is then separated from the liquids by centrifugation. Mackerel oil has been chosen as high quality marine fish oil in broodstock nutrition for successful embryonic and early larval development (Bórquez *et al.,* 1997), . The fatty acid profile of mackerel oil was favoured, when compared to other marine fish oil compositions (Table 5), due to the higher level of DHA and the ratio of DHA/EPA and EPA/ARA, which were desirable for use in a marine broodstock diet.

Table 5: The major fatty acids in fish oil (% Total lipid)

Fatty acid	Mackerel oil (Bórquez et al., 1997)	Capelin oil (Waagbø et al., 1995)	Sardine oil (Waagbø et al., 1995)
16:0	18.66	12.1	14.8
18:0	16.3	1.3	2.1
16:1n-7	4.4	-	-
18:1n-9	11.1	9.3	9.6
20:5n-3 (EPA)	7.9	7.7	13
22:5n-3	1.4	0.6	1.6
22:6n-3 (DHA)	12.2	6.8	10.8
18:2n-6 (linoleic)	1.9	5.2	4.1
20:4n-6 (ARA)	1.4	0.2	0.8
22:5n-6	1.4	-	-
Sum saturated	49.6	20	24.1
Sum monoenes	16.5	52	37.5
Sum n-3	21.5	20.3	29.8
Sum n-6	4.7	5.6	5
Total PUFAs	26.2	25.9	34.8
n-3:n-6 PUFA	4.5	3.6	6
ARA: EPA	0.18	0.025	0.06
DHA: EPA	1.54	0.88	0.83

Overall aims

➢ To study halibut broodstock FER and feeding behaviour in order to design a high quality commercial diet.

➢ To set up control of the experimental diets in order to define protein and lipids intake of halibut broodstock.

➢ To investigate the effect of arachidonic enriched diets on halibut broodstock weight variation, FER and survival

2 Materials and Methods

2.1 Skretting sausage
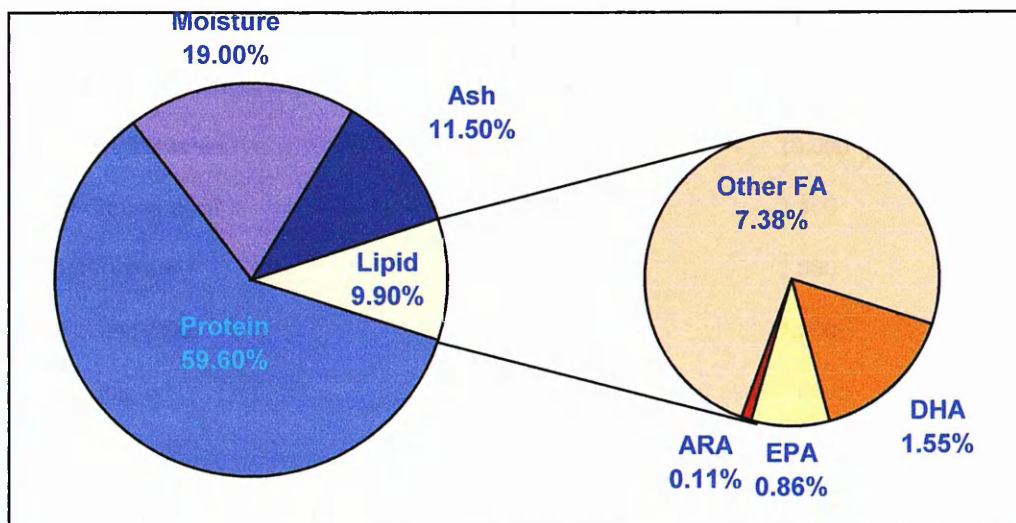
Figure 7: Skretting sausage raw material analysed nutrient content

The Skretting sausage raw material biochemical analysis is shown Figure 7. Fishmeal (Table 6) was a fish blend produced from the North hemisphere, either from Norway or Iceland, species predominantly including capelin and herring but also potentially, blue whiting, sand eel and sprat. Fish were dried at low temperature.

Table 6. Skretting sausage raw material formulation inclusion %

	Marine broodstock mix	Marine broodstock mix +squid
Fishmeal	72.325	65.792
Fish protein concentrate, Soprapeche Special G	9.167	9.167
Squid meal, Peruvian	0	10.000
Prawn / Shrimp meal	7.500	7.500
Krill hydrolysate	2.013	2.000
Wheat gluten (2003 only)	7.500	7.500
Vitamin premix*	1.000	1.000
Mineral premix*	0.450	0.450
Selenium from Se enrich yeast	0.500	0.500
Vitamin C, 35%	0.257	0.257
Vitamin E premix*	0.750	0.750
Astaxanthin, Carophyll Pink 8%	0.018	0.018
Nucleotides from yeast	0.200	0.200

*Vitamin and mineral premixes were commercial formulation supplied by Skretting,

Northwich, UK see Table 7 above for more detail.

Table 7. Analysed micronutrients content

	Marine broodstock mix	*Marine broodstock mix +squid*
Astaxanthin (ppm)	22.500	21.200
Vitamin A (iu/kg)	21000.000	32000.000
Vitamin D (mg/kg)*	3000.000	3000.000
Vitamin E (mg/kg)	414.500	660.500
Vitamin C (mg/kg)	836.000	1038.000
Selenium (mg/kg)	4.600	2.100
Calcium (mg/kg)	29141.667	30602.767
Phosphorus (mg/kg)	18070.600	17754.700
Iron (mg/kg)*	60.700	60.700
Zinc (mg/kg)*	228.000	228.000
Copper (mg/kg)*	5.967	5.967

*Nutritional value estimated on the known nutrient composition of the raw material

2.1.1 Halibut broodstock moist sausage

Sausages were made on site. The fishmeal and the mackerel oil, according to the diet type, were mixed in a mixer bowl. Water was slowly added until reaching a consistency that passed easily through a hydraulic sausage fillers. The sausages were held together by Devro Handlink collagen casing and kept in freezer. Then according to the daily percentage of feed intake / tank biomass, sausages were cut into portions of suitable size (10-15 cm in length and 5 cm in diameter) and defrosted before feeding to the fish.

Table 8 Moist Skretting sausage composition

	May 2001- May2002	May 2003
Fish meal	12.5 kg	12.5 kg
Mackerel oil	1.5l	1.5l
Water	12.4l	12.4l
Binder	750g (spawning season), 1.1 kg (feeding season)	Added by manufacturer

2.1.2 Binder inclusion in the diet

A trial with three different types of binder was set up in December 2001. Algibind (seaweed alginate), micronised ground wheat and wheat gluten were added to raw sausage mix in order to improve the palatability of the diet for the fish and to prevent breakdown in the water. Algibind was included at 5% and 10%, extruded wheat at 5% and 10% and wheat gluten at 5%, 7.5% and 10%.

2.2 Arachidonic acid enrichment

The Dutch company DSM Food Specialties produces arachidonic acid by a natural fermentation process from a "food grade" fungus *Mortiellia alpina*. DSM markets ARA under the brand VEVODAR®. In order to increase the level of lipid in the sausage from 10% to 20%, mackerel oil supplied by Skretting was added to the fishmeal. The mackerel oil was previously enriched with arachidonic acid (VEVODAR®) according to the level required in the experimental diet. Mackerel oil was added up to 1.5 l to 120ml and 200ml of

pure arachidonic acid to respectively obtain the 0.4% diet and 0.6% ARA diet. The mackerel oil was stored in white plastic jerrycans and kept frozen.

2.3 Diet sampling for biochemical analyses

Moist sausages were made on site according to fish food intake. For every new bag of raw material used, a sausage duplicate was sampled for protein, lipid and fatty acid composition. During the three-year experiment, the fishfarm used different types of diet for their own broodstock including Inve marine species broodstock diet in 2001 and 2002 and Skretting Vitalis® halibut broodstock diet in 2003. These two diets were also sampled to compare composition with diets used for the project.

2.3.1 Protein analyses

In March 2002, a triplicate of each diet prior to binder inclusion and a triplicate of each diet after binder inclusion were analysed by Kjeldahl protein using the Tecator Kjeltec system (Chapter 2, section 2.2).

2.3.2 Lipid extraction

In order to calculate the percentage of lipid in the diet used for the trial, once a year for the three years, lipids were extracted from four 1g samples of each diet according to Folch *et al.* (1957) (Chapter 2, section 2.3). Another 2g were sampled and dried overnight in an oven at 105°C to obtain percentage of moisture in each diet.

2.3.3 Fatty acid analyses

0.5mg of total lipids for each sampled diet were prepared by acid catalysed transesterification as described in chapter 2, section 2.5.

2.4 Calculations

The effect of diet on halibut broodstock growth was studied with weight variation of individual fish from their initial weight at the beginning of the trial.

FER = Feed efficiency ratio = wet weight gain / dry feed intake.

Dry feed intake= (feed offered- feed waste) x % moisture

2.5 Statistical analysis

Weight variation of halibut broodstock females and males in each treatment and fatty acid concentration of the experimental diets and production diets used at the fishfarm were pooled for statistical analyses using SPSS version 11.5 for windows. Data were tested for normality using Kolmogorov and Smirnov method. Once normality and variance homogeneity were validated, data were analysed by ANOVA and the means compared by Tukey *post-hoc* (Puri, 1996). The significance level was set at $p<0.05$. All results are given as mean ± standard deviation (S.D.)

3 Results

3.1 Protein and binder level in the diet

The addition of 5% or 10% Algibind to the diet increased the binding of the mix but also increased hardness making it difficult to pass through the sausage maker. Moreover, the processing of the Algibind needed several stages of mixing before being ready to use in the mix, making the whole sausage making process more time consuming. Micronised wheat added at 5% and 10% had no effect on the consistency of the mix. When added at 10% wheat gluten gave the expected improved elasticity to the mix. The mix was easier to pass through the sausage maker and the sausage stayed bound when fed to the fish. No more leakage through the fish gills was observed and water quality in the experimental tanks was improved when previously most of the diet was being rejected through the fish gills. Percentage protein for each trial diet is shown in Table 9. There were no significance differences before and after adding 10% wheat gluten to the diet. The mean percentage protein in trial diets was 61.53±0.94.

Table 9 Percentage protein in dry halibut broodstock diet. Values are mean ± S.D., n=3 in all groups.

Diet	Without binder	With 10% wheat gluten
Control diet	61.18 ±0.33	59.92 ±0.31
Squid diet	62.62 ±0.15	63.09 ±0.07
0.4% ARA diet	61.11 ±0.27	61.44 ±0.05
0.6% ARA diet	61.44 ±0.12	61.45 ±0.16

3.2 Fatty acid composition of the diet

The fatty acid composition of the trial diet is shown in Table 10. The 0.6% ARA and control diets had significantly lower levels of linoleic acid (18:2n-6) than the other experimental diets. The 0.6% ARA diet had a higher level of γ-linoleic acid (18:3n-6) and a lower level of linolenic acid (18:3n-3). As expected, the level of ARA (20:4n-6) decreased from the 0.6% ARA diet, Vitalis diet, and 0.4% ARA diet and the level was the lowest, with no significant differences, in the squid, control and Inve diets. Over the three years of the trial, the percentage ARA in the diets (**Error! Reference source not found.**) was not significantly different except for the 0.4% ARA diet. They were no differences in the n-3: n-6 ratio (Table 10) as well as the DHA/EPA ratio (Figure 9) in all diets. According to the level of ARA addition to the diet, the EPA/ARA ratio was lower in the diet supplemented with ARA and the Vitalis® diet compared to all other diets and the lowest EPA/ARA ratio was observed in the 0.6% ARA diet.

Table 10: Mean fatty acid composition of the diet in μgFA/mg lipid. Values are mean ± S.D., $n=12$ in each trial group and $n=2$ in Skretting and Inve diet. Means with different superscripts within the same rows are significantly different ($p<0.05$).

	Control diet	Squid diet	0.4% ARA diet	0.6% ARA diet	INVE diet	Skretting Vitalis
Total lipid (%)	20.07±1.73	17.83±1.56	19.56±2.37	19.54±1.69	17.87±2.74	19.14±0.17
14:0	26.05±3.97	27.81±4.76	29.54±2.35	26.57±4.20	22.76±0.21	21.90±0.08
16:0	99.31±16.28	99.85±16.05	106.56±10.04	99.47±15.53	103.27±3.68	106.26±0.76
16:1n-9	0.86±0.28	1.22±0.38	1.02±0.48	0.92±0.29	1.10±0.06	1.68±0.11
16:1n-7	29.88±4.68	28.52±5.05	30.77±2.92	28.59±4.67	29.77±0.76	29.24±0.78
18:1n-9	82.15±13.58	79.64±13.94	86.80±8.44	82.01±13.28	86.02±3.75	88.31±1.53
18:1n-7	20.46±3.19	18.52±3.60	19.90±1.96	18.99±3.64	20.76±1.03	20.68±0.36
20:1's	36.49±5.57	37.34±5.76	39.45±4.02	37.01±6.45	35.66±0.42	38.27±0.56
22:1's	47.03±8.32	47.21±8.30	50.29±5.29	47.15±8.12	47.05±0.82	43.84±1.09
18:2n-6	**9.23±1.66** [b]	**13.01±3.46** [a]	**12.53±3.29** [a]	**11.29±1.92** [b]	**9.30±0.06** [b]	**31.79±0.68** [a]
18:3n-6	**0.80±0.05** [c]	**0.96±0.16** [c]	**1.67±0.31** [b]	**2.08±0.29** [a]	**0.80±0.20** [c]	**1.10±0.47** [c]
20:4n-6	**4.71±1.06** [c]	**5.27±0.99** [c]	**14.24±2.58** [b]	**19.44±3.64** [a]	**5.09±0.00** [c]	**16.81±1.23** [ab]
18:3n-3	**5.68±0.90** [b]	**6.32±0.89** [a]	**5.94±0.79** [b]	**5.28±0.46** [b]	**5.62±0.20** [b]	**7.82±0.42** [a]
18:4n-3	12.46±2.88	13.63±3.03	12.70±1.79	11.95±1.73	13.10±0.81	13.53±1.14
20:3n-3	0.96±0.21	1.06±0.19	0.94±0.14	0.85±0.15	1.02±0.01	1.06±0.07
20:4n-3	4.09±0.90	4.48±0.92	4.18±0.70	3.83±0.66	4.33±0.13	4.20±0.38
20:5n-3	44.93±10.73	46.90±10.27	45.35±7.56	43.43±7.73	48.92±0.87	46.06±3.86
22:5n-3	6.79±1.62	7.68±1.64	6.93±1.37	6.45±1.20	7.28±0.25	7.13±0.79
22:6n-3	75.76±19.31	83.25±18.72	76.72±15.03	72.62±14.85	82.53±2.88	77.49±8.63
Σ Sat	148.78±22.96	152.90±24.25	164.35±16.04	154.10±23.60	150.69±4.33	155.98±0.84
Σ MSat	234.51±38.01	230±39.69	246.92±23.71	231.68±38.61	239.08±5.29	237.26±5.04
Σ PUFA	176.24±40.22	193.65±39.99	193.28±34.46	189.26±34.18	189.25±3.92	221.18±17.61
Σ n-3	151.45±36.50	164.11±35.67	153.52±27.33	145.13±26.77	163.59±4.72	158.11±15.31
Σ n-6	**18.43±3.05** [d]	**23.31±4.13** [bcd]	**33.19±6.58** [abc]	**37.87±6.35** [ab]	**18.78±0.24** [dc]	**55.76±1.81** [a]
Σ n-3:n-6	8.22	7.04	4.63	3.83	8.71	2.84
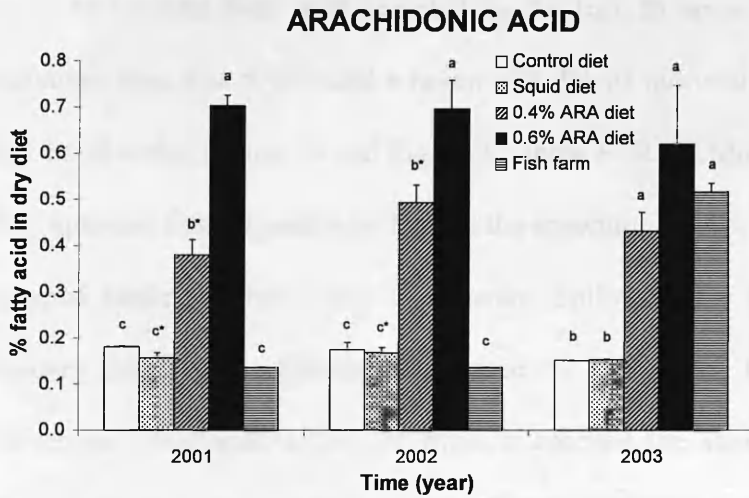
ARACHIDONIC ACID

Figure 8. Arachidonic acid concentration (Mean ± S.D., $n=4$) in dry trial diet through the three years of the project. Columns assigned a different letter are significantly different ($p<0.05$). * at a given year denotes significant difference between years within each experimental treatment. Fish farm diet: Inve diet in 2001 & 2002, Vitalis diet in 2003.

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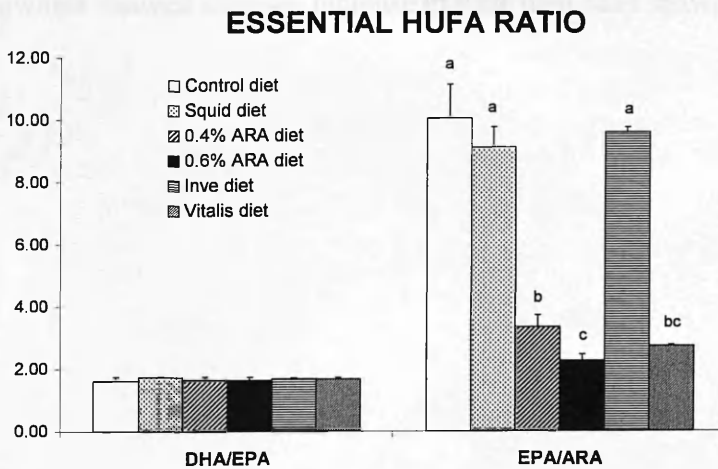
Figure 9. DHA/EPA ratio and EPA/ARA ratio (Mean ± S.D., n=4) in trial diet and production diet used at the fish farm (2001 & 2002: Inve diet, 2003: Vitalis diet). Columns assigned a different letter are significantly different (p<0.05). There was no significant difference among DHA/EPA ratios.

3.3 Feed efficiency ratio (FER)

In all tanks diets were well accepted by the fish. In order to feed the fish to satiation, feed was distributed a ration of 1.3% of individual body weight per day in all tanks. Figure 10 and Figure 11 show FER for May spawner fish and July spawner fish respectively. During the spawning season halibut broodstock stopped feeding or had a low feed intake. Following the binder inclusion in January 2002, feed efficiency increased in every trial tank. The FER in November 2002 was 0.42±0.07 while it reached the same value earlier, in October 2003, indicating the positive effect of the addition of binder to the diet. After the second spawning season, May and July spawner fish fed ARA enriched diets had a higher FER than fish fed squid or control diet. There were no significant differences between May and July spawner FERs. However, July spawners showed a slower increase in FER than May spawners in 2003.
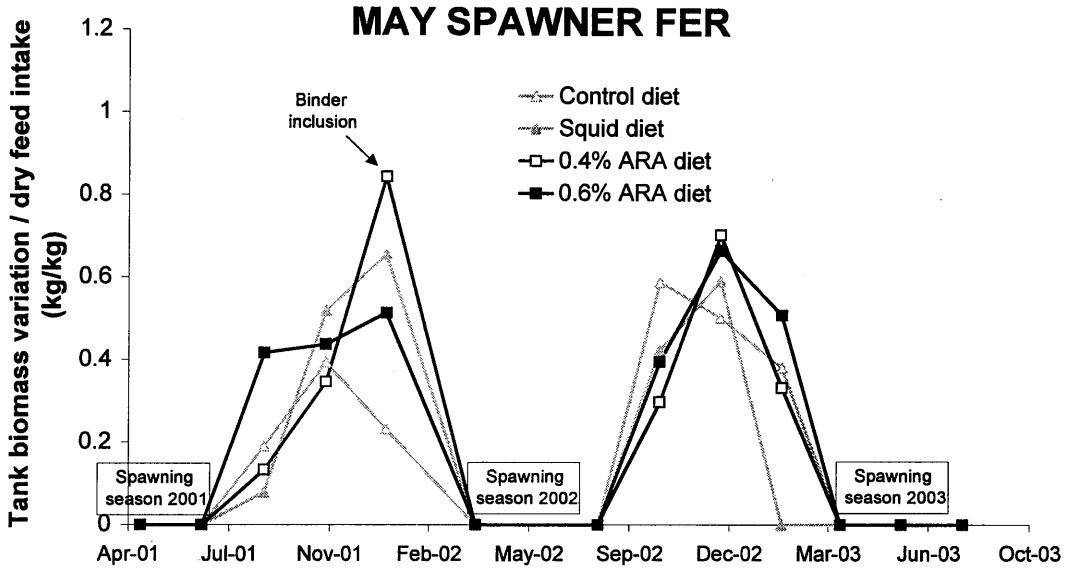
Figure 10. FER in May spawning Atlantic halibut broodstock fed experimental diets over the 3-year trial
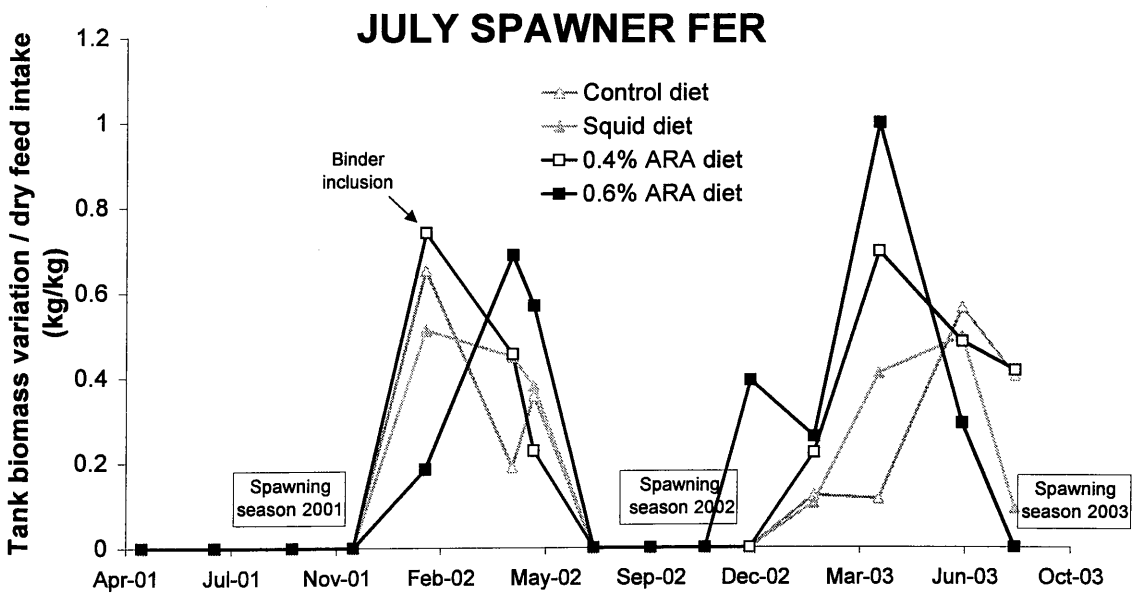
Figure 11. FER in July spawning Atlantic halibut broodstock fed experimental diets over the 3-year trial

3.4 Halibut broodstock survival and weight variation

For all experiments, the small number of mortalities was mainly due to handling stress and was not an effect of diet in May spawners (Table 11), but a tendency towards higher mortalities was observed in July spawners fed the control and 0.6% ARA diets (Table 12).

Table 11: May spawner female and male number, means ±S.D. initial and final weight (kg) and survival over the three year trial according to diet.

Diet		MAY SPAWNER			
		Control	**Squid**	**0.4% ARA**	**0.6% ARA**
Female	Number	15	15	15	15
	Initial weight	10.77±1.66	12.07±2.35	12.46±2.72	13.03±1.99
	Final weight	13.4±2.31	14.34±3.10	15.35±3.90	16.44±2.88
	Survival	100%	100%	93%	93%
Male	Number	5	5	5	5
	Initial weight	6.74±0.77	6.82±0.98	7.37±0.75	6.90±0.92
	Final weight	7.17±1.34	6.85±1.3	8.03±0.46	8.5±1.99
	Survival	60%	80%	80%	80%

Table 12: July spawner female and male number, means ±S.D. initial and final weight (kg) and survival over the three year trial according to diet

Diet		JULY SPAWNER			
		Control	Squid	0.4% ARA	0.6% ARA
Female	Number	15	15	15	15
	Initial weight	15.78±2.15	14.97±1.89	14.22±1.22	14.09±1.31
	Final weight	15.04±2.04	14.79±2.79	14.09±1.97	13.89±3.16
	Survival	73%	73%	87%	80%
Male	Number	5	5	5	5
	Initial weight	7.30±0.87	7.16±1.23	6.68±0.59	7.14±1.61
	Final weight	7.50±1.28	8.24±0.86	7.00±0.75	7.07±0.99
	Survival	60%	100%	100%	40%

As for the FER, weight variations follow the spawning season. Halibut females spawning in May (Figure 12) lost 2.2±0.8 kg at each spawning season. Female July spawner weight variation (Figure 14) was 4.3±0.9 kg for the first spawning season and 1.7±1.3 kg for the second. Halibut female mean weight was twice the male weight for the same age fish. Male May spawners (Figure 13) had smaller weight variation than females and lost 0.6±0.3 kg every spawning season. Male July spawner weight variation (Figure 15) was 1.3±1.09 kg for the first spawning season and 0.6±0.6 kg for the second spawning season. Therer were no significant effects of diet on weight variation over the two different spawning seasons for male and female halibut broodstock.

FEMALE MAY SPAWNER
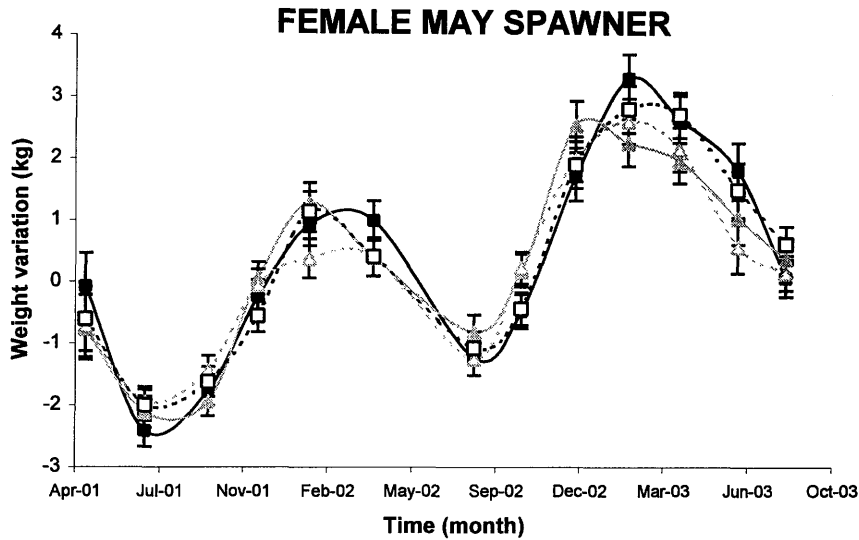
Figure 12. Weight variation (mean ± S.E.) for Atlantic halibut broodstock females fed with control diet (--△--), squid diet (——▲—), 0.4% ARA diet (--□--) and 0.6% ARA diet (——■—), over the 3 year trial. No significant differences were found among the experimental diets.

MALE MAY SPAWNER
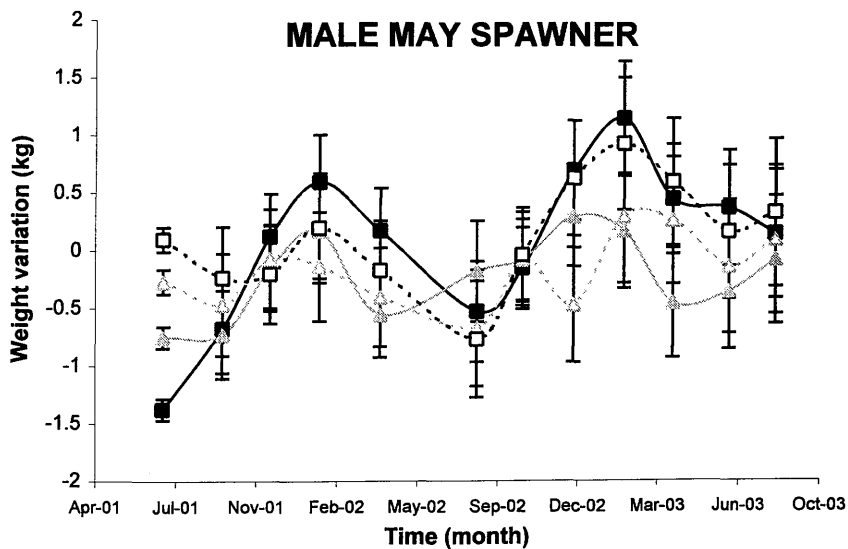
Figure 13. Weight variation (mean ± S.E.) for Atlantic halibut broodstock males fed with control diet (--△--), squid diet (——▲—), 0.4% ARA diet (--□--) and 0.6% ARA diet (——■—) over the 3 year trial. No significant differences were found among the experimental diets.
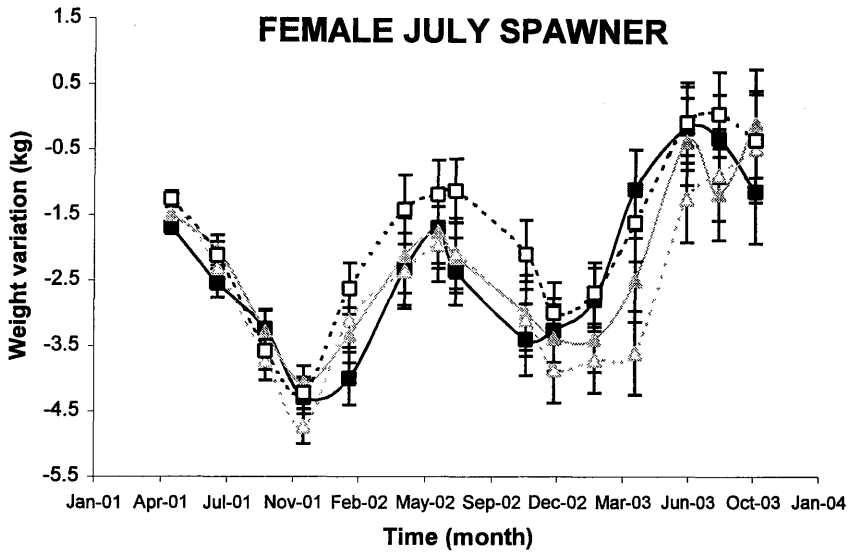
Figure 14. Weight variation (mean ± S.E.) for Atlantic halibut broodstock females fed with control diet (--△--), squid diet (--▲--), 0.4% ARA diet (--□--) and 0.6% ARA diet (—■—), over the 3 year trial. No significant differences were found among the experimental diets.
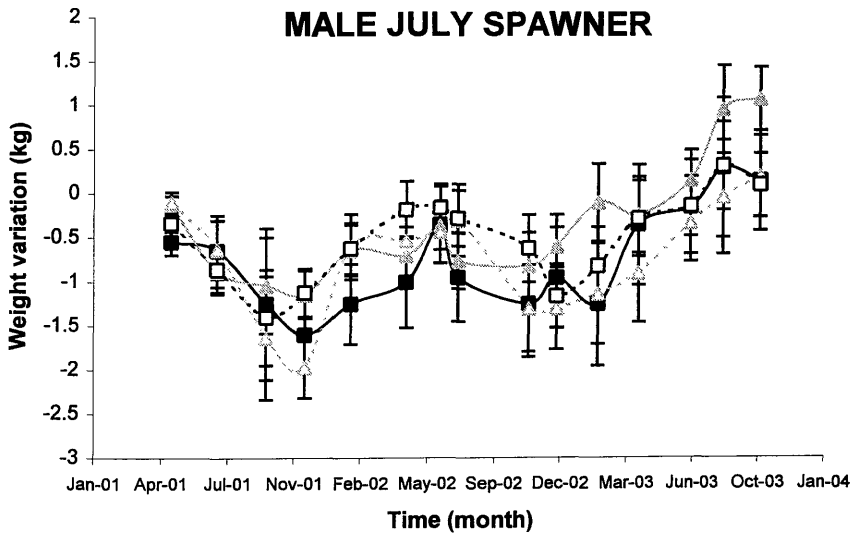
Figure 15 Weight variation (mean ± S.E.) for Atlantic halibut broodstock males fed with control diet (--△--), squid diet (▲), 0.4% ARA diet (--□--) and 0.6% ARA diet (—■—) over the 3 year trial. No significant differences were found among the experimental diets.

4 Discussion

4.1 Diet design and diet proximate composition

At the end of the first spawning season in July 2001, fish started to increase their feed intake significantly from 0 to 0.06% body weight per day. When halibut were fed the trial moist sausage ingestion reduced the sausage to a powdery form. Some of this material was expelled through the fish gills creating concern about water quality and actual feed intake. Even if binders such as alginates and guar gum can have detrimental effects on various digestive processes by accelerating gastrointestinal transit time and depressing the apparent digestibility of protein and fat in rainbow trout (Fagbenro & Jauncey, 1994), reduced leaching of dry matter was achieved with the inclusion of 2% alginate and 35% water in salmon food (Hillestad *et al.*, 1999). Moreover, the digestibility of protein extracts (gluten) of corn and wheat was similar to that of fishmeal in silver perch (Allan *et al.*, 2000). Wheat gluten showed high apparent digestibility coefficient (ADC) values for protein, energy and organic matter in European seabass (Robaina *et al.*, 1999). Atlantic salmon showed increased ADCs for fat and energy when fed a diet where fishmeal crude protein was replaced with wheat gluten crude protein (Storebakken *et al.*, 2000). The use of wheat gluten, as a natural antioxidant, instead of the synthetic antioxidant butylated hydroxytoluene (BHT) has shown a positive effect on oxidative stability. This treatment significantly improved the oxidative stability of the resultant product without any apparent flavour and

palatability defects (Al-Neshawy & Al-Eid, 2000). The exchange of fishmeal

for wheat, with constant fat level, had small, negative effects on fat

digestibility in Atlantic halibut (Grisdale-Helland & Helland, 1998). Therefore,

in November 2001, it was decided to test the inclusion of different types of

binders in the trial diet. Alginate, micronised wheat and wheat gluten were

tested. The optimum elasticity of the sausage was obtained with 10% of wheat

gluten binder inclusion. Subsequently, no more sausage breaking or powdery

reduction was observed. It was possible to assume the full ingestion of the

sausage by the fish and a significant improvement in water quality. The use of

wheat gluten as a binder was a necessary improvement of the diet and did not

significantly change the level of protein or total lipid in the diet.

Table 13: Marine species broodstock diet proximate protein and lipid composition (% dry weight)

	Crude protein	Lipid
Seabream (Almansa *et al.,* 1999)	46	13
Seabass (Asturiano *et al.,* 2001)	53	22
Halibut (Mazzora *et al.,* 2003)	61	17

Diet composition was regularly sampled and analysed in order to kdetermine

whether the formulation of the diet was stable. The protein levels of $61.5 \pm$

0.94% and the total lipid levels of $19.01 \pm 2.05\%$ in the diets were similar to

those used in other marine species broodstock diets (Table 13). These values

also reflect the proportions observed when halibut are fed on frozen fish. There was no significant difference in percentage of total lipid in the diets over the three year experiment. This lipid percentage is also in accordance with other marine fish species requirements. Commercial halibut broodstock diets, such as Inve and Vitalis, used at Otter Ferry Seafish Ltd have a similar proximate composition to the trial diets. The percentage moisture in all diets was not significantly different and was around 48%.

4.2 Fatty acid composition of the diet

Two important issues have been raised in marine larval fish nutrition, the high dietary level of DHA required for larval neural development (Bell *et al.,* 1995) and the importance of the dietary ratio of EPA/ARA for determining eicosanoid actions (Sargent *et al.,* 1999b). The two issues are inexorably linked because all dietary oils rich in DHA (marine fish oils) contain substantial but variable amounts of EPA and also minor and variable amounts of ARA. Therefore, it can be difficult to practically achieve the desired blend of dietary PUFA to provide a pre-determined dietary balance. To investigate the first issue it was decided to use mackerel oil for the experiment due to the high level of DHA present. The optimal EPA/ARA ratio for eicosanoid production is likely to be much more species-specific. One of the objectives of this project was to find the best EFA ratio for Atlantic halibut broodstock, keeping in mind, that this will also determine the deposition of EFA in eggs and yolksac larvae.

The 0.4% and 0.6% of ARA in the diet was tested by adding Vevodar® ARA to the mackerel oil. Throughout the three-year trial there were no significant differences in DHA concentrations.

4.3 Feed intake

The addition of binder to the diet increased fish feed intake and improved water quality. This positive effect can be observed through an increased FER in January 2002, when it was decided to add 10% wheat gluten to diets. Halibut broodstock considerably reduced their feed intake four months prior to the spawning season and even stop feeding two months prior to spawning. In order for the broodstock fish to gain energy for somatic growth and recovering properly from the stressful spawning season, it is important to get a high feed intake as early as possible after the spawning season. This translated into a rapid increase in May spawner FER. In contrast, it is interesting to note that July spawners were slower to return to a higher FER in 2003. Atlantic halibut broodstock fed with vitamin-enriched whole herring, by hand, to satiation twice a week show that consumption followed a clear maturational-related annual cycle with feeding gradually increasing following spawning, reaching maximum levels three to four months after spawning and then decreasing steadily to reach lowest levels during spawning (Björnsson *et al.*, 1998).

4.4 Weight variation

When observed in the wild, the Atlantic halibut carcass does not seem to be overly affected by the energy expenditure involved in the seasonal accumulation of reproductive tissues and in spawning, particularly in females where no significant sacrifice of body weight was observed (Haug & Gulliksen, 1988). This observation does not appear to be applicable to domestic halibut broodstock. The present study showed considerable body weight variation over the spawning season with greater weight variation observed in females than in males. The halibut liver depletion observed in wild broodstock during the spawning season (Haug and Gulliksen, 1988) agreed more with the fish weight variation observed during the project. A linear model adequately describes the average weight gain of farmed halibut within the size-range from 2 to 12 kg for females and 2-7 kg for males (Björnsson, 1995). However, when halibut started to spawn, they were losing weight, and their growth was no longer following a linear model. Thus, for the present project, growth was analysed through fish individual weight variation from their initial weight at the beginning of the trial. The average weight of wild Atlantic halibut at 50% maturity, kept in a Norwegian fishfarm was 3.2 and 12.7 kg for males and females, respectively. After the males became mature their growth rate was less than half that of the females (Björnsson, 1995). Experimental males and females are first generation domesticated fish at Otter Ferry Seafish and it was then difficult to compare them to wild fish. However, the difference between

male and female size is similar. The background of the two different spawning groups can explain the weight variation differences between May and July female spawners. May spawners had already been in smaller experimental tanks prior to the project and were moved earlier (November 2000) to the trial tanks compared to the July spawners (January 2001). July spawners were moved at the beginning of the trial, at a time when they were supposed to reduce feed intake prior to spawning season and they did not return to normal feed intake until the end of the spawning season in September 2001. This resulted in a longer starvation period (9 months) and a mean weight loss of 4.3±0.9 kg during this period. There were no significant differences when comparing diet effects on female and male weight variation. However, when looking at May female and male spawners and female July spawners, a general positive effect on weight variation may be observed when fish were fed ARA enriched diets.

Chapter 4 Halibut broodstock reproduction

1 Introduction

Atlantic halibut is a long-lived species and multiple batch spawner. They produce many batches of eggs over a relatively short period of time, usually a matter of weeks. The large number of fish with running milt and roe caught at the bottom on the spawning grounds suggests that halibut remain on the seabed during the spawning season and spawn at depths of 300-700m, in total darkness (Neilson *et al.*, 1993). Maturity is size-dependent in wild females; the 50% maturity level is reached at an age between 13 and 14 years (Haug & Tjemsland, 1986). Halibut males mature at an earlier age (2 years) and smaller size than females (5 years). Analysis of gonadosomatic index (GSI) and fork length (FL) data has indicated that a minimum length of 80 cm and 115-120 cm, for males and females respectively, is required for sexual maturation of halibut from Newfoundland waters (Kohler, 1967; Methven *et al.*, 1992). These observations are further supported by the lack of detectable sex steroids in captive halibut from the same range of length (Methven *et al.*, 1992). In captive halibut, males and females become sexually mature at an average weight of 3.2 kg and 12.7 kg respectively (Björnsson, 1995). Gonad growth, fecundity and egg viability are known to be very susceptible to environmental factors, such as temperature, nutrition and stress factors (Kjørsvik, 1990). The natural spawning season in Norwegian water occurs from January to March

(Kjørsvik *et al.*, 1987). In captivity, reproduction timing can be controlled by several parameters, the most effective control for Atlantic halibut being photoperiod. Female halibut can release eggs without external hormonal manipulation; however, these are generally unfertilised when retrieved from the holding tank (Shields *et al.*, 1999b). For this reason, gametes are collected separately by stripping and returned to the hatchery for fertilisation using a wet procedure with milt activated in seawater before mixing with the eggs. For Atlantic halibut, stripping of individual fish is necessary in order to do experiments such as investigating a nutritional effect or selective breeding, and will consequently be the appropriate method in the domestication process (Rabben, 1987). This involves the development of reliable methods for individual marking of fish. Marking fish could also be used to accelerate the stripping process and to avoid stripping the same female several times or to miss a mature female (Berge 1990).

1.1 Halibut male gamete production

Atlantic halibut have high total ejaculate volumes from 1 to 92 ml per stripping and sperm motility duration after seawater activation is 60-70 s (Billard *et al.*, 1993; Suquet *et al*, 1995). Spermatogenesis is the developmental process that provides spermatozoa. The final differentiation of spermatids into flagellated spermatozoa occurs during spermiogenesis (Schulz *et al.*, 1999). The germinal compartment in Atlantic halibut testis appears to be organised in branching lobules of the unrestricted spermatogonia type, because spermatocytes with

spermatogonia were found throughout the testis (Weltzien *et al.*, 2002). The sperm concentration in halibut ranges from 11.9 x 10^9 to 37.2 x 10^9 spermatozoa/ml, 1-2 times higher than reported for other teleost species (Fauvel *et al.*, 1999). Higher sperm densities in halibut are likely to be an adaptation to their spawning location, fecundity and egg size. The combination of producing large volumes of large eggs in a dark environment may require exceptionally high sperm densities to ensure reproductive success (Tvedt *et al.*, 2001). Spermatogenesis is regulated by extrinsic (endocrine and paracrine) and intrinsic cues. Spermatogenesis ceases upon androgen deprivation, hence the importance of steroidogenic gonadotropins regulating Leydig cell androgen production (Schulz *et al.*, 1999). For Atlantic halibut, spermiation commences about 1-2 months prior to female ovulation. This asynchronous production frequently results in reduced sperm production as indicated by higher spermatocrit levels later in the spawning season, while some females still produce viable oocytes needed to be fertilised. Poor quality milt at this time adversely affects fertilisation and, thus, production capabilities (Powell *et al.*, 1998). The sperm of most teleost fish differs from that of mammals in four important aspects: it is immotile on ejaculation; motility is induced on contact with water; it remains fully motile for less than 2 min; and has no acrosome. Methods of assessment of sperm quality must consider these factors, since the parameters measurable will be affected by the technique by which motility is induced, and by the time after induction of motility at which quality is

measured. The two most obvious parameters that are useful in assessing sperm quality are motility and duration of movement. In fish, the trajectory of sperm is generally more curved than in mammals and fish sperm can move three-dimensionally in the aqueous medium. Since the velocity of fish sperm decreases rapidly with time, the duration of progressive movement will also have a significant influence on the ability of sperm to enter the egg. It is also important to note a further difference to mammals, in that sperm of teleost fish do not simply have to meet the egg and enter via an acrosome reaction; they must find the single point of entry, the micropyle, on the surface of the egg within the very short time available (Kime *et al.*, 2001).

1.1.1 Halibut sperm collection

In fish, two methods of stripping milt can be used. One consists of collecting sperm into a syringe after drying the urogenital pore. The other involves catheterising the ureter and emptying the urinary bladder by gently squeezing the fish belly, subsequently collecting sperm by stripping, after rinsing with distilled water and drying the urogenital pore. Although it is not possible to catheterise turbot or halibut sperm duct without injuring the males, as urine has a deleterious affect on the quality of turbot spermatozoa, it is necessary to empty the urinary bladder before stripping, in order to significantly decrease milt contamination (Dreanno *et al.*, 1998).

1.1.2 Milt quality assessment: spermatocrit

A common method of assessing sperm quality has been simply to mix eggs and sperm and measure fertilisation or hatch rate. However, an earlier step and more individual milt quality parameters will be useful in a commercial hatchery. Spermatocrit is a well-established technique for estimating sperm density in fish. It is easier and faster than counting spermatozoa, and of interest for reproductive studies in aquaculture species (Tvedt *et al.*, 1999). Spermatocrit level, or packed sperm cell volumes, are indicative of the condition of viscosity and dispersion property of the milt (Martin-Robichaud *et al.*, 2001). Duration of centrifugation had a significant effect on spermatocrit, when considering all time intervals, nevertheless, there was no statistically significant difference among halibut male spermatocrit after centrifugation for 30 minutes. Based on this result, 40 min centrifugation was used for subsequent spermatocrit determination (Tvedt *et al.*, 2001).

1.2 Halibut female production

Spawning females usually have hyaline as well as non-hyaline oocytes in their ovaries. For halibut in Northern Norway, the mean diameter of the opaque oocytes increased continuously from September to January, with the first maturing oocytes being transformed into hyaline phase in January. Fluid intake of oocytes started in ovaries, with mean mature and opaque oocyte diameters of 1.70-2.05 mm, and the hyaline eggs ranged from 2.25-3.37 mm (Haug & Gulliksen, 1988). Sequential biopsies revealed that group-synchronous batches

of oocytes underwent a rhythmic cycle of hydration, such that the water content of the pre-hydrated oocytes increased from approximately 63% of wet mass to 90% in the ovulated eggs. The driving force of the oocyte hydration is a transient hyperosmolality of the yolk, which is due mainly to the liberation of free amino acids. These mechanisms of oocyte hydration are responsible for pre-adapting the pelagic eggs of teleosts to the hyperosmotic condition of seawater in which they will spawn (Østby et al., 1999; Finn et al., 2002). Individual fish release multiple batches of pelagic eggs according to their ovulatory rhythms during the spawning season. Halibut of 20-60 kg have been reported to give 6-16 egg batches during one spawning season, with mean ovulatory rhythms of 70-90h (Haug & Gulliksen, 1988).

1.2.1 Female stripping and overripening

Scientific literature shows that the timing of the halibut strip is of great importance. However, the number of fish to be stripped is increasing each year with the addition of farmed broodstock to the wild catch broodstock, making the management of the stripping process more difficult. Following ovulation unfertilised eggs undergo a process of ageing, commonly described as over-ripening. During overripening, eggs present a series of morphological and compositional changes as well as a progressive loss in quality or viability. The period of optimum ripeness, which is defined as the time when the highest fertilisation and egg and larval survival are achieved, varies with different

species of fish, from 1h in Tilapia (Bromage *et al.,* 1994) to 20 days in Coho

salmon (Fitpatrick *et al.,* 1987). Flatfish generally show periods of optimum

ripeness which are intermediate in duration (Bromage *et al,* 1994).

Discoloration or non-transparency, irregular shape, chorion breakdown, fusion

of cortical alveoli and dimpled appearance of the cytoplasm characterised egg

overripening. Timing of stripping of individual females is considered important

in order to get an acceptable yield of halibut eggs. Knowledge of the intervals

between each ovulation for individual female halibut is important in order to

optimise the stripping time (Norberg *et al.,* 1991) The time after ovulation at

which the optimum egg quality is achieved is between 4 and 6 h for Atlantic

halibut ((Kjørsvik *et al.,* 1990; Holmefjord, 1991; Bromage *et al.,* 1994).

1.2.2 Female fecundity

The fecundity of fish can be defined as the number of oocytes produced, either

over a lifetime, or during a single spawning season (Horwood *et al.,* 1989) in

relation to body weight/size. Halibut have a large potential for egg production,

their fecundity is very high: in the wild, a 1.95m female can produce up to 7

million eggs per spawning season (Haug & Gulliksen 1988).

1.3 Environmental and endocrine control of reproduction

1.3.1 Temperature

Temperature fluctuations in land-based hatcheries are commonly observed.

However, temperature control through water chilling is effective in improving

egg viability and a necessary feature of halibut broodstock management in Scotland. The spawning performance of an ambient stock is generally poorer than that of a chilled stock. The ambient stock showed a shorter and delayed spawning season coupled with lower fertilization and hatching rates which fell dramatically once the water temperature exceeded 8°C and resulted in reduced egg production (Brown *et al.*, 1995). Water temperature management is one of the main economic issues in Atlantic halibut farming. Broodstock fish under photoperiod control spawn from January to October and it is therefore imperative to chill the water below 6°C over the summer season in order for the female to produce viable eggs.

1.3.2 Photoperiod

An important fish characteristic is seasonality, with timing of developmental and maturational events synchronised with seasonal changes in climate, day length and food supplies. This coordination and the associated internal processes of control ensure that young fish are produced when environmental conditions are the most suitable for their survival. Although a number of environmental factors have been implicated as possible proximate cues, including photoperiod, temperature, rainfall, food supplies and pheromones, it is the seasonally changing pattern of day length which is probably responsible for the cueing and timing of reproduction in the majority of temperate fish species. Photoperiod manipulation was first used on farms to adjust spawning

time in order to produce out-of-season supplies of seed (Bromage *et al.*, 2001). Total blackout is recommended as at many times in the year the artificial light regime will be different from the ambient one. For most farms, heavy-duty polythene or butyl linings over a simple metal, plastic pipe or wooden framework provides a cheap and effective method of blacking-out broodstock enclosures (Bromage *et al.*, 2001). Hatcheries can use photoperiod to manipulate maturation and spawning time to produce all-year-round supplies of eggs and fry. Manipulation of the annual photoperiod cycle had some clear consequences for spawning activity of female halibut (Jonassen *et al.*, 1999; Norberg *et al.*, 2001). It appears that day length increasing at an accelerated rate until July, and at a normal rate after that, was perceived as an effective environmental cue by females for the timing of sexual maturation (Björnsson *et al.*, 1998). The reproductive sensitivity of Atlantic halibut to annual photoperiod cycles indicates that this species responds readily to photoperiod information. Indeed, halibut broodstocks show an endogenous circannual rhythm which seems biologically advantageous for synchronizing sexual maturation of an oceanic bottom-living species often encountered at depths from 300 to over 1000 m. It may also appear surprising that photoperiod entrains an endogenous rhythm for a species living in deep water where photoperiod cues are bound to be extremely limited because light penetration is very low. However, it is known that the Icelandic stock enters shallow coastal waters in late spring following spawning, the fish staying on shallow 'feeding

grounds' during summer and into autumn. Therefore, wild halibut can be expected to perceive relatively strong photoperiod cues for around half of the year, including the decrease in day length in autumn (Björnsson *et al.*, 1998).

1.3.3 Hormonal induction of maturation

Individual fish response to a range of environmental, seasonal, behavioural and physiological cues is mediated by the interaction of several chemical substances with hormonal function produced at different levels in the hypothalamus-pituitary-gonad axis and acting on different target tissues (Zohar, 1989). Several methods are available to induce final oocyte development and ovulation in female, and spermiation in male captive fish, including injection of follicle stimulating hormone (FSH) or luteinizing hormone (LH) or gonadotropin hormones (GTH) (Zohar, 1988). Pituitary extracts contain human chorionic gonadotropin (hCG) and gonadotropin-releasing hormones (GnRH) (Carrillo *et al.*, 1999). GnRH stimulates pituitary production of gonadotropin (GTH), which subsequently stimulates the production of gonadal steroid hormones. Androgens such as testosterone and 11-ketotestosterone, and gonadal progestogens such as $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$-P) are thought to play a key role in spermatogenesis and spermiation processes (Schulz *et al.*, 1999; Vermeirssen *et al.*, 1999). Recently, hypothalamic hormones such as a native GnRH or their analogues have gained favour among commercial fish producers (Zohar &Mylonas,

2001). GnRH oligopeptides are smaller (10 amino acids), easier to prepare, and more effective at inducing maturation than other peptides such as GTH glycoprotein (200 amino acids). As they are natural peptides controlling gonadogenesis, they are more reliable and effective without causing any harmful side effects. In addition, GnRHa analogues of both mammalian and salmon (sGnRHa) forms are more potent and degrade more slowly than natural hormones. In males, cultured fish GnRH has been used to stimulate, or enhance, or synchronise spermiation in many species using a slow-releasing implant, GnRH analogue (D-Arg^6-Pro^9-NEt salmon GnRH; Ovaplant®; 150 µg; sGnRHa) (Powell *et al.*, 1998).

1.4 Artificial fertilisation

The study of artificial insemination is particularly important in species whose gametes are hand-stripped, since this may decrease the variability of reproduction yield noted in commercial hatcheries. Standardization of the insemination protocol is a pre-requisite in improving the fertilisation process. Halibut eggs stored at 1 to 3°C exhibit good fertilisation after 64h with a loss of fertilisation capacity of about 10-20%. Egg storage temperatures below 0°C result in less viable ova (Martin-Robichaud & Rommens, 1998, 1999). Halibut egg fertilisation success is largely independent of sperm density within a range of $9x10^5$ to $5x10^8$ spermatozoa/egg (Tvedt *et al.*, 2001). This ratio can be successfully lowered to 10^4 spermatozoa/egg, but this is still higher than the

sperm densities necessary for successful fertilisation in other teleost species (Suquet *et al.,* 1995; Vermeirssen *et al.,* 2000). The hardening of the egg chorion is due to an enzyme reaction during the activation process, and the egg's ability to sustain mechanical resistance (egg strength or chorion hardness) is better in good quality eggs than in poor quality eggs of cod, lumpsucker and halibut (Kjørsvik, 1990). The egg's potential to produce viable fry is determined by several physical, genetic and chemical parameters, as well as the initial physiological processes occurring in the egg. If one of the essential factors is lacking, or is incomplete, egg development will fail at some stage. Thus, egg quality should be determined when the egg has left the female fish and the fertilisation process is complete (Kjørsvik, 1990). In order to be of value to the fishfarmer, methods of assessment must be simple to perform and should be capable of being carried out early in egg development to avoid occupying hatchery facilities and staff time with what may turn out to be unproductive batches of eggs (Bromage & Roberts, 1995).

1.5 Chapter aims

➢ Standardisation of Atlantic halibut broodstock stripping frequency, stripping process and egg artificial fertilisation protocols

➢ To determine the effect of photoperiod and temperature on experimental halibut broodstock

➢ To study the effect of ARA enriched diets on halibut female reproductive performance (fecundity, egg volume, egg fertilisation rates)

➢ To study the effect of ARA enriched diets on halibut male reproductive performance (spermatocrit)

➢ To determine the effect of Ovaplant® on halibut milt.

2 Materials and Methods

2.1 Fish tagging

Halibut Female and male broodstock were individually tagged with microchips. However, the scanner was not waterproof, so to be able to read the broodstock tag without damaging the scanner, the female had to be up at the surface. Many females had to be checked in order to find the target one. The process was long, inefficient, and stressful for the fish and it was determined that an external tag was necessary.

Dorsal fin

Ventral fin

Nylon tag (Avery Dennison supplier) with three differents coloured platic tubes glued on it.
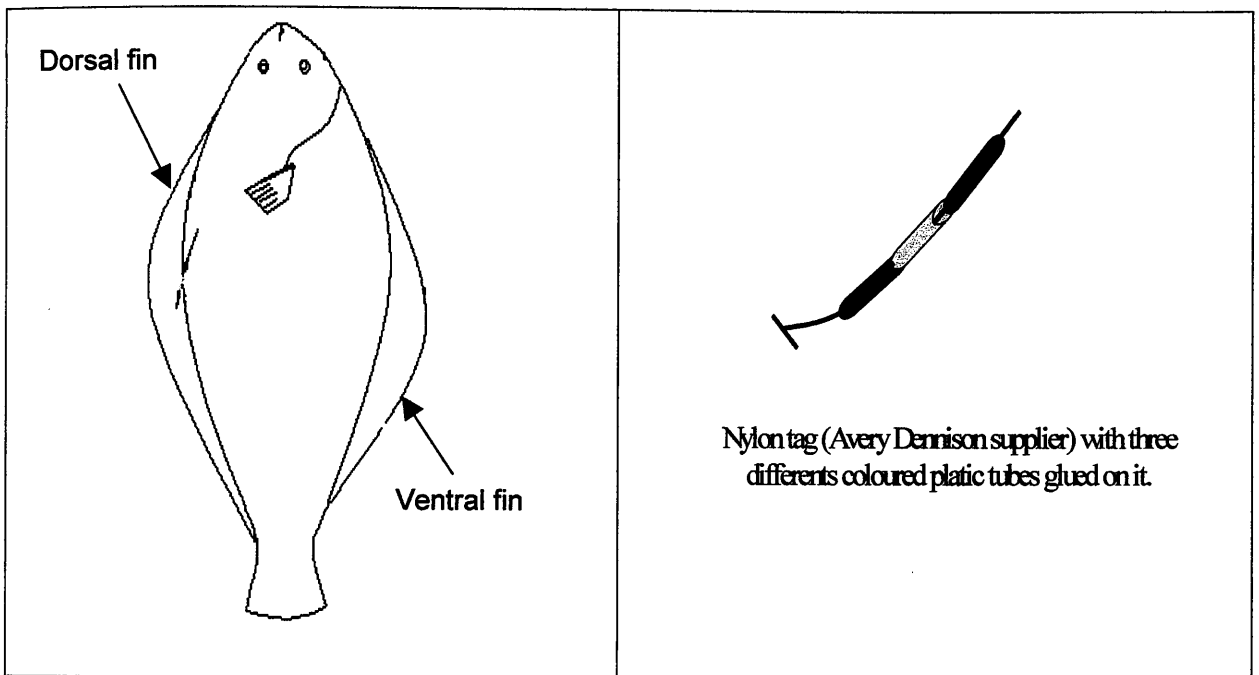
Figure 16. External colour tag on halibut broodstock

In 2001 a coloured plastic band was placed around the tail of mature halibut, but a few weeks after beginning the stripping process 60% of the bands fell off and 80% of the broodstock showed significant wounding around their tail caused by the friction of the band. In 2002, at the start of the spawning season, halibut female and male broodstock were tagged with plastic anchored tags (see Figure 16) to accelerate the stripping process and to either avoid stripping the same female or male several times or to miss a mature female. A flow anchor tag was used that consisted of three different colours of soft PVC tubes about 1.5 mm in diameter and 20 mm long attached to a rigid 10 cm nylon string. One end of the nylon string is a little thicker and "T" shaped. A tagging

gun similar to those designed for price-tagging clothes is used to inject the tag

into the halibut. The tag is located on the upper side of halibut at the base of the

dorsal fin to avoid damaging vital organs (Figure 16).

2.2 Temperatures and photoperiod

Over the three spawning seasons of the experiment, seawater was chilled below

6°C a month prior to the spawning season. During the spawning season water

temperature (Figure 17) was kept below 6°C. Sea water was pumped from a

depth of 20m and then passed through a header tank and then through two

80cm sand filters (15 micron).

Filtered water then went through a cold recovery exchanger (two titanium

plates) decreasing water temperature with previously chilled wastewater. The

partially chilled water than passed through a chilling heat exchanger based on

glycol circulation (0°C), decreasing the water temperature to 5°C and feeding

directly into the broodstock tank. Tanks were covered with lightproof covers

over broodstock enclosures and provided with artificial lighting controlled by

automatic time clocks (Figure 18).
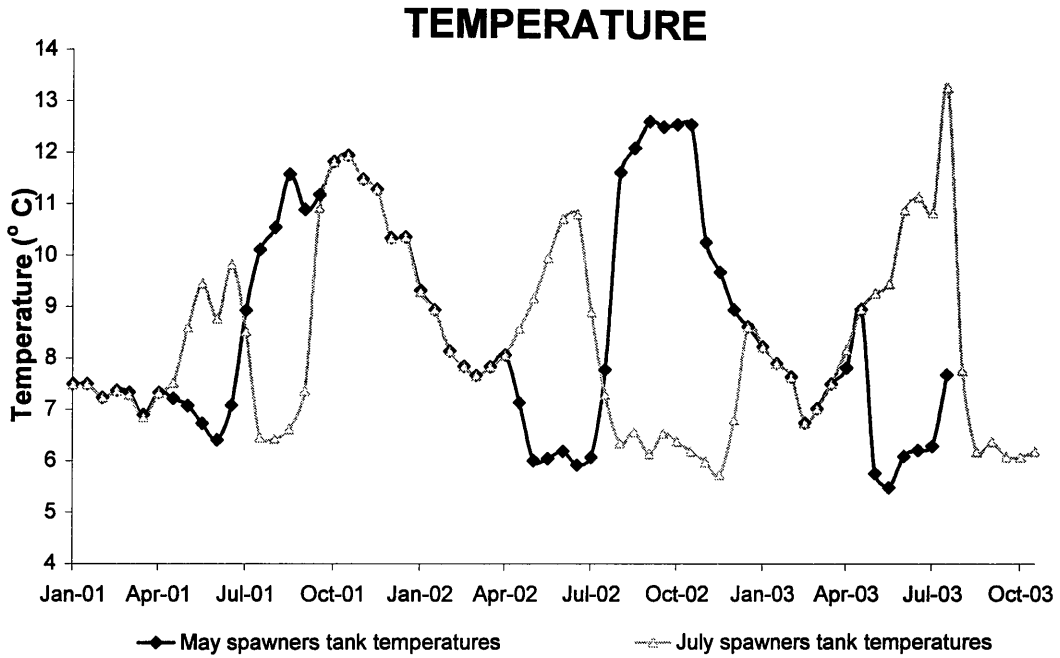
TEMPERATURE

Figure 17. Halibut broodstock tank water temperature over the three year project
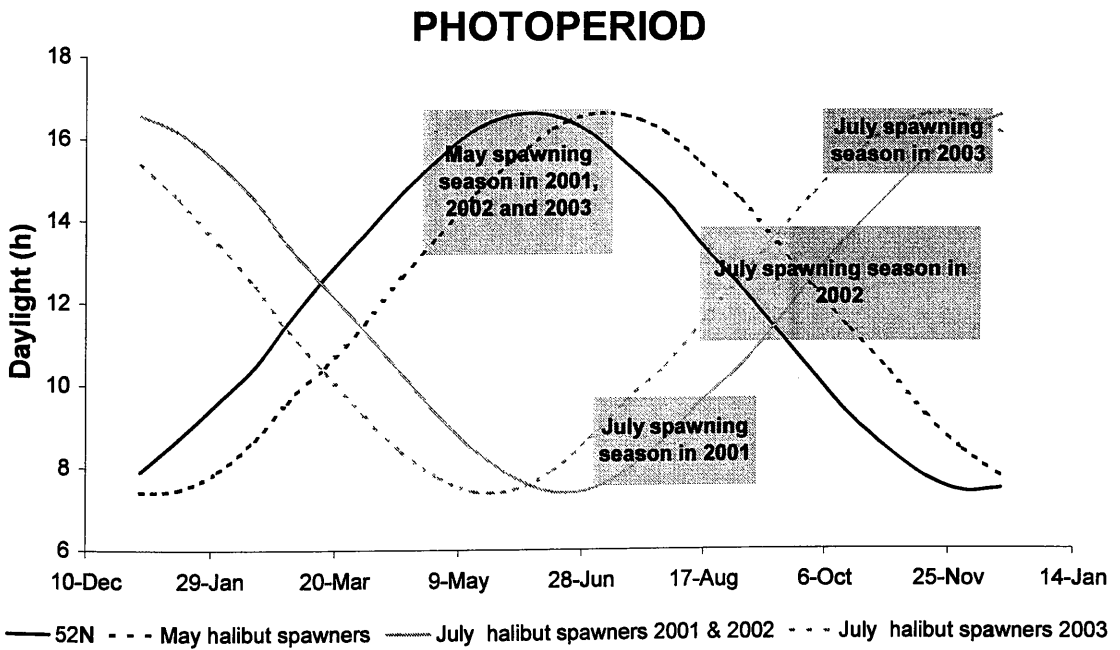
PHOTOPERIOD

Figure 18. Halibut broodstock experimental photoperiod over the three year project.

2.3 Halibut female production

2.3.1 Fish timing

Fish in 2001 were stripped nearly every day apart from weekends in order to assess the state of ripeness of the halibut female. However, females were rarely ready for stripping. The whole process was time inefficient and stressful for both farmers and fish. In order to reduce fish handling stress and to have efficient stripping routines set up, it was decided to stop individual assessment and to strip halibut every 3 days by improving broodstock fish tagging.

2.3.2 Female stripping
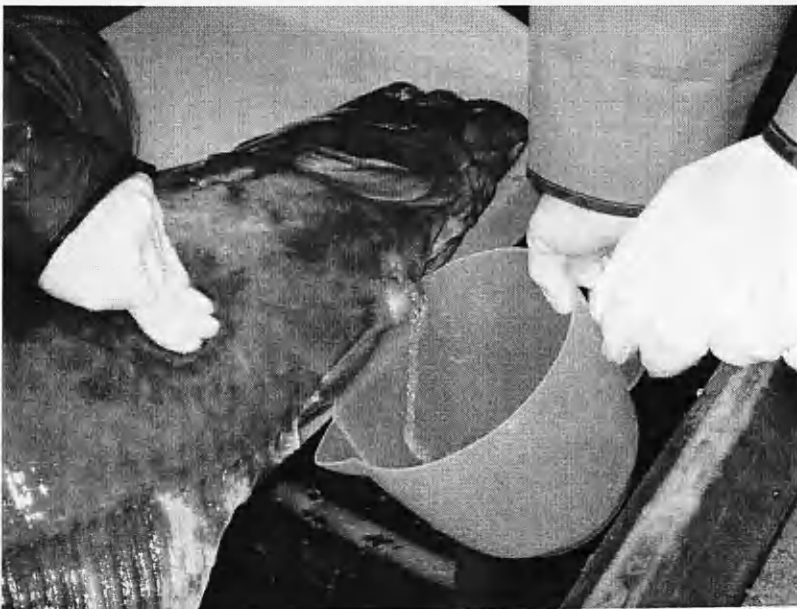
Picture 2. Halibut female stripping

Stripping was accomplished by lifting the broodstock female from the water and placing it on a table, then applying pressure on their abdomen in a

backward to forward movement with a flat hand (Picture 2). Eggs were collected in a 2l plastic jug previously kept in sodium hypochlorite and rinsed with seawater. The females were kept hydrated during the process by watering them with a jug of seawater but avoiding eggs coming into contact with the seawater. Jugs were labelled and kept in a cool box to protect them from sunlight and temperature variation during the stripping process (Picture 3). The cool box was then brought into the cold dark egg room where individual batches of eggs were used for artificial fertilisation. Relative fecundity was the individual number of eggs produced per female body weight (eggs/kg). Number of egg was estimating from the volume stripp in the jugg as 40000 eggs/l following fishfarm protocols.

Picture 3. Halibut eggs kept in cool box in between stripping process and moving to the dark, cold, egg room

2.4 Halibut male gamete production

2.4.1 Male stripping

Picture 4. Halibut milt collection

For each stripping two halibut males from each tank were placed on a stripping board. The urogenital pores were dried with adsorbent paper to remove excess seawater. Milt was expressed directly into 60 ml Sterilin disposable polypropylene sterile containers by applying gentle abdominal pressure (Picture 4). When applying pressure, urine and faeces can be released through pores situated forward of the genital pore and it is important to avoid any contamination and make sure that only sperm goes into the collection vessels. 60 ml containers were labelled with fish pit tag number, tank number, date, and

kept in a cool box to be protected from sunlight and temperature variation during the stripping process. The cool box was then brought into the egg room where milt was used for artificial fertilisation. Prior to artificial fertilisation sperm motility was tested. One drop of milt was mixed with one drop of treated seawater on a slide and observed under a microscope. If motility was not activated by seawater, milt would not have kept for the fertilisation process. However, over the three spawning seasons, all the freshly stripped milt showed good motility.

2.4.2 Halibut male spermatocrit

Spermatocrit was determined using milt sampled into Vitrex microhaematocrit tubes (soda lime glass 75 mm length, 1.1-1.2 mm inner diameter) and centrifuged at 5500 x g for 40-min (Sarsted MH2 centrifuge). Triplicate samples of halibut milt for each male stripped were run. Percentage of sperm over seminal liquid was measured with a haematocrit reader.

2.5 Males implanted with Ovaplant®

To study the opportunity to extend the milt production period, males were implanted with Ovaplant®, an exclusive trade name of Syndel International Inc. When more than half of the males showed spermatocrit exceeding 80%, two males were randomly selected in each trial tank and implanted. Halibut

males were sedated with 1ml.l^{-1} of phenoxyethanol then placed on a stripping board. To inject Ovaplant® all equipment was cleaned with ethanol and the implant injected, using the injection needle and gun supplied (Ralgun pellet injector), into the dorsal sinus. Based on the size of the fish the dose was about 13μg/kg.

2.6 Artificial fertilisation and fertilisation rates

Just after the stripping process, the cool box containing egg jugs and milt boxes were brought into the egg room. Egg handling was carried out under low intensity red filtered light and at similar temperatures to those of the broodstock tanks. According to the number of jugs, 10L plastic buckets were filled up with 5L of UV treated seawater. For artificial fertilisation individual female egg batches were mixed with the milt from two different males both originating from the same diet treatment. 1ml of milt was added to 10L of seawater and mixed thoroughly, then one jug containing one individual female egg batch was emptied into the bucket and these were covered with another 1ml of milt from another male and mixed gently. After 30 minutes eggs settled at the bottom of the bucket as they are negatively buoyant in 33‰ salinity seawater allowing careful disposal of the mixing water contaminated with milt and poor in oxygen. Sufficient water for the eggs to stay well hydrated (around 1L) was kept in the bucket. At this stage eggs were sampled with a reversed 10 ml plastic pipette and placed into glass beakers containing 200 ml of UV

treated seawater. Enough eggs from individual batches were collected in order to obtain a single layer of eggs at the bottom of the beaker. Eggs reaching the eight-cell stage (12h after fertilisation at 5°C) were sampled for determination of fertilisation rate by examination under a dissecting microscope (16x). 200 eggs were counted and classified as fertilised, unfertilised or dead. Fertilised eggs were defined by the presence of dividing cells. Unfertilised eggs were either completely clear without inclusions or clear with only the germinal disk evident. Dead eggs were characterised by the presence of opaque spots of coagulated yolk. After sampling the eggs, buckets were slowly filled up with 8L of UV treated seawater and 250 ml of saturated salt solution to maintain viable eggs in the water column. Buckets were kept overnight in a dark cold room. Only eggs with fertilisation rates higher than 50% were placed in the conical tank (see egg incubation Chapter 71.1.1) for incubation.

2.7 Statistical analysis

Female fecundity, fertilisation rates and milt spermatocrit were pooled for statistical analyses using SPSS for windows version 11.5 (Apache software foundation). Data were tested for normality using the method of Kolmogorov and Smirnov. When normality was established, data were analysed by ANOVA and the post-test means comparison was performed by LSD (Puri, 1996). The significance level was set at $p < 0.05$. All results are given as mean ± standard deviation (S.D.)

3 Results

3.1 Spawning seasons

During the experiment, May spawner broodstock under artificial photoperiod started to show signs of maturity in February, just after the shortest day length period. Over the three spawning seasons (Figure 19) individual halibut females started to spawn at the same date and over a similar period. From May to July, the number of spawning females was highest. Through the whole experiment female broodstock fed the 0.6% ARA diet had almost a two week delay in spawning season onset compared to females fed the other trial diets. The temperature curves followed the photoperiod curves and were kept under 8°C during each spawning season.

July spawners in 2001 started to show signs of maturity in February as for May spawners, corresponding to the longest day length under their artificial photoperiod (Figure 20). In 2002 females started to mature in May, which is closer to the shortest day length. The photoperiod was modified in 2003 and females started to mature in June and started to spawn in August. The temperature curve did not follow the photoperiod curve. Water temperatures before the spawning season were high being 10°C in 2002 and 13°C in 2003 and then decreased rapidly to spawning temperature.

MAY HALIBUT SPAWNERS
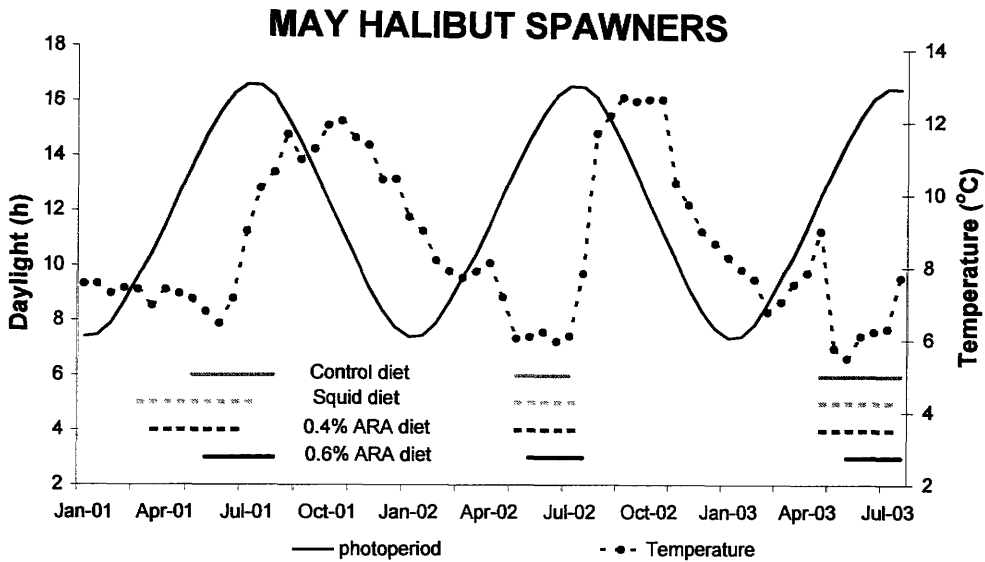
Figure 19: Halibut broodstock May spawners actual spawning seasons according to diet, over the three year trial in relation to photoperiod and temperature control. Horizontal bar represent the spawning period.

JULY HALIBUT SPAWNERS
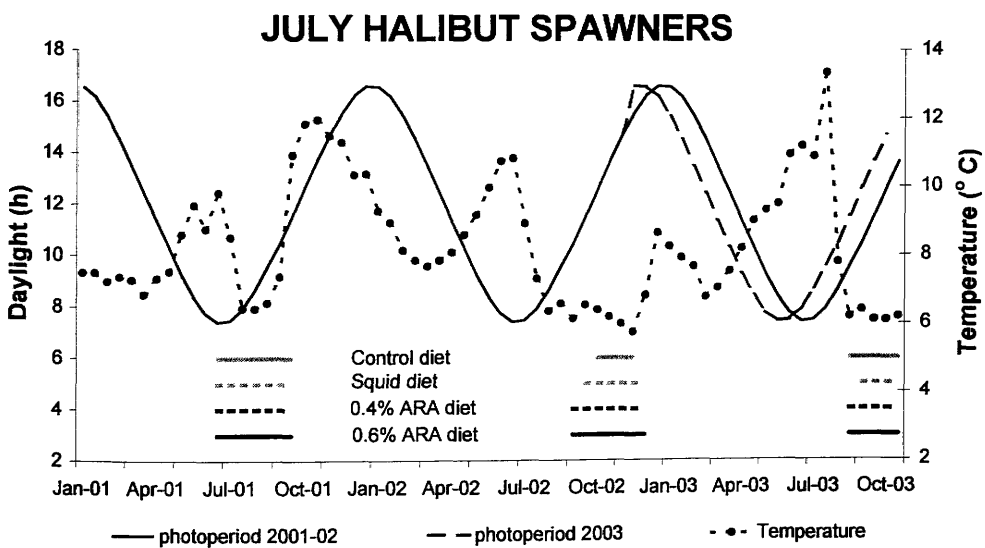
Figure 20: Halibut broodstock July spawner actual spawning season according to their diet over the three year trial in relation with photoperiod and temperature controlled. Horizontal bar represent the spawning period.

3.2 Halibut female production and fecundity

In 2001 diet had no effect on overall results for female halibut. Halibut May spawners showed a higher number of mature females than July spawners (Table 14) through the three spawning seasons. The number of July spawning mature females was the lowest in 2003. Halibut May spawners produced nearly twice as many eggs as the July spawners with respectively 537L and 277L over the three-year trial.

Table 14. Number of halibut broodstock females spawning in different tanks fed the different experimental diets (15 females per tank).

	May spawner			July spawner		
	2001	2002	2003	2001	2002	2003
Control diet	13	12	14	6	10	3
Squid diet	12	10	11	9	9	3
0.4% ARA diet	12	12	12	10	9	4
0.6% ARA diet	11	10	10	9	6	7

In 2002 and 2003 female May spawners fed the 0.4% ARA diet had higher egg production (Figure 21) than females fed the other experimental diets. Female May spawners fed the 0.6% ARA diet showed the lowest egg production. Female July spawner egg production (Figure 22) was the lowest in 2003 except for females fed the 0.6% ARA diet.

MAY SPAWNERS EGG PRODUCTION
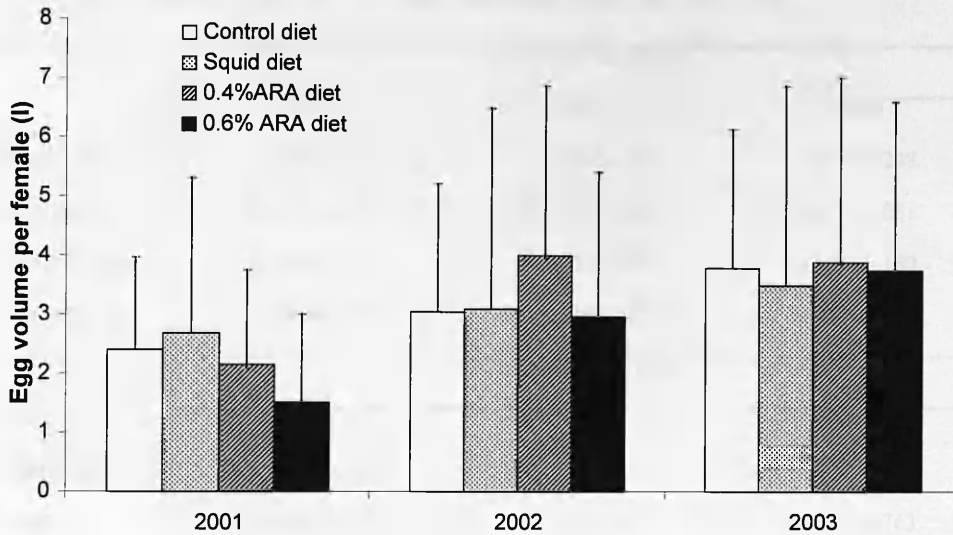
Figure 21: Halibut broodstock female May spawners egg production (mean ± S.D., n=15) according to diet over the three year trial. No significant difference observed between each treatment.

JULY SPAWNER EGG PRODUCTION
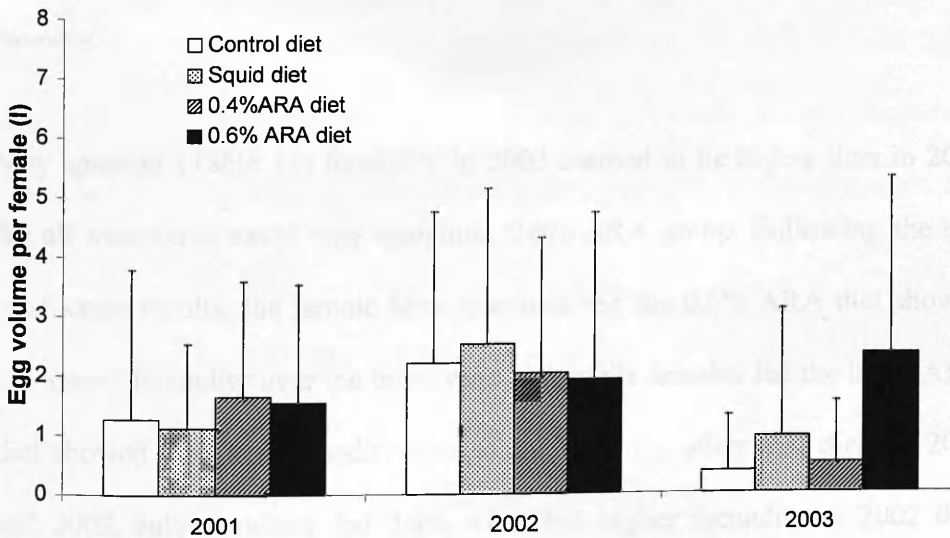
Figure 22: Halibut broodstock female July spawners egg production (mean ± S.D., n=15) according to diet over the three year trial. No significant difference observed between each treatment.

Table 15: Halibut female relative fecundity (eggs / kg, mean ± SD) over the three year trial according to diet. No significant diet effects were observed.

	MAY SPAWNERS		
	2001	2002	2003
Control diet	11,289±5,087	13,622±6,383	11,874±6,248
Squid diet	10,895±6,893	12,597±8,000	12,174±8,051
0.4% ARA diet	9,147±5.322	14,380±3,895	13,090±8,180
0.6% ARA diet	6,427±4,087	10,653±5,301	11,451±4,493
	JULY SPAWNERS		
	2001	2002	2003
Control diet	8,120±7,096	9,150±6,423	4,984±4,277
Squid diet	5,984±4,059	12,393±6,585	14,594±9,763
0.4% ARA diet	8,652±6,472	10,337±6,037	7,227±2,098
0.6% ARA diet	8,141±7,189	14,235±7,388	13,667±6,562

Relative fecundity was calculated only with females producing eggs. Due to high individual variation in female production, there were no significant diet effects on halibut female fecundity.

May spawner (Table 15) fecundity in 2002 seemed to be higher than in 2003 for all treatments exept may spawning 0.6% ARA group. Following the egg production results, the female May spawners fed the 0.6% ARA diet showed the lowest fecundity over the three year trial, while females fed the 0.4% ARA diet showed a higher fecundity than females fed the other trial diets in 2002 and 2003. July spawners fed 0.6% ARA had higher fecundity in 2002 than females fed the other trial diets. In 2003, fecundity was highest in female July spawners fed squid.

3.3 Halibut egg fertilisation rates

Table 16. percentage of egg batches with fertilisation rates higher than 10% over the three year trial according to diet

	May spawner			*July spawner*		
	2001	2002	2003	2001	2002	2003
Control diet	35%	52%	73%	0%	32%	46%
Squid diet	39%	47%	70%	0%	61%	60%
0.4% ARA diet	42%	61%	71%	0%	48%	43%
0.6% ARA diet	31%	38%	49%	0%	57%	49%

Over the experiment only batches of eggs with fertilisation rates higher than 10% were assessed (Table 16). For May spawners only females fed the 0.6% ARA diet showed significant differences in fertilisation rates (Figure 23) between years 2001 and 2003. In 2002 the fertilisation rates of females fed the 0.4% ARA diet were significantly higher than of females fed the 0.6% ARA diet. There were no significant diet effects on May spawner fertilisation rates in 2001. No significant differences were observed between control and squid and the ARA enriched diets.

As far as the July spawners are concerned, in 2001 all egg batches harvested displayed fertilisation rates below 10% and were therefore not assessed. In 2002 and 2003 diet did not have significant effect on July spawner fertilisation rates. However, in 2002, as for the May spawners, females fed the 0.4% ARA diet seemed to have a higher fertilisation rate than females fed the other trial diets.

MAY HALIBUT SPAWNERS
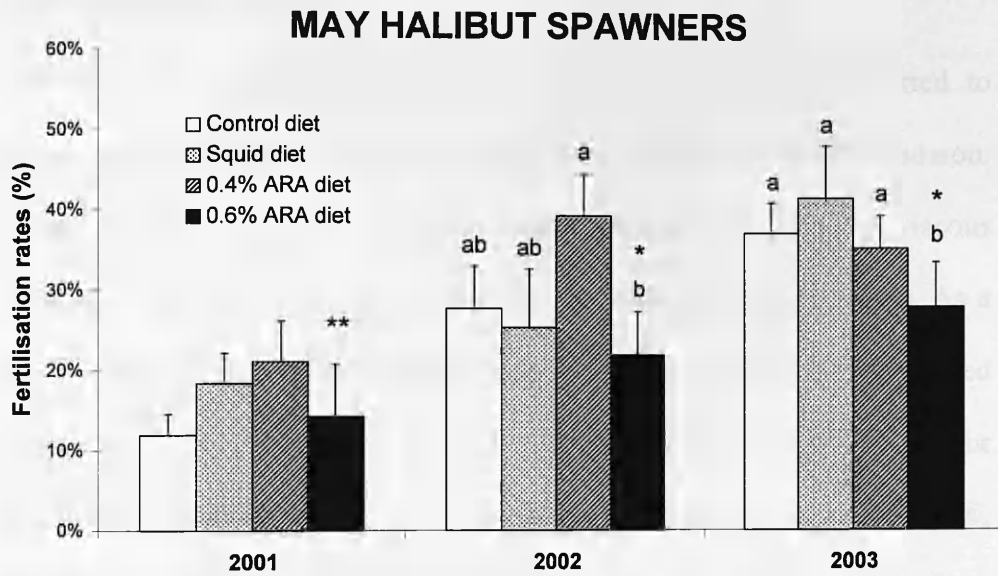
Figure 23. Halibut female fertilisation rate (mean ± S.D.), according to diet, over the three-year trial. Superscript denotes significant difference between years (*) and treatement at each year (letter).

JULY HALIBUT SPAWNERS
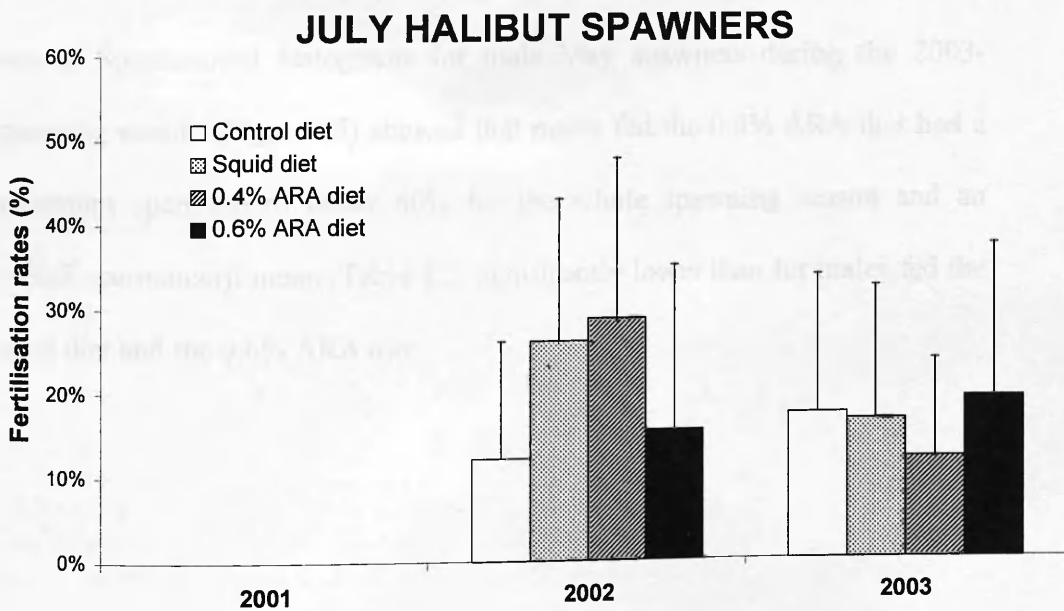
Figure 24. Halibut female fertilisation rate (mean ± S.D.), according to diet, over the three-year trial.

3.4 Milt production

Over all three project spawning-seasons, Atlantic halibut males started to produce milt one-month prior to the beginning of the female spawning season. At the end of the reproductive season, males were producing milt so viscous that it was very difficult to collect the volumes required to fertilize eggs. As a direct result, in 2001 males stopped producing milt before females stopped spawning. In 2002 males were injected with Ovaplant® and supplied milt for the full May spawning season. In 2002, males were implanted with Ovaplant®, therefore the diet effect on halibut spermatocrit was only studied in 2003. Eggs produced by July spawners were of such poor quality and of so limited numbers that a decision was made not to attempt fertilisation.

In 2003 males fed the 0.4% ARA diet produced milt for the full May spawning season. Spermatocrit histograms for male May spawners during the 2003-spawning season (Figure 25) showed that males fed the 0.4% ARA diet had a maximum spermatocrit under 60% for the whole spawning season and an overall spermatocrit mean (Table 17) significantly lower than for males fed the squid diet and the 0.6% ARA diet.
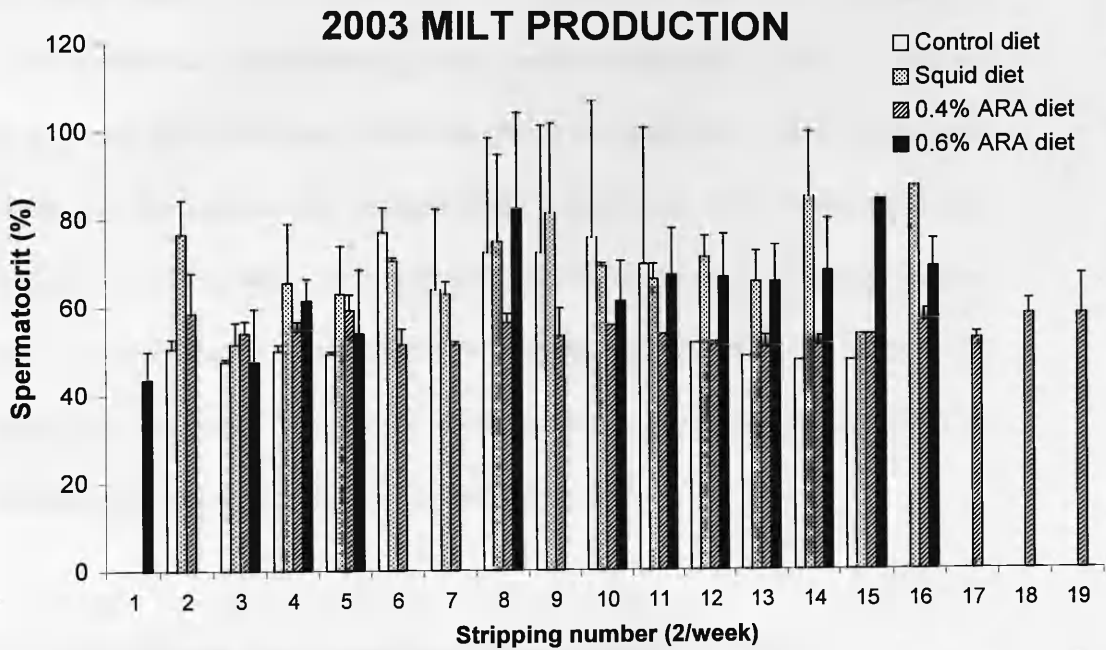
Figure 25. May halibut male spawners spermatocrit according to diet during the 2003 spawning season. Values are expressed as mean ± S.D. (n=2)

Table 17. Mean ± SD (n=40), halibut male spermatocrit in 2003

Diets	Spermatocrit (%)
Control diet	60±12 [bc]
Squid diet	71±11 [a]
0.4% ARA diet	55±3 [c]
0.6% ARA diet	66±11 [ab]

Means with different superscripts are significantly different (p<0.05).

In contrast males fed the squid and 0.6% ARA diets had higher spermatocrit closer to the end of the spawning season with a maximum of 88% and stopped to give milt three strippings before the end of the spawning season. In addition, males fed the control diet reached high spermatocrit early in the spawning season. Therefore, when collecting milt later in the season, stripping pressure as to be such that it was impossible to avoid contamination by urine when sampling the milt. This urine contamination explains the decrease in spermatocrit toward the end of the spawning season .

3.5 Ovaplant® effect on halibut milt production

There were no significant differences between spermatocrits of control and implanted fish before injection (Figure 26). After six strippings spermatocrits in all experimental tanks were higher than 60%. Subsequently males implanted with Ovaplant® produced milt with a lower spermatocrit, milt was very watery at first. Implanted males produced milt until the end of the spawning season while control males stopped producing milt six strippings (18 days) before implanted males.

SPERMATOCRIT IN MALE MAY SPAWNERS IN 2002
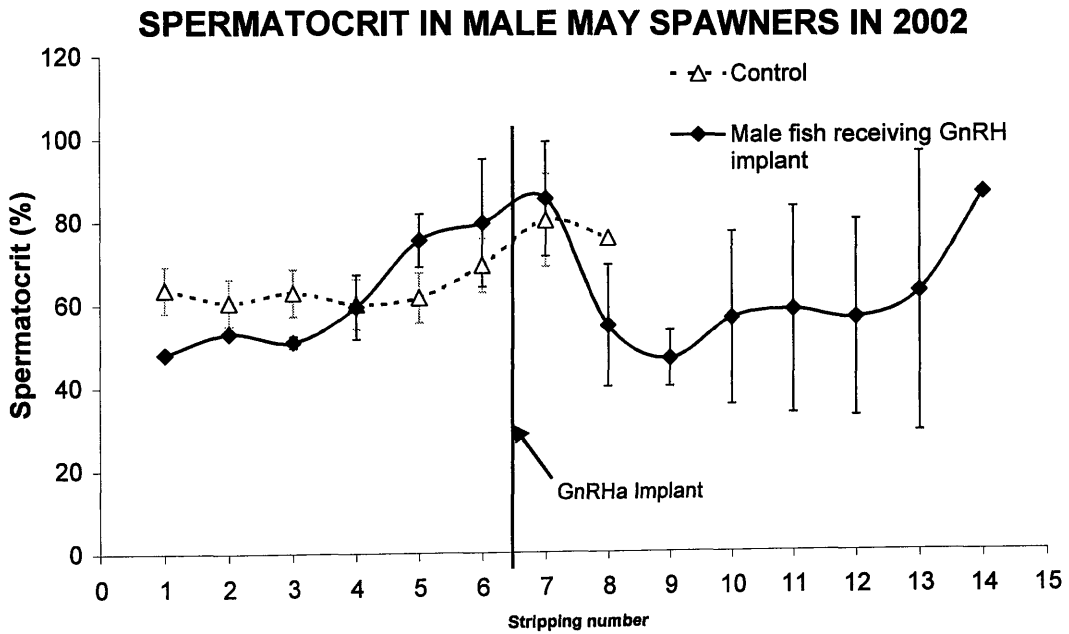
Figure 26. Halibut spermatocrit for fish either implanted with GnRH or not over the spawning season (mean ± S.D.). No significant differences were observed between treatments until stripping number 7

4 Discussion

4.1 Photoperiod & temperature

Table 18 shows results of halibut annual photoperiod cycle being gradually advanced by four months in an advanced group, and gradually delayed by four months in a delayed group. Spawning period was recorded from first to last appearance of eggs (Björnsson *et al.*, 1998).

Table 18: Norwegian farmed Atlantic halibut broodstock photoperiod manipulation (Björnsson *et al.*, 1998)

	First spawning season	*Second spawning season*	*Third spawning season*	*Fourth spawning season*
Control group	12 Mar to 6 Jun	28 Feb to 25 May	24 Feb to 24 May	22 Feb to 14 May
Advanced group	12 Mar to 25 May	7 Dec to 18 Feb	9 Nov to 23 Jan	22 Nov to 25 Jan
Delayed group	30 Mar to 6 Jun	27 Jun to 13 Aug	13 Jul to 21 Sep	15 Jul to 5 Oct

The manipulation of the annual photoperiod cycle had some clear consequences for female spawning activity. Annual changes in photoperiod are a major environmental cue for timing of spawning in Atlantic halibut. Delaying spawning in halibut by photoperiod manipulation is not as disruptive as rapidly advancing it. The disruption of spawning caused by the advanced photoperiod is likely to be due to physical constraints of sexual maturation, where adequate energy reserves need to be accumulated for rapid gonadal growth. As was

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shown by Björnsson *et al.* (1998), a minimum time of nine months was required for fully successful gonadal development between spawning seasons. Under the conditions of the present study, female May spawners showed a positive adaptation to artificial photoperiod. Spawning season windows were such that it was possible to define the starting spawning day for each individual female within two or three days. However, female May spawners fed the 0.6% ARA diet were almost two weeks late during the three spawning seasons when compared to fish fed the other trial diets. Female July spawners did not properly adapt to artificial photoperiod such that through the three years of the experiment none of the females spawned in the same period. This effect could be due mainly to the low food intake or the duration of the inter-spawning interval in 2001. In three-spined sticklebacks, low ration resulted in an increase in the number of days until the next spawning (Ali & Wotton, 1999a). In Baltic herring, the timing of individual maturation cycles is primarily determined by feeding conditions prior to spawning (Rajasilta, 1992). After 6 months of feeding seabass broodstock with a half food ration growth rates decreased, spawning time was delayed and eggs, as well as newly hatched larvae were smaller than those obtained from fish fed full rations (Cerdá *et al.,* 1994). Another explanation of the poor reproductive performance of female July spawner and non-adaptation to photoperiod could be the temperature pattern. Temperatures experienced during the breeding season affected timing of ovulation and egg quality of common wolfish (Tveiten *et al.,* 2001).

4.2 Female volume and fecundity

Broodstock nutrition affects fish egg quality (Bromage & Roberts, 1995). More specifically n-3 HUFA are one of the most important dietary nutrients for marine species broodstock, since they require n-3 HUFA as EFA (Bruce et al., 1999; Furuita et al., 2000, 2002). Recently attention has also been paid to n-6 fatty acids, especially ARA. Significant improvement in egg quality has been seen in cultured European seabass broodstock fed increased levels of ARA (two fold) in pelleted dry feeds (Bell et al., 1997: Bruce et al., 1999). A significant improvement in fertilisation rates was also observed in Atlantic halibut broodstock fed diets containing 2% of total fatty acids as ARA compared to those containing 0.5% or 1.0% ARA (Bromage et al., 2001, Mazorra et al., 2003). Japanese flounder total egg production over the spawning season was highest in fish fed a 0.6% ARA diet, although reproduction seemed to be negatively affected by a high dose of ARA (1.2%) (Furuita et al., 2003).

In the present study halibut broodstock fed the 0.4% ARA diet produced a higher volume of eggs in 2002 and showed a higher fecundity than broodstock fed other experimental diets. However, when fish were fed a 0.6% ARA diet a generally negative effect on egg production was observed as observed in Japanese flounder (Furuita et al., 2003). It appears that a 0.6% dietary ARA could be a too high level in terms of halibut ARA requirement. The lower egg

production from female July spawners in 2001 was probably due to handling and moving stress. In fact,63% of females in May 2001 showed wounds on their non-pigmented side after being moved from 13m tanks to smaller experimental tanks (5m). They were probably moved to the trial tanks at a bad time just when fish were reducing feed intake before maturation resulting in a longer starvation period (nearly 6 months). Food restriction itself can seriously affect spawning success. Female halibut with suppressed food intake due to physical distress, such as small holding tanks or parasites, have been found to reabsorb gonads and skip a spawning season (Haug, 1990). A reduction in feeding rate has been reported to cause inhibition of gonadal maturation in several fish species (Horwood et al., 1989). Exposure of juvenile turbot to low rations during vitellogenesis, covering the 4 months immediately prior to spawning, led to a drop of 70% in mean ovary weight, and was associated with poor growth of the vitellogenic oocytes or, in a third of cases, the absence of vitellogenic oocytes (Bromley et al., 2000). Food level can also significantly affect fecundity in plaice. The lack of granular oocytes was not due to atresia, but to an early decision not to proceed with gonad development (Horwood et al., 1989). Other species, such as female three-spined sticklebacks, are able to adjust their food intake to compensate for short periods of food deprivation allowing them to maintain their reproductive performance at first spawning (Ali & Wotton, 1999b). The chorion appearance (such as wrinkled egg membranes) and egg shape are also seen to deviate in poor quality eggs

(Bromage & Roberts, 1995). In 2001 most females showed signs of egg resorbtion characterised by increased production of either wrinkled or dead eggs.   -

4.3 Fertilisation rates

Atlantic halibut aquaculture has been hampered by a failure to obtain consistently high fertilisation rates (Bromage & Roberts, 1995). In most egg quality investigations, fertilisation rates have been used as important criteria. Survival to specific developmental stages and final production of fry have also been used as measures of egg quality (Trippel et al., 1999). In sea bass hatching rates were significantly correlated in two consecutive years indicating that reproductive performance may be repeatable. Most of the parental effects on early performance in sea bass are derived from the female (Saillant et al., 2001). Improved fertilisation was obtained in sea bass fed artificial diets containing fish oil (high in EFA), in comparison to those of fish fed artificial diets containing maize oil (low in EFA) (Bell et al., 1997). In the present study the main objective was to optimise the EFA levels, particularly ARA, which were available to the developing eggs and yolk sac larvae. In 2002 female May and July spawners showed a higher fertilisation rate when fed the 0.4% ARA diet although female May spawners had a significantly lower fertilisation rate when fed the 0.6% ARA diet.

Variable results in 2003 were mostly explained by individual halibut female variation in reproductive performance. For unknown reasons some females

spawned in 2002 but did not in 2003. Even if females fed the 0.4% ARA diet showed improvement in reproductive performance assessed through fecundity, volume of egg and fertilisation rates, no clear pattern of ARA effects on halibut females emerged during the study, although several hypotheses can be made. 0.4% ARA could advance the optimal level of halibut production and young females spawning for the first time could immediately give good quality eggs. Better results for July spawners fed 0.6% ARA in 2003 could be due to an improvement in the ability of fish to resist stress caused by the artificial photoperiod and unstable temperature pattern. To conclude, ARA should be used as a reproduction enhancer at a levels defined by species-specific requirements and added to the diet intermittently.

4.4 Male spermatocrit

The highly significant relationship found between spermatocrit and sperm density in Atlantic halibut by Tvedt *et al.*, 2001 and in non-flatfish teleost species (Cieresko & Dabrowski, 1993) allows in the present study the use of spermatocrit as a simple and rapid estimator of sperm density. During the experiment it was noted that male milt with 50% spermatocrit was easier to strip than milt with a spermatocrit >60% (thick milt). The former was also easier to mix with seawater during artificial fertilisation. Low spermatocrit (<40%) milt was watery and low sperm density was of concern. In 2001 artificial fertilisation at the end of the spawning season was difficult because

males ran out of milt before females stopped spawning. In 2002 males were implanted with hormone and produced milt through the whole spawning season but milt produced just after Ovaplant® implantation had a lower spermatocrit (<40%). Even though seawater activation produced milt that was 100% motile, sperm density was of concern.

In 2003 there was no need for hormonal implants as males fed the 0.4% ARA diet showed an extended milt production period with spermatocrit values in the range 50 ± 5%. European sea bass males fed PUFA-enriched diets with a lower concentration of EPA and a lower *n-3: n-6* ratio, exhibited a longer spermiation period and higher milt volume and density as compared to males fed trash fish (Asturiano *et al.,* 2001). In 2003 males fed the 0.4% ARA diet gave milt within the 50% spermatocrit range and until the end of the spawning season. In contrast males fed squid or and 0.6% ARA had spermatocrits >60% early in the spawning season and stopped giving milt 2 weeks before the end of the spawning season. Males fed the control diet presented spermatocrit >60% from the beginning of the spawning season; consequently it was impossible to collect non-urine-contaminated milt during stripping. As a direct consequence a lower spermatocrit value was obtained and, consequently, milt quality was compromised.

4.5 Male hormone injection

GnRH-A has been used to stimulate the reproductive system of male and female winter yellowtail flounder (Harmin & Crim, 1989; Harmin *et al.,* 1995)

and induced ovulation of high quality eggs in this cold-water batch spawning teleost (Larsson *et al.*, 1997). By injecting Ovaplant® as a single implant dose 3 -4 weeks prior to normal spawning, spawning of fish has been advanced and synchronized (Powell *et al.*, 1998; Martin-Robichaud *et al.*, 2001). Ovaplant® uses a safe controlled release compound and has been tested in Atlantic salmon and other fish species (Powell *et al.*, 1998). In Atlantic halibut the duration of good quality, expressible, milt availability was successfully extended within 7 days post-implant compared to control fish that showed signs of "drying up". It has been shown that levels of androgens fall following GnRHa treatment while levels of progestogens rise briefly with a significant increase in milt fluidity (Vermeirssen *et al.*, 2000). As observed in the present study, treated male halibut broodstock reliably produced copious quantities of sperm with spermatocrit levels similar to values observed at the beginning of the season (Martin-Robichaud *et al.*, 2001).

Chapter 5 Atlantic halibut milt biochemical quality

1 Introduction

Knowledge about the biology of freshwater fish sperm is important, especially its quality and cryopreservation potential for domesticated species such as trout, salmon or carp (Leray & Pelletier, 1985; Billard *et al.,* 1995). However, milt from marine species and the relationship between dietary lipid and sperm quality has received little attention although this might equally be an important factor for fertilised egg viability. Little is known about the physiological mechanisms involved in regulation within the fish testis. At the cellular level PUFAs and their cyclooxygenase and lipoxygenase metabolites can have different modulatory effects on gonadal metabolism of steroids (Asturiano *et al., 2000*).

1.1 Importance of lipid in fish milt

The fact that sperm of one species of fish exists under hypertonic environmental conditions, while the sperm of other species exists under hypotonic conditions, illustrates the adaptive abilities of organisms and their cells, developed in the course of evolution (Drokin, 1993). The ability of fish sperm to be motile in water is largely determined by membrane properties. .

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One of the most important structural elements involved in regulation of these properties is the phospholipid and fatty acid composition (Drokin, 1993).

1.1.1 Lipid class

The majority of lipid in vertebrate sperm cells is polar lipid mainly phosphoglyceride present in the plasma membrane whereas there is very little neutral lipid (Poulos et al., 1975). It is known that PC is an important component of all animal cell membranes. Peculiarities in the phospholipid composition of sperm of marine and freshwater fish are related, apparently, to the permeability of their membranes. Phospholipids can act as ionophores in the cell (Poulos et al., 1975; Drokin, 1993). PS is a phospholipid which disorders or fluidises membranes while PI actively participates in regulation of cell function. They are important for the activation of membrane enzymes, motility and performance of sperm activation reactions. As suggested by Drokin (1993), SM is the most saturated phospholipid and an increase in content would make the sperm membrane more rigid. LysoPC affects membrane integrity of spermatozoa (with the exception of acrosomal reaction) and that is why its presence is normally unfavourable and regarded as pathological. Lipid compositions of different fish sperm showed that molar ratios of cholesterol to phospholipids are determined by habitat salinity (Drokin, 1993). At another physiological level, membrane phase behaviour may be important in controlling fusion of the sperm plasma membrane with the egg. PE, a type of phospholipid with a small head group, and PS, an anionic

PUFA-rich phospholipid, form unstable bilayers reverting to reverse hexagonal phase. Such lipids are fusogenic and could therefore be important in promoting fusion of the sperm plasma membrane with the egg (Brown, 1994).

1.1.2 Fatty acid composition of fish milt

The fatty acid composition of fish tissue reflects dietary lipid to a great extent and it is relatively easy to manipulate the fatty acid composition of key tissues such as neural and reproductive tissue by dietary input (Bell *et al.,* 1996). Polyunsaturated fatty acids (PUFAs) of the *n-3* series, particularly EPA and DHA, influence gonadal hormone levels and fecundity in the European sea bass (Asturiano *et al.,* 2000). Only a few studies describe the influence of *n-6* series PUFAs (i.e. AA: 20:4n-6) on teleost reproduction. Male eels with induced spermiation show an increase of monounsaturated fatty acids whereas EPA, DHA and PUFAs were significantly reduced (Pérez *et al.,* 2000).

1.1.3 Arachidonic acid

Arachidonic acid (ARA) is released from membrane phospholipids in response to hormone stimulation in a great number of tissues (Knight *et al.,* 1995). Once released it can either act as a second messenger in activating protein kinase C or can be converted mainly to eicosanoids. These compounds although relatively labile are formed in a wide range of tissues. Metabolites of arachidonic acid form a seemingly endless array of biological messengers (Knight *et al.,* 1995).

1.2 Eicosanoids

1.2.1 Reproduction priming pheromones

During spawning a number of fish species are known to synchronize reproduction by the release and detection of pheromones (Stacey & Goetz 1982; Stacey *et al.*, 1994). One group of compounds that is considered to have such a function are the F-series prostaglandins (PGFs) (Sorensen *et al.*, 1995). It is becoming increasingly evident that prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$), functions as a blood-borne signal in many species of fish synchronizing ovulation with the expression of female sexual behavior and then acting as a precursor for sex pheromone production (Sorensen *et al.*, 1995). In goldfish, PGFs function as potent olfactory stimulants that induce typical male sexual behaviour (Sorensen *et al.*, 1988 & 1989). PGFs have been shown to function as reproductive pheromones in Atlantic salmon (Waring & Moore, 1995). Nevertheless, PGFs have not yet been definitely identified using biochemical means in the gonad, blood, or holding water of any fish. This is of particular concern because these compounds, which are traditionally thought to have autocrine and paracrine activity, are notoriously diverse and labile, and binding antibodies are often non-specific. PGF$_{2\alpha}$ and its metabolite 15-ketoprostaglandin $F_{2\alpha}$ (15K- PGF$_{2\alpha}$) are the most potent forms of PGF (Sorensen *et al.*, 1988). Most studies on the function of PGFs in fish reproduction have focused on their role in the female. Thus, PGF$_{2\alpha}$ plays a role in stimulating ovulation and eliciting female sexual

behaviour and in addition the breakdown products of $PGF_{2\alpha}$ have sex pheromone activity (Stacey & Goetz 1982; Sorensen & Goetz, 1993).

1.2.2 Spermatogenesis and stimulation of testosterone production

The reproductive cycle of male Atlantic halibut is characterized by distinct seasonal variations in absolute and relative testicular size in relation to developmental stage and in plasma levels of sex steroids such as testosterone (T) and 11-Ktestosterone (11-KT) associated with different phases of reproductive activity (Weltzien et al., 2002). 11-KT and T are considered as the most important androgens in control of teleost reproduction. In Atlantic halibut, 11-KT occurred in higher concentrations than T during all stages of testicular development, generally with levels at least four-fold higher than those for T. In addition to their role in stimulating spermatogenesis, 11-oxygenated androgens, and particularly 11-KT, are important stimulators for reproductive behaviour and secondary sexual characteristics (Weltzien et al., 2002). Eicosanoids may play a role in the regulation of testicular steroidogenesis in fish as ARA acts in a time and dose-related manner to stimulate T production. The steroidogenic actions of ARA were shown to be dependent on metabolism of cycloxygenase products as cycloxygenase inhibitors strongly attenuate this effect (Wade & Van Der Kraak, 1993). Several cycloxygenase metabolites, most notably PGE_1 and PGE_2, cause a marked stimulation of T production. The relative potency of various PGs is in the sequence $PGE2 = PGE_1 >> PGI_2 > PGF_{2\alpha}$. Prostaglandins exert their actions

by binding to cell surface receptors linked to G-proteins which, in many cases, are of the Gs type, which activate adenylate cyclase. Eicosanoids may act as intratesticular modulators of testicular function. The stimulatory effects of ARA and PGE_2 are mediated, at least in part, via the production of cAMP. Although ARA also stimulated T production in goldfish ovarian follicles it appears that the mechanisms of action in this tissue differs from those seen in the testis (Wade & Van Der Kraak, 1993). Prostaglandins, being labile in circulation, exert their action locally. To influence testicular steroidogenesis PGs must be synthesized within the testis and studying PGs concentration in fish sperm could increase knowledge of the physiological process. There was a clear need for further work on the involvement of PGs in male reproductive function in halibut and to determine if there are functional consequences in altering 20:4n-6 levels in halibut broodstock male diets on milt lipid class, fatty acid profile and $PGF_{2\alpha}$ production.

1.3 Overall aims

➢ To find a relationship between halibut milt quality and lipid content.

➢ To study the effect of ARA enriched diets on halibut milt total lipid, lipid composition and fatty acid profile.

➢ To study the effect of ARA enriched diets on halibut milt $PGF_{2\alpha}$ concentration.

2 Materials and Methods

2.1 Halibut milt sampling

After artificial fertilisation, the remaining milt was moved from the egg room to the fishfarm laboratory where spermatocrit and sampling were carried out. Each individual stripping of halibut male milt collected was individually sampled in a 2 ml plastic vial, labelled with fish identity, tank number and sampling date, and kept on site in a freezer. At the end of the spawning season, frozen milt samples were brought back to the Stirling University Nutrition Laboratory for biochemical analyses. Over the three-year trial more than 300 samples were collected and the selection of samples for biochemical analyses is shown in Table 19. In 2001 samples from each trial diet were randomly selected to perform lipid analyses.

Table 19. Milt samples analyse over the three-year trial.

	2001	2002	2003
Total lipid extraction	16	12	20
Lipid class analyses	-	12	20
Fatty acid analyses	16	40	48
$PGF_{2\alpha}$	-	12	20

In 2002 samples of control and implanted males from each trial diet were randomly selected for lipid analyses and samples of control and implanted males from each trial diet were randomly selected for milt determination of $PGF_{2\alpha}$ concentration. In 2003 milt samples from each trial diet were randomly selected and split in two, half for lipid analyses and half was used for determination of milt $PGF_{2\alpha}$ concentration.

2.2 Biochemical analyses

It was more convenient to move frozen milt into glass tubes in order to weigh the sample. 18 ml of chloroform-methanol 2:1 (v/v) was added to each sample, and then Folch extraction, lipid class analyses and fatty acid analyses were carried out as described in Chapter 2, sections 2.3, 2.4 and 2.5 respectively. Eicosanoid extraction and $PGF_{2\alpha}$ immunoassay was carried out as described in chapter 2, sections 2.6.1 and 2.6.2 respectively. In order to express $PGF_{2\alpha}$ concentration per mg of protein, 200 µl of each sample just to prior eicosanoid extraction were processed as indicated in chapter 2 section 2.1 for Lowry protein measurement.

2.3 Statistical analyses

Halibut milt total lipid, lipid class and fatty acid composition were pooled for statistical analyses using SPSS for windows version 11.5 (Puri, 1996). Data were tested for normality using the method of Kolmogorov and Smirnov. Data were then analysed by ANOVA and means comparison performed by *post-hoc*

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Tukey when comparing diet effect and the three-year trial data. Data were

analysed by Leven's test for equality of variance and variances T-test when

comparing 2002 and 2003 data. All results are given as mean ± standard

deviation (S.D.). Total lipid and spermatocrit relationships were analysed for

2002 and 2003 separately using linear regression and ANOVA. Relationships

between ARA and PI in milt were obtained from the quadratic model curve

estimation and validated by ANOVA ($p<0.05$): PI= aARA^2+bARA+c, where

a, b and c are constants estimated by non-linear regression. The significance

level was set as $p<0.05$

3 Results

3.1 Total lipid content

There were no significant diet effects on halibut milt total lipid (Table 20) over the three-year trial. In 2001 total lipid was significantly lower in males fed the control diet when compared to 2002 and 2003. Mean percentage of total lipid in halibut milt was 1.24±0.28%. Halibut milt total lipid was significantly correlated with spermatocrit (Figure 27). The regression value for 2002 (r^2=0.57, n=12) was similar to 2003 (r^2=0.59, n=20) and both were significant at p<0.05.

Table 20. Percentage total lipid in milt from males fed the trial diets through the three years of the project.

	2001	2002	2003
Control diet	1.04±0.01% [a]	1.19±0.23% [b]	1.28±0.21% [b]
Squid diet	0.97±0.06%	1.50±0.45%	1.27±0.15%
0.4% ARA diet	0.97±0.05%	1.56±0.70%	1.10±0.11%
0.6% ARA diet	1.53±0.09%	1.24±0.04%	1.21±0.08%

Values are mean ± S.D., n=2 in 2001, n=3 in 2002, n=5 in 2003 in trial group. Means with different superscripts within the same row are significantly different (p<0.05)
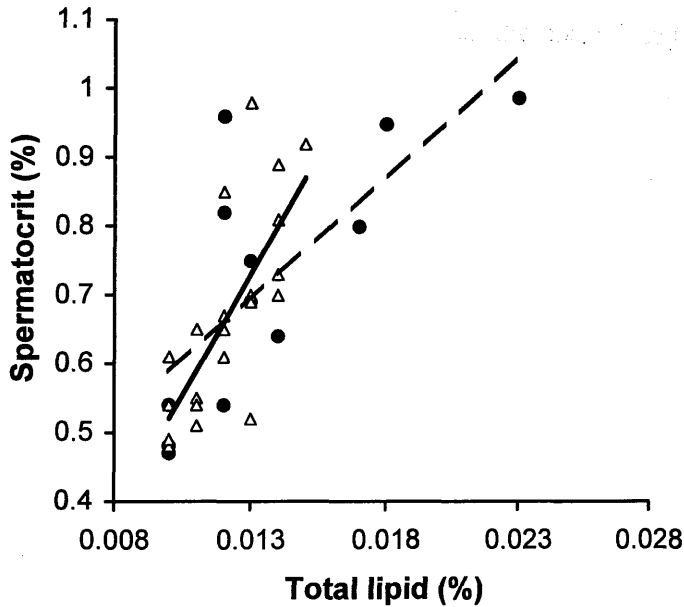
Figure 27. Relationship between total lipid and spermatocrit in halibut male milt in 2002 (●) and in 2003 (Δ). The curves were fitted by linear regression ($p<0.05$). 2002 (—), $y=35.68x+0.23$ ($r^2=0.57$) and 2003 (---), $y=70.147x-0.23$ ($r^2=0.59$) were obtained.

3.2 Lipid class

Nine lipid classes were identified (Figure 28) in the sperm of Atlantic halibut.

In 2002 a significant diet effect on milt lipid class (Figure 29) was observed for

PE and CL+PG. PE was higher in males fed the control diet than in males fed

the 0.6% ARA diet.
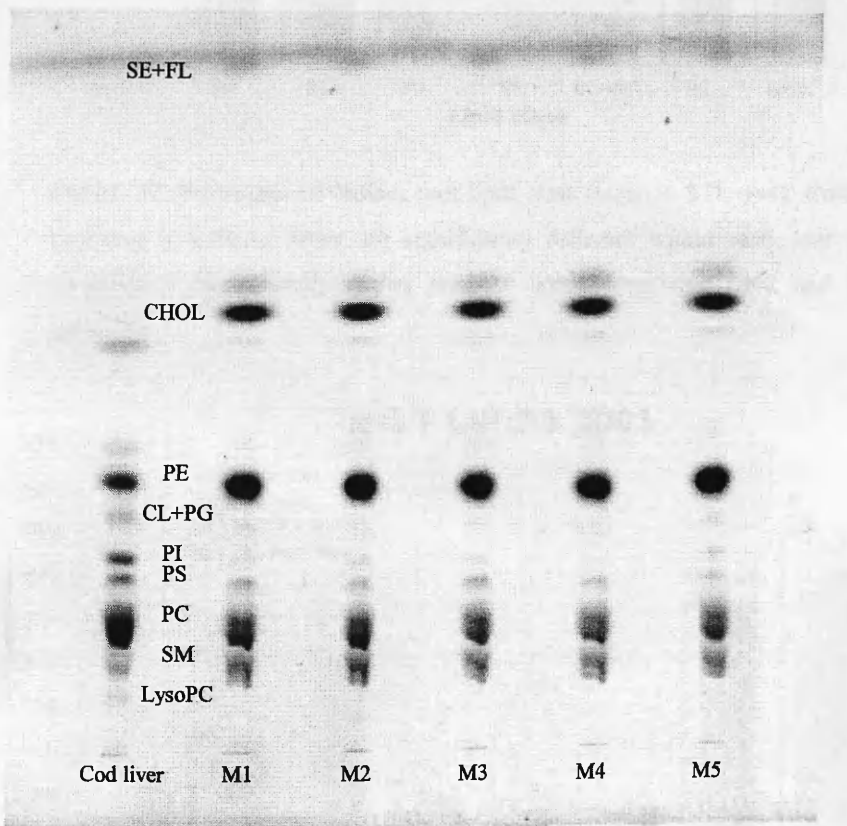
Figure 28. Halibut male milt (M: individual sample) and cod liver total lipid HPTLC separation. Nine lipid classes were identified: LysoPC: Lysophosphatidylcholine. SM: Sphingomyelin. PC: Phosphatidylcholine. PS: Phosphatidylserine. PI: Phosphatidylinositol. CL+PG: Cardiolipid + Phosphoglycerol. PE: Phosphatidylethanolamine. Chol: Cholesterol. SE+FL: Sterol+ solvent front line.
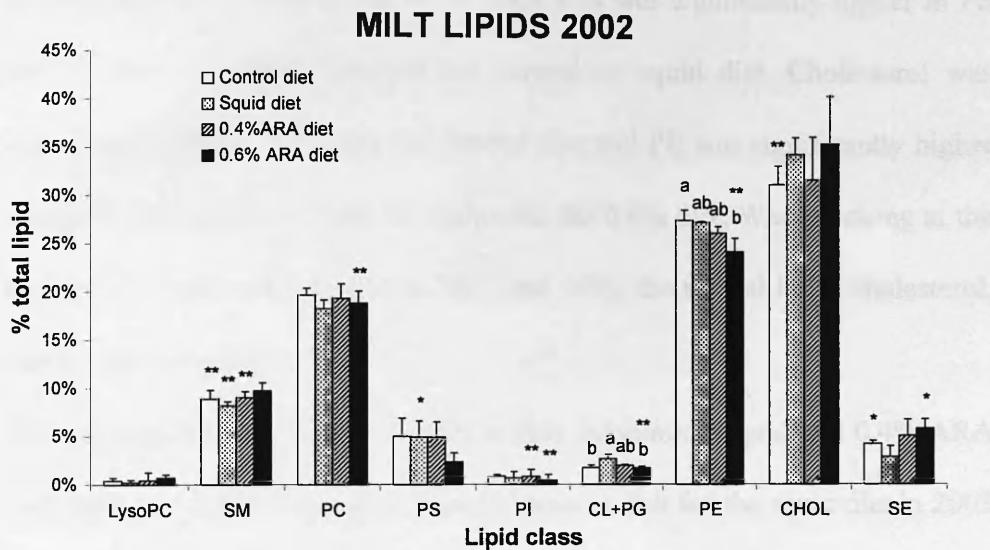
MILT LIPIDS 2002

Figure 29. Percentage of halibut milt lipid class (mean ± S.D. *n*=4). Columns assigned a different letter are significantly different within each year class (*p*<0.05). * Significantly higher than ** when comparing 2002 and 2003 (*p*<0.05)
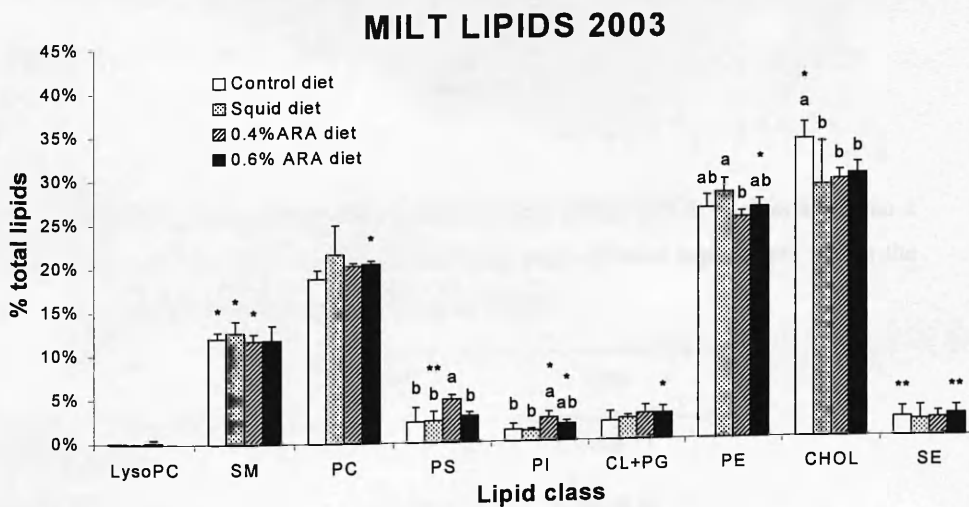
MILT LIPIDS 2003

Figure 30. Percentage of halibut milt lipid class (mean ± S.D. *n*=4). Columns assigned a different letter are significantly different within each year class (*p*<0.05). * Significantly higher than ** when comparing 2002 and 2003 (*p*<0.05)

In 2003 milt from fish fed the 0.4% ARA diet was significantly higher in PS and PI than milt from fish fed the control or squid diet. Cholesterol was significantly higher for males fed control diet and PE was significantly higher for males fed squid diet than for males fed the 0.4% diet. When looking at the general milt lipid class profile in 2002 and 2003, the neutral lipid, cholesterol, had the highest level.

SM was significantly higher in 2003 in fish fed control, squid and 0.4% ARA diets than in 2002. PS was significantly lower in fish fed the squid diet in 2003 than in 2002. PI was significantly higher in males fed the ARA enriched diets in 2003. PE was significantly lower in males fed the 0.6% ARA diet in 2003. When comparing the sum phospholipids to cholesterol ratio, there were no significant diet effects except for males fed the control diet where the ratio was higher in 2003 than in 2002.

Table 21. Σ phospholipid to cholesterol ratio (Σ PL: CHOL) Values are mean ± S.D., $n=3$ in 2002, $n=5$ in 2003. Means with different superscripts within the same row are significantly different ($p<0.05$).

	2002	2003
Control diet	1.72±0.18[a]	1.43±0.11[b]
Squid diet	1.50±0.12	1.94±0.63
0.4% ARA diet	1.66±0.31	1.80±0.08
0.6% ARA diet	1.34±0.31	1.71±0.08

3.3 Fatty acid analyses

The most abundant fatty acids in Atlantic halibut milt were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), eicosaenoic acid (20:1n-9) linoleic acid (18:2n-6), ARA (20:4-n6), EPA (20:5n-3) and DHA (22:6n-3) (Table 22). In 2001 male May spawners were not fed the trial diet prior to the spawning season and male July spawners were fed for three months prior to the spawning season. When fed the 0.4% ARA diet halibut milt showed significantly higher levels of 18:1n-9 compared to control diet and significantly higher 20:1n-9 compared to control diet and 0.6% ARA diet as well as a higher level of saturated fatty acids. ARA concentration was significantly higher in fish fed 0.6% ARA. In 2002 (Table 23) most of the fatty acids in fish fed the control diet were significantly higher than in milt of fish fed the 0.6% ARA diet, except for ARA. In 2003 (Table 24) 18:1n-9 was significantly higher in milt of males fed the 0.4% ARA diet than for males fed the squid diet. 18:2n-6 in milt was significantly higher in males fed squid diet than males fed ARA enriched diets. There were significant effects of diet on level of EPA in milt. The highest level was observed in fish fed control diet and the lowest in fish fed the 0.6% ARA diet. There were no significant dietary effects on milt DHA concentration in 2003 but in 2002 DHA was significantly higher in milt from males fed the control diet compared to males fed the 0.6% ARA diet.

Table 22. Fatty acid composition of Halibut milt with respect to trial diet in 2001
(μg fatty acids/ mg total lipid)

Fatty acid	Control diet	Squid diet	0.4% ARA diet	0.6% ARA diet
14:0	16.65±3.54	16.45±2.03	16.62±3.04	15.91±2.99
16:0	172.28±8.51	177.76±10.25	189.22±4.24	170.79±19.75
18:0	47.47±7.48	45.58±2.20	55.30±8.49	40.59±4.64
16:1*n*-9	1.40±0.45	1.42±0.59	1.50v0.63	1.38±0.24
16:1*n*-7	17.55±3.23	19.86±2.25	16.88±3.46	20.34v2.78
18:1*n*-9	102.81±7.84[b]	109.33±10.11 [ab]	118.27±6.02 [a]	107.48±6.95 [ab]
18:1*n*-7	37.97±2.86	35.69±1.45	39.53±2.86	36.76±1.62
20:1*n*-9	32.95±1.76 [b]	34.07±0.77 [ab]	38.09±3.11 [a]	32.42±2.10 [b]
22:1*n*-11	8.72±0.62	8.39±2.47	8.69±0.80	9.44±0.80
18:2*n*-6	22.75±2.11	24.15±2.09	23.21±1.39	25.08±2.41
18:3*n*-6	1.05±0.13	0.74±0.50	1.13±0.09	0.93±0.04
20:4*n*-6 (ARA)	26.17±1.92 [b]	23.29±0.78 [b]	24.98±2.55 [b]	31.66±1.27 [a]
18:3*n*-3	3.87±0.69	3.48±0.51	3.67±0.67	3.54±0.54
18:4*n*-3	1.55±0.30	1.60±0.32	1.25±0.32	1.71±0.12
20:3*n*-3	0.82±0.55	1.13±0.11	1.18±0.12	0.67±0.95
20:4*n*-3	2.82±0.44	2.41±0.31	2.76±0.45	2.83±0.03
20:5*n*-3 (EPA)	76.84±8.58	72.12±7.75	81.03±9.80	72.00±3.46
22:5*n*-3	9.24±0.57	8.98±0.57	9.46±1.43	7.36±0.01
22:6*n*-3 (DHA)	241.05±23.85	228.08±21.79	250.08±22.01	222.86±21.05
Total	854.32±53.05	845.09±44.44	914.39±44.51	836.98±66.51
Σ Sat	240.31±12.43 [b]	243.23±10.44 [ab]	265.10±7.42 [a]	231.21±28.11 [b]
Σ MSat	208.97±10.85	215.91±10.27	230.17±13.61	216.61±15.54
Σ PUFA	403.96±32.09	384.82±28.62	417.86±32.25	388.01±22.78
Σ n-3	336.60±31.85	318.38±26.57	349.83±30.70	311.81±23.14
Σ n-6	59.01±4.57	56.69±3.21	58.58±4.57	66.49±1.88
Σ *n*-3: *n*-6	5.73±0.76	5.62±0.40	5.99±0.63	4.70±0.48

Values are mean ± S.D., *n*=4 in trial group. Means with different superscripts within rows are significantly different (*p*<0.05).

Table 23. Fatty acid composition of Halibut male milt fed trial diets in 2002 (µg fatty acids/ mg total lipid)

Fatty acid	Control diet	Squid diet	0.4% ARA diet	0.6% ARA diet
14:0	16.18±2.05 [a]	13.46±1.92 [ab]	13.35±2.84 [ab]	13.13±1.86 [b]
16:0	164.72±19.32	153.14±16.28	154.31±21.47	152.15±26.27
18:0	45.54±7.16	42.25±4.57	49.83±6.51	46.35±9.57
16:1*n*-9	2.68±0.31 [a]	2.36±0.30 [ab]	2.52±0.40 [ab]	2.21±0.30 [b]
16:1*n*-7	17.61±3.27	16.23±2.66	15.44±3.06	14.99±2.03
18:1*n*-9	103.93±16.77	104.85±14.35	103.33±13.76	92.35±17.75
18:1*n*-7	36.75±4.35 [a]	33.07±3.97 [ab]	34.35±5.03 [ab]	29.94±4.81 [b]
20:1*n*-9	34.00±3.82 [a]	27.85±3.29 [bc]	31.49±2.99 [ab]	26.89±3.71 [c]
22:1*n*-11	7.78±2.59	6.62±1.12	7.83±0.96	7.67±1.19
18:2*n*-6	21.93±3.55 [a]	20.71±3.15 [ab]	18.25±2.27 [bc]	16.71±2.38 [c]
18:3*n*-6	0.96±0.3	0.77±0.35	0.77±0.2	0.67±0.43
20:4*n*-6 (ARA)	26.22±3.17 [b]	23.88±3.94 [b]	42.91±13.51 [a]	53.56±10.37 [a]
18:3*n*-3	2.27±0.94	2.08±0.39	1.84±0.66	1.78±0.65
18:4*n*-3	0.87±0.28	0.39±0.35	0.97±0.58	0.89±0.81
20:3*n*-3	1.10±0.15 [a]	0.79±0.34 [b]	0.99±0.22 [ab]	0.89±0.18 [ab]
20:4*n*-3	2.49±0.42 [a]	2.07±0.40 [ab]	2.18±0.61 [ab]	1.72±0.34 [b]
20:5*n*-3 (EPA)	65.60±7.84 [a]	58.92±10.96 [ab]	51.92±9.92 [b]	38.56±9.48 [c]
22:5*n*-3	8.75±1.48 [a]	7.11±1.36 [ab]	7.15±1.32 [ab]	5.60±1.15 [b]
22:6*n*-3 (DHA)	217.46±28.95 [a]	193.36±34.78 [ab]	190.91±41.97 [ab]	166.60±40.23 [b]
Total	810.61±89.46	741.01±93.71	762.79±120.58	703.03±124.26
Σ Sat	230.76±26.73	212.10±22.12	221.33±30.17	215.32±37.11
Σ MSat	212.36±27.69	199.98±26.66	204.25±26.90	184.00±28.42
Σ PUFA	366.25±44.57	327.05±54.35	336.13±70.54	302.90±65.56
Σ *n*-3	299.13±37.88 [a]	265.01±47.28 [ab]	256.01±47.28 [ab]	216.39±51.40 [b]
Σ *n*-6	57.67±7.36 [bc]	53.09±6.47 [c]	69.92±16.34 [ab]	78.23±13.72 [a]
Σ *n*-3: *n*-6	5.21±0.56 [a]	4.96±0.42 [a]	3.69±0.29 [b]	2.74±0.22 [c]

Values are mean ± S.D., *n*=10 in trial group. Means with different superscripts within the same rows are significantly different ($p<0.05$).

Table 24. Fatty acid composition of Halibut male milt fed trial diets in 2003 (µg fatty acids/ mg total lipid)

Fatty acid	Control diet	Squid diet	0.4% ARA diet	0.6% ARA diet
14:0	13.13±2.25	11.85±2.22	13.95±2.39	12.37±2.12
16:0	159.57±19.80	159.12±24.30	162.69±19.51	160.75±22.77
16:1n-9	2.35±0.49	2.56±0.73	2.60±0.87	2.64±0.85
16:1n-7	16.96±2.36	16.24±3.21	17.31±2.77	17.11±2.30
18:0	54.55±7.53 [ab]	49.89±8.09 [b]	61.16±10.68 [a]	55.76±8.20 [ab]
18:1n-9	111.48±14.88	110.18±23.37	117.30±15.93	108.14±16.45
18:1n-7	42.63±5.81	36.18±7.25	42.43±5.03	36.65±5.00
20:1n-9	34.04±3.72	30.59±6.05	32.13±3.39	30.43±3.95
22:1n-11	9.23±0.98 [a]	7.19±1.44 [b]	8.11±0.93 [ab]	7.68±0.84 [ab]
18:2n-6	28.05±3.55 [ab]	32.73±6.04 [a]	23.30±3.12 [b]	25.23±5.36 [b]
18:3n-6	1.10±0.18	1.05±0.28	1.06±0.23	0.99±0.26
20:4n-6 (ARA)	27.19±1.83 [c]	24.85±4.80 [c]	54.27±6.32 [b]	66.83±13.97 [a]
18:3n-3	2.93±0.19	2.74±0.54	2.51±0.61	2.46±0.37
18:4n-3	0.94±0.25	0.89±0.40	1.09±0.21	1.06±0.22
20:3n-3	1.13±0.12	1.11±0.24	1.07±0.17	1.07±0.15
20:4n-3	3.14±0.45	2.67±0.66	2.88±0.51	2.57±0.39
20:5n-3 (EPA)	84.70±10.68 [a]	71.17±14.70 [ab]	71.12±10.84 [ab]	63.17±13.49 [b]
22:5n-3	9.73±1.11	8.59±1.82	9.13±1.31	8.46±1.38
22:6n-3 (DHA)	230.92±19.42	221.24±43.97	226.27±30.46	218.16±36.11
Total	869.78±88.51	823.83±145.48	884.42±107.02	855.98±114.54
∑ Sat	230.87±28.93	224.20±33.04	241.03±29.09	232.07±31.05
∑ MSat	228.19±28.81	212.00±42.91	229.95±28.49	212.96±27.14
∑ PUFA	409.39±34.91	386.70±75.72	412.45±53.31	409.29±57.48
∑ n-3	334.01±30.72	308.79±61.68	314.46±42.96	297.37±51.50
∑ n-6	66.08±4.72 [c]	68.01±12.40 [c]	88.31±10.48 [b]	102.86±12.36 [a]
∑ n-3: n-6	3.11±0.23 [a]	2.86±0.15 [b]	2.06±0.98 [c]	1.05±0.66 [d]

Values are mean ± S.D., $n=12$ in trial group. Means with different superscripts within the same rows are significantly different ($p<0.05$).

When looking at ARA concentration in halibut milt (Figure 31) over the three-year trial, the 0.6% ARA diet had significantly the highest-level compared to the other trial diets except the 0.4% ARA diet in 2002. No significant differences were observed in ARA concentration between the control diet and the squid diet moreover there were no significant differences between 2001, 2002 and 2003. In 2002 and 2003 males fed ARA enriched diets had significantly higher milt ARA levels than males fed either control or squid diet. Males fed the 0.4% ARA diet had significantly increased ARA concentration in milt during the three-year trial. Males fed the 0.6% ARA diet had a significant increase between 2001 and 2002 but not in 2003.

The DHA/EPA ratio in halibut milt (Figure 32) was significantly higher in 2002 and 2003 in males fed the 0.6% ARA diet when compared to males fed the other trial diets. No significant diet effect was observed in 2001. In 2003 the DHA/EPA ratio was significantly lower in fish fed the control diet when compared to the other trial diets. Males in 2002 fed ARA enriched diets had a higher DHA/EPA ratio than in 2001 and 2003. During the last spawning season, males fed control diet showed a significant decrease in DHA/EPA ratio.

In terms of the EPA/ARA ratio in halibut milt (Figure 33) an inverse diet effect can be observed. Through the three spawning seasons, males fed 0.6% ARA showed a significantly lower EPA/ARA ratio compared to males fed the other trial diets except in 2003 compared to the 0.4% ARA diet. In 2002 and 2003

there was a significant effect of ARA enriched diets on the EPA/ARA ratio in milt. The EPA/ARA ratio was significantly lower in 2002 in milt of males fed control and squid diets when compared to 2001 and 2003.

There appeared to be a relationship between total lipid ARA and PI as a percentage of total lipid. Halibut milt in 2003 showed a significant curvilinear relationship fitted to a second-order polynomial function (Figure 34). Using a derived function of fitted curve equation, the curve optimum was obtained, 51.4 µg ARA/mg total lipid for 2.3% PI in total lipid of halibut milt.
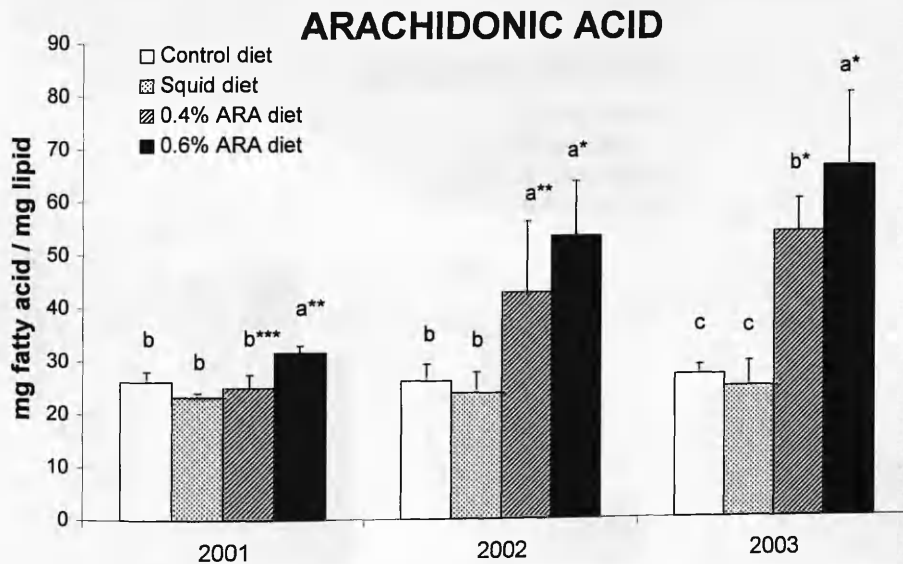
Figure 31. Arachidonic acid concentration (mean ± S.D.) in milt of halibut males fed trial diets over the three-year project. Columns assigned a different letter are significantly different (p<0.05). * Significantly higher than ** between years within each experimental treatment.
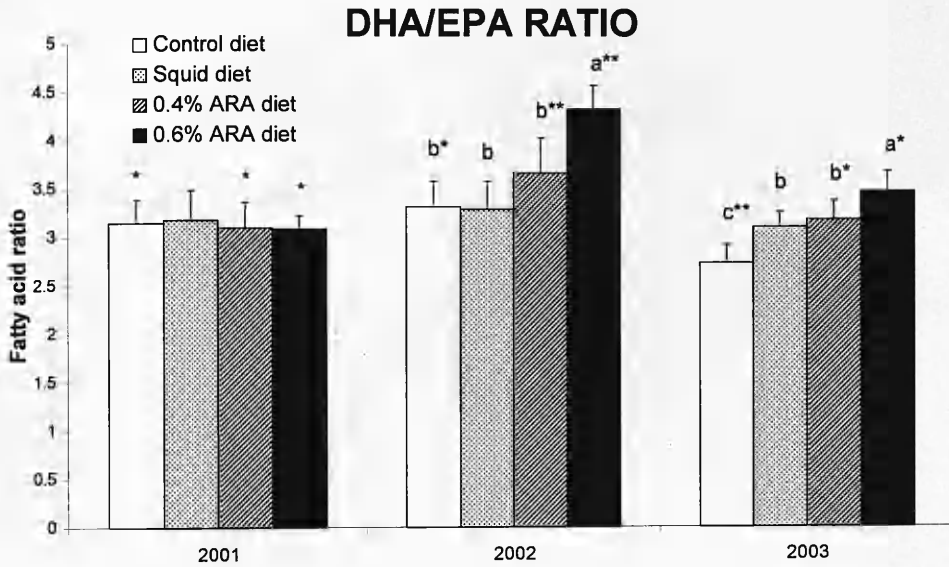
DHA/EPA RATIO

Figure 32. DHA/EPA ratio (mean ± S.D.) according to diet over the three-year trial. Columns assigned a different letter are significantly different (p<0.05). * Significantly higher than ** between years within each experimental diet
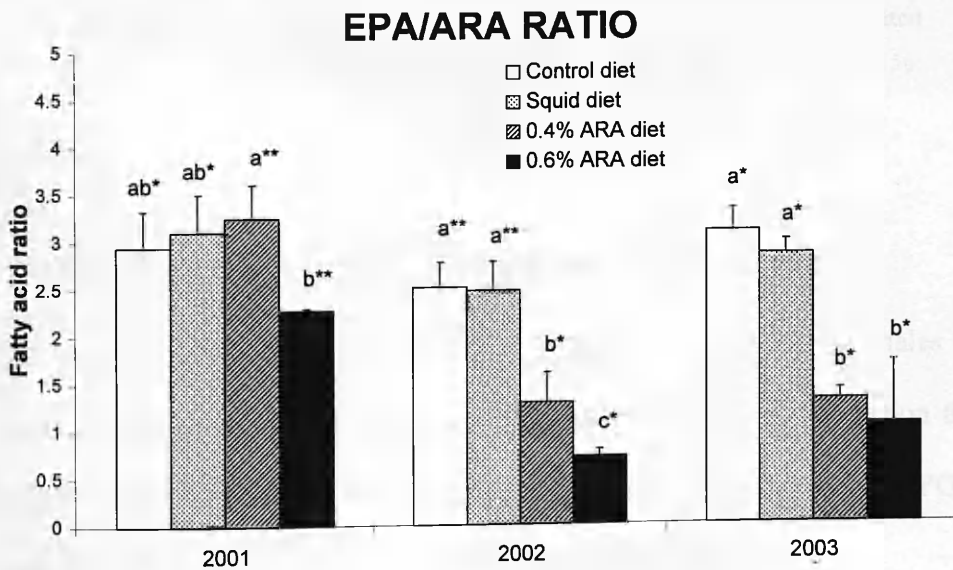
EPA/ARA RATIO

Figure 33. EPA/ARA ratio (mean ± S.D.) according to diet over the three-year trial. Columns assigned a different letter are significantly different ($p<0.05$). * Significantly higher than ** between years within each experimental diet.

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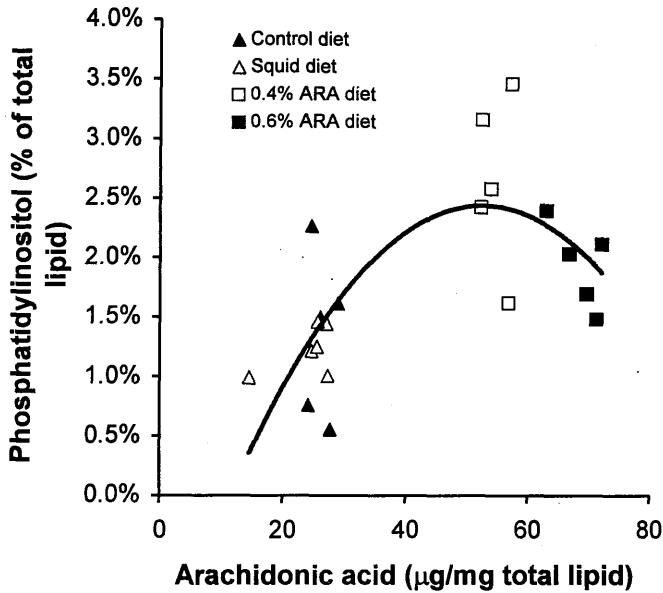
Figure 34. Relationship between arachidonic acid concentration and phosphatidylinositol percentage in halibut male milt total lipid. Curve was fitted by non linear regression ($p<0.05$) $PI=-1.46.10^{-5}\ ARA^2 + 0.00153\ ARA - 0.0156$. ($r^2=0.517$).

3.4 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) concentration in halibut milt

No significant diet effect was observed in 2002. In 2003 halibut males fed ARA enriched diets had a significantly higher $PGF_{2\alpha}$ milt concentration than males in 2002 (Figure 35). Moreover, when fed the 0.4% diet, male milt $PGF_{2\alpha}$ was significantly higher than in males fed the control diet.
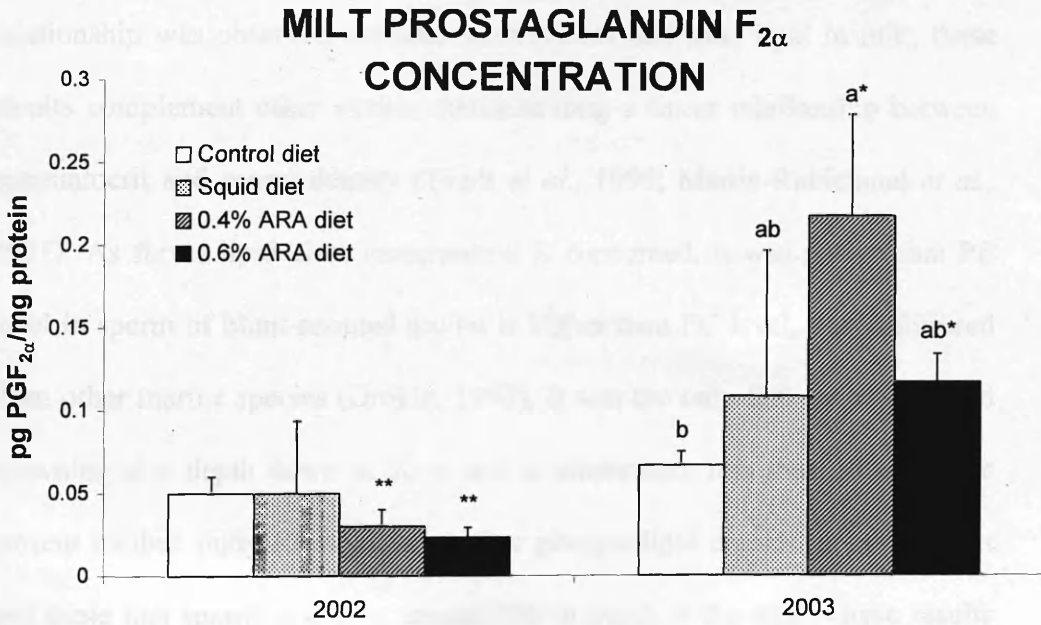
Figure 35. Mean ± S.D. for $PGF_{2\alpha}$ milt concentration according to halibut male diet in 2002 and 2003. Columns assigned a different letter are significantly different ($p<0.05$). * Significantly higher than ** between years within each experimental diet.

4 Discussion

4.1 Lipids in Atlantic halibut milt

There were no significant effects of trial diet on total lipid percentage during the three consecutive years and this was 1.24± 0.28% in halibut milt. A linear relationship was observed between spermatocrit and total lipid in milt, these results complement other studies demonstrating a linear relationship between spermatocrit and sperm density (Tvedt *et al.,* 1999; Martin-Robichaud *et al.,* 2001). As far as lipid class composition is concerned, it was shown that PE level in sperm of blunt-snouted mullet is higher than PC level, which differed from other marine species (Drokin, 1993). It was the only fish species studied spawning at a depth down to 50 m and a temperature less than 10°C. In the present halibut study PE dominates other phospholipid classes in halibut milt and these fish spawn at 6°C at around 300 m depth in the wild. These results could confirm Drokin's (1993) hypothesis on the relationship between spawning habits and PE, PC dominance. In this study we have shown that PC is the second highest phospholipid in halibut milt and it is also known to protect sperm from osmotic and cold stress. This may be viewed as one of the reasons for cryoresistance of marine fish sperm (Drokin, 1993). Halibut are a cold-water species and a higher level of cholesterol was observed when compared to other individual lipid classes. When comparing the cholesterol content of the sperm plasma membrane from trout reared at 18°C and 8°C to those reared at 13°C, the cholesterol/ protein and cholesterol/ phospholipid

ratios were lower for the 18°C reared fish (Labbé & Maisse, 1996). This agrees with the concept of homeoviscous adaptation and it can be interpreted as an active adaptation of the membrane to preserve its fluidity as temperature drops. Cholesterol was significantly higher in males fed control diet in 2003 and these males showed a higher spermatocrit (60±12%) and ran out of milt earlier than fish fed the other trial diets. Lower cholesterol content in the trout sperm plasma membrane is correlated with improved fertilising ability of spermatozoa after cryopreservation, emphasising the possible relationship between low cholesterol content and membrane stability towards thermal stress (Labbé & Maisse, 1996). The level of PI and PS in milt was significantly higher in males fed the 0.4% ARA diet in 2003 when compared to males fed other trial diets and this may highlight the preferential incorporation of ARA in PI and PS

4.2 Arachidonic acid: a reproductive enhancer

The halibut milt fatty acid composition fitted well-established patterns noted in other tissues and species, overlaid with some specific characteristics of sperm (Table 25). It is interesting to note that seabass (Asturiano *et al.*, 2001) and halibut fed the 0.4% ARA diets had an identical AA: EPA ratio whilst seabass levels of EPA and ARA are lower. European eels milt had a lower level of DHA and a higher level of total saturated fatty acids characteristic of freshwater species (Pérez *et al.*, 2000). PUFA are very abundant among the phospholipids of sea bass sperm (Bell *et al.*, 1996). Sea bass milt from males

fed a commercial diet had elevated 20:5n-3 and decreased 20:4n-6 compared to wild fish, reflecting the fish oil component of the diet (Bell *et al.,* 1996). As expected, a significant increase in milt ARA concentration was observed in males fed ARA enriched diets from 2001 to 2003. These concentrations are directly related to the level included in the diet. Fish fed the 0.6% ARA diet had the highest ARA content and the fastest increase in ARA, as was already observed in 2001 in fish fed only three months prior to the spawning season, even when these males had relatively little feed intake. This indicates rapid and selective uptake of ARA into tissues, especially cells related to reproduction. It was interesting to note that there was no significant difference in ARA concentrations for males fed the 0.6% ARA diet between 2002 and 2003, while the difference was significant for males fed the 0.4% ARA diet. This might characterise the maximum ARA uptake in milt. A significantly lower level of EPA in milt of males fed the 0.6% ARA diet was observed but not in males fed the 0.4% ARA diet. In terms of milt production and spermatocrit, fish fed the 0.4% ARA diet showed better reproductive performance when compared to males fed the other trial diets. This emphasises the importance of the balance between EPA/ARA in reproductive performance.

As shown in previous studies on seabass sperm, the dietary lipid markedly altered the fatty acid composition especially with respect to 20:4n-6 and 20:5n-3, and especially in PI, which caused dramatic changes in the ARA/EPA ratios in that lipid class (Bell *et al.,* 1996).

Table 25: Fatty acid composition of milt from different fish species (% total fatty acid)

Fatty acid	Sea bass (Asturiano *et al.*, 2001)	European eel (Pérez *et al.*, 2000)	Halibut fed trial control diet	Halibut fed trial 0.4% ARA diet
Saturated				
16:0	21.2	30.6	18.3	18.4
18:0	6.2	8.55	6.2	6.9
Total	29.8	40.77	26.5	27.3
Monounsaturated				
16:1n-7	1.2	1.34	1.9	1.9
18:1n-7	2.3	2.41	4.9	4.8
18:1n-9	8.4	10.13	12.8	13.3
20:1n-9	1.3	1.98	3.6	3.4
22:1n-11	0.3	0.11	0.1	0.1
Total	14	16.3	26.2	26.0
PUFA n-3				
20:5n-3 (EPA)	4.7	9.23	9.7	8.0
22:5n-3	1.3	0.86	1.1	1.0
22:6n-3 (DHA)	40.1	9.94	26.6	25.6
Total	46.5	20.56	38.4	35.6
PUFA n-6				
18:2n-6 (linoleic)	0.6	2.39	3.2	2.7
20:4n-6 (AA)	3.3	4.39	3.1	6.1
22:5n-6	1.0	0.44	0.5	0.4
Total	5.6	8.33	7.6	9.9
Total PUFAs	52.1	30.31	47.1	46.7
n-3:n-6 PUFA	8.2	2.47	5.1	3.6
AA: EPA	0.7	0.48	0.3	0.7
DHA: EPA	8.6	1.06	2.7	3.2

When studying the relationship between PI and ARA in halibut milt, the curve suggested the establishment of an optimum milt ARA concentration of around 51.4 µg ARA/mg lipid. This corresponds to the observed halibut male milt ARA concentration range when fed with the 0.4% ARA diet. There was no

significant relationship between ARA and PS and/or ARA and PE concentration in halibut milt.

4.3 PGF

In fish several studies have demonstrated the role of PGs on gonadal steroidogenesis especially in ovulation and spermiation and on sexual behaviour through a pheromonal role. Analysis of the physiological and molecular mechanisms which control reproduction in molluscs reveals a prostaglandin-dependent regulation of spawning in abalones (Morse, 1986). *In vitro* studies on steroidogenesis in goldfish testis found that 20:4n-6 stimulates testosterone production, whereas n-3 PUFA, particularly 20:5n-3, may function as an inhibitory regulator (Wade *et al.,* 1994). The inhibitory actions of 20:5n-3 and 22:6n-3 were at least partly due to the inhibition of PGE_2 formation from 20:4n-6 (Wade *et al.,* 1994). PGE_2 has also been reported in the testis of flounder and tuna (Wade & Van Der Kraak, 1993). Unesterified 20:5n-3 and 22:6n-3 were found to inhibit gonadotropin-stimulated testosterone production *in vitro* in a dose-related manner in ovarian follicles from goldfish and rainbow trout, whereas 20:4n-6 was only weakly inhibitory. However, 20:4n-6 was the only one of the three HUFA to increase basal testosterone level via cAMP production (Mercure & Van Der Kraak, 1995). Essential PUFAs significantly modulate testicular prostaglandin production *in vitro* and therefore may have important effects on steroidogenesis and spermiation in the male European sea bass (Asturiano *et al.,* 2000).

PGE_2 is derived from ARA via a cycloxygenase and therefore the availability of ARA is the limiting factor for production of PGE_2 and $PGF_{2\alpha}$. This has been demonstrated extensively in the ovary of teleost fish (Stacey & Goetz, 1982; Sorensen *et al.*, 1988; Van Der Kraak *et al.*, 1989). PGFs ($PGF_{1\alpha}$ and $PGF_{2\alpha}$) are not only potent odorants in mature male brown trout but also have a reproductive priming effect on these fish, increasing plasma concentrations of 17,20 β-dihydroxy-4-pregnen-3-one (17,20 βP) and levels of expressible milt (Moon *et al.*, 2003). Atlantic halibut male spawning occurs at the peak of gonadal activity. This was evidenced by the finding that plasma T levels were higher in stage IV in males from whom milt was easily stripped, than from other stage IV males (Weltzien *et al.*, 2002). In this study a significantly higher level of $PGF_{2\alpha}$ in milt was observed in males fed 0.4% ARA. This result confirmed the link between the 0.4% ARA diet and enhancement of halibut male reproductive performance through a favorable spermatocrit, a highest level of PI and an increase of ARA level in milt as well as a highest $PGF_{2\alpha}$ concentration. There is an evident benefit of using ARA enriched diets on male reproductive performance as indicated by the 2003 results for males fed the 0.4% ARA diet.

Chapter 6 Atlantic halibut egg quality

1 Introduction

The egg's potential to produce viable fry is determined by several physical, genetic and chemical parameters, as well as the initial physiological processes occurring in the egg. If one of the essential factors is lacking, or is incomplete, egg development will fail at some stage. Thus, egg quality should be regarded as determined when the egg has left the female fish and the fertilisation process is completed (Kjørsvik *et al.,* 1990).

1.1 Vitellogenesis

The females of egg-laying vertebrates, including most species of fish, enter a phase of oocyte maturation in preparation for ovulation and spawning. Under the control of the hypothalamus and the pituitary gland, the growing follicles synthesise and excrete, into the blood circulation, sex steroid hormones that govern oocyte developmental processes (Norberg, 1995; Riple *et al.,* 1999). One of the primary target organs for these steroids, particularly 17β-estradiol (E_2), is the liver. This organ, which possesses highly specific binding proteins for E_2, in turn responds to such hormonal stimulus by the synthesis and export via the blood of vitellogenin (Hyllner *et al.,* 1994). Vitellogenin constitutes the carrier for various classes of compounds accumulated by the developing oocyte. While the backbone of the vitellogenin molecule is a protein chain of substantial size (molecular weight 250-600 kd), it also carries copious amounts

of lipid material, carbohydrate components, phosphate groups, and mineral salts. Following highly selective uptake into the oocyte, the vitellogenin is broken up and accumulated as egg-specific yolk constituents, such as phosvitin and lipovitellin (Cerdá *et al.,* 1994). The early larval developmental stages of many species of fish entail long periods without exogenous nourishment, for example 120 °C/days (dd) for halibut prior to their first active exogenous feeding (Blaxter *et al.,* 1983). Therefore maternal production of vitellogenin and deposition of adequate supplies of yolk are essential to subsequent embryonic and larval survival. The bulk of the *(n-3)* PUFA-rich phospholipid in eggs is located in lipovitellin. For vitellogenesis to proceed normally in turbot, a plentiful exogenous source of food is obligatory during the vitellogenic phase (Bromley *et al.,* 2000). Vitellogenesis was identified as the period during ovogenesis where EFA are incorporated most effectively into developing European sea bass oocytes (Navas *et al.,* 1997)

1.2 Biochemical composition of halibut eggs

During oocyte development in vertebrates an acellular envelope is formed outside the oolemma. The terminology and biological functions of the egg envelope vary in different vertebrate groups. In teleosts the envelope is often referred to as the vitelline envelope, and forms a tough protective coat around egg and embryo. The egg envelope plays several roles: attraction of spermatozoa, prevention of polyspermy and protection of eggs/embryo.

Lysosomal enzymes such as cathepsin D and cathepsin L are involved in proteolytic cleavage during oocyte maturation of seabream eggs. The second proteolytic process is the main one responsible for the hydration process which is fundamental for acquiring buoyancy (Carnevali *et al.*, 1999). The biochemical composition of a healthy egg reflects the embryonic nutritional requirements for growth. Some components are known to be "essential" for an organism (i.e. the organism is unable to synthesize the nutrient) and these components have to be present in optimum amounts to satisfy biological demands. Biochemical egg quality assessment is therefore a good tool to determine whether eggs are of good quality prior the fertilisation process. During embryogenesis and larval development of most fish species, growth and energy storage is dependent on endogenous yolk reserves transferred by the broodstock. Thereafter, larval growth and survival depends on the availability of exogenous food in sufficient quantity and of adequate quality after yolk resorption (Kjørsvik *et al.*, 1990).

1.2.1 Protein and essential amino acids (EAA)

As shown in seabream, dietary essential amino acids (EAA) control fecundity and egg and larval quality, mainly via the synthesis and selective uptake of yolk constituents (Harel *et al.*, 1995). Pelagic eggs of marine teleosts have a high content of free amino acids (FAA), mainly found in the yolk (Rønnestad *et al.*, 1992). The protein content of developing halibut eggs appears to be

related to egg size and neither protein nor FAA content varied significantly between similar weight and size eggs (Finn *et al.*, 1991).

1.2.2 Lipids

Egg lipids have several critical roles in the development of fish embryos. They provide major energy storage, play a structural role in membranes, act as chemical messengers and serve as a source of micronutrients. Teleost oocytes accumulate large amounts of lipids in addition to the polar lipids accumulated through the vitellogenin molecule. In spawned eggs several classes of lipids are represented, where strong variations exist among different fish species. Requirements, reproductive strategy, habitat of fish at spawning time and lipid accumulation vary. Depending on the preferred type of lipid accumulated in the eggs three strategies can be distinguished:

> Equally high levels of polar lipids and triglycerides (rainbow trout, sole & whitefish)

> Accumulate mainly polar lipids, 75% to 90% (Baltic herring, roach, turbot, halibut)

> Accumulate large amounts (>80%) of wax and sterol esters in the so-called egg oil globules (gourami, sea bass, striped bass, perch, burbot)

Lipids are mainly found in two compartments of the egg. The lipoprotein yolk contains mostly phospholipid along with some neutral lipids whereas the oil is composed of neutral lipids such as triacyglycerols and steryl and/or wax esters. The oil, if present, may be in the form of tiny droplets or a large, discrete

globule (Wiegand *et al.*, 1999). The main phospholipid in eggs is invariably

phosphatidylcholine, and eggs with short and long incubation times have low

and high levels of triacylglycerols respectively (Tocher & Sargent, 1984a). The

physiological advantages of accumulating large amounts of wax esters in eggs

may be explained by the fact that in addition to serving as an energy supply,

they will play an important role in buoyancy control for the embryo and

developing larva. Marine teleost eggs exhibit an internal yolk osmotic value

which is much lower than the surrounding medium, and this is maintained by

the low permeability of the vitelline membrane. Higher yolk osmolarity levels

are reported for poor quality eggs of cod, probably resulting in a longer and

incomplete cortical reaction in these eggs (Kjørsvik *et al.*, 1990). Changes in

specific gravity may partly be due to osmoregulatory variations in egg batches,

and buoyancy for pelagic eggs is often better with good quality eggs. For a

number of species, including halibut, no such correlations of buoyancy or of

any other morphological characteristics, with quality, has been consistently

reported (Riis-Vestergaard *et al.*, 1982; Haug *et al*, 1990). Freshwater spawners

,such as the salmonids, generally shed relatively large eggs with large lipid

reserves containing substantial amounts of triglyceride as well as

phospholipids, and with long incubation periods up to 20 weeks. They generate

large larvae that are easy to feed with artificial dry diets. In contrast, many

marine fish generally shed small eggs with relatively modest reserves of lipid,

composed principally of phospholipids, and with short incubation periods of

around 20 days. They generate small larvae that are very difficult to feed with defined diets (Bromage & Roberts, 1995).

1.2.3 Fatty acid compositions

There is a high degree of consistency among populations in the egg content of fatty acids (Wiegand *et al.*, 1999). Essential fatty acids (EFA), vital for early survival and development of newly hatched larvae, are determined by the lipids derived directly from dietary input of broodstock in the period preceding gonadogenesis (Kjørsvik *et al.*, 1990; Sargent, 1995). Fatty acids are mobilised from the neutral lipid reserves of fish adipose tissue during gonadogenesis and transferred via the serum to the liver where they are assembled into the egg-specific lipoprotein, vitellogenin. Vitellogenin synthesis can be induced in Pacific herring (Koya *et al.*, 1999) and Atlantic halibut (Norberg *et al.*, 1995) by repeated injections of 17β estradiol (E_2). The spawning Atlantic cod ovary is very active in terms of vitellogenesis as shown by the significant levels of E2 in blood plasma (Kjesbu *et al.*, 1996). This is a specialized very high-density serum lipoprotein (VHDL) consisting of approximately 80% protein and 20% lipid. Phospholipids and triacylglycerols account for about two-thirds and one-third of the lipids respectively, and because of its abundance in phospholipids the vitellogenin is rich in (*n-3*) PUFA, especially DHA. Vitellogenesis is identified as the period in the sea bass reproductive cycle during which EFA are incorporated most effectively into the developing oocytes (Navas *et al.*, 1997). Polyunsaturated fatty acids (PUFA) are considered a structural

component during organogenesis and precursors of physiologically active molecules such as prostaglandins and other eicosanoids (Bell *et al.*, 1992; Sargent, 1995). As suggested by Czesny *et al.* (2000), egg fatty acid profile can be a viable tool in discrimination of different sturgeon populations with respect to caviar.

1.2.3.1 *n-3* HUFA: EPA and DHA

Up to 60% of the FFA mobilised, preferentially saturated and monounsaturated fatty acids, can be catabolised to provide metabolic energy for egg lipoprotein biosynthesis. The remainder, preferentially (*n-3*) polyunsaturated fatty acids (HUFA) and especially docosahexaenoic acid (DHA, 22:6(*n-3*)), are incorporated into the phospholipid-rich vitellogenin. (*n-3*) HUFA are composed principally of DHA and eicosapentaenoic acid (EPA, 20:5(*n-3*)) in a ratio of approximately 2:1 (Sargent, 1995). The fatty acids of both phospholipids and triacylglycerols (TAG), including their (*n-3*) PUFA, are catabolised to provide metabolic energy for the developing egg and early larva, but the chief role of (*n-3*) PUFA is in the formation of cellular membranes. Because of the unusual richness of DHA in neural cell membranes, this fatty acid appears to have a critical role in the formation of the brain and the eyes, which constitute a large fraction of the embryonic and larval body mass (Tocher & Harvie, 1988). This observation is illustrated by the high levels of phosphoglycerides rich in DHA in fish tissue especially neural tissues; some

40% of the dry matter of fish brain is lipid, some 10% of which is DHA-rich phosphatidylethanolamine (Sargent, 1995).

1.2.3.2 Arachidonic acid

As shown in previous studies arachidonic acid (ARA; 20:4n-6) levels are significantly lower in cultured striped bass eggs compared to wild stock. Phosphatidylinositol (PI) was the dominant phospholipid found in all striped bass eggs from all origins as opposed to phosphatidylcholine (PC) which is usually the dominant phospholipid. These data indicate that PI and AA might have important and as yet unidentified roles in fertilisation and embryonic development in these fish (Gallagher *et al.*, 1998).

1.3 Overall aims

➢ To understand if lack of fertilisation in eggs is due to biochemical and especially lipid deficiency

➢ To identify variations betweens different geographical and time origins in egg lipid composition

➢ To study the effect of enriched ARA diet fed to broodstock on lipid composition of eggs (transfer and effect on quality and fertilisation process), variation in time (from 2001 to 2003) and between May and July spawners.

2 Materials and Methods

2.1 Origin of eggs

In the present study, eggs were obtained from three different sources:

- Samples of Atlantic halibut eggs spawned in 1996 and in 2001 at St Andrews Biological Station, Canada, by 1994 wild catch broodstock fed frozen fish enriched with a mixture of vitamins and fish oil. In 1996 lipid extraction and fatty acid profile acquisition took place in St Andrews nutrition laboratory. In 2001 eggs were sent to the Stirling nutrition laboratory for lipid extraction and fatty acid analyses as described in chapter 2 section 2.3 and 2.5

- Samples of Atlantic halibut eggs spawned in 2001 at Otter Ferry Seafish Ltd. by 1994 wild catch broodstock fed INVE diet.

- Samples of Atlantic halibut eggs spawned in 2001, 2002 and 2003 at Otter Ferry Seafish Ltd. and part of the dietary trials.

Table 26. Halibut egg samples analysed over the three-year trial

Halibut eggs biochemical lipid analyses			Samples
1996	St Andrews F1		12
2001	May	Egg before fertilisation	20 batches (10 eggs / batch)
		Fertilised eggs	20 batches (10 eggs / batch)
		Non fertilised eggs	20 batches (10 eggs / batch)
	July		No production
	Otter Ferry F1	Egg before fertilisation	20 batches (10 eggs / batch)
		Fertilised eggs	20 batches (10 eggs / batch)
2002	May		48=>12 batches x 4 diets (10 eggs / batch)
	July		48=>12 batches x 4 diets (10 eggs / batch)
	St Andrews F1		12 batches (10 eggs / batch)
2003	May		48=>12 batches x 4 diets (10 eggs / batch)
	July		48=>12 batches x 4 diets (10 eggs / batch)

Individual egg batches were collected in a jug by stripping. Eggs were transported to a dark cold room in a closed cool box to avoid any UV and temperature damage. Individual egg batches were then treated according to the protocols (Figure 36) set-up during the project. Around 10 ml of eggs was sampled, using the top end of a 10 ml plastic pipette, from every jug and placed in a petri dish containing UV treated seawater. 10 eggs were then sampled from each petri dish and observed under a microscope in order to avoid dead eggs in the sample. Live eggs were placed in a 2 ml glass vial containing 1ml of chloroform: methanol (v:v 2:1)+0.01% BHT.

EGG ASSESSMENT

Control   **Squid**   **Low ARA**   **High ARA**

Stripping

Eggs: Females were stripped every 3 days. Between 1 and 5 strips were expected per day strip and per tank. Eggs were collected in one jug for each female

10 eggs sampled from each jug

Fertilisation

10L bucket

In fridge at 3.5°C: Beaker to check individual fertilisation rate

One bucket for one jug. Left overnight, then distributed according to fertilisation rate in egg conical

Eicosanoid trial (4 cells, 8 cells, 16 cells), in duplicate

10 fertilised eggs/10 non fertilised eggs (2001)

Incubation

Figure 36: Atlantic halibut egg quality assessment protocol used at Otter Ferry Seafish for the three year project.

Following the fishfarm protocol eggs were fertilised as described in chapter 4, section 2.6. Beakers from the trial diets were kept in a fridge at 3.5°C in order to obtain first division early the following morning. When checking fertilisation under the microscope, 10 fertilised eggs and 10 unfertilised eggs were sampled and placed in separate 2 ml glass vial containing 1ml of chloroform: methanol (v:v 2:1)+0.01% BHT.

2.2 Eggs before fertilisation, fertilised eggs and non fertilised egg collection

Twenty wild broodstock individual egg batches sampled before fertilisation were randomly selected and lipid extracted. Lipid class and fatty acid analyses were carried out on these samples and, from the same batch, fertilised egg samples. Twenty egg batches from May spawners were randomly sampled before fertilisation. Lipid were then extracted and analysed for lipid class and fatty acid composition. Analyses were also performed on both fertilised and non-fertilised eggs sampled from the same batch. Sampling is summarized in Table 26.

2.3 Atlantic halibut eggs from trial broodstock

For each spawning season, four egg samples from four different females spawning in May and four others spawning in July were randomly selected for each diet. In order to study the diet effect on egg quality, egg samples were then pooled in two different groups according to their fertilisation rate. When

fertilisation rate was higher than 35% eggs were included in the incubated egg group (inc) and lower than 35%, eggs were included in the non-incubated egg group (ni). Sampling is summarized in Table 26.

2.4 Wild broodstock: Scottish and Canadian Atlantic halibut eggs

Egg sample results were randomly selected from fatty acid analyses performed in 1996 on St Andrews broodstock individual egg batches. As indicated in chapter 2 section 2.3 and 2.5, lipid extraction and fatty acid analyses were carried out on samples from 2002 St Andrews individual egg batches and 2001 Otter Ferry individual egg batches samples. Sampling is summarized in Table 26.

2.5 Statistical analyses

Atlantic halibut egg total lipid, lipid class and fatty acid compositions were pooled for statistical analyses using SPSS for windows version 11.5. Data were tested for normality using the method of Kolmogorov and Smirnov. When the normality test was validated, data were analysed by ANOVA and the post-test means comparison was performed by Tukeys test when comparing eggs before fertilisation, fertilised eggs and non-fertilised eggs, diet effect and the three-year trial data (Puri, 1996). Data were analysed by Leven's test for equality of variance and variances T-test when comparing 2002 to 2003 data, eggs before fertilisation to fertilised eggs and incubated egg group to non-incubated egg group data (Puri, 1996). The significance level was set at $p < 0.05$.

3 Results

3.1 Eggs before fertilisation, fertilised eggs and non fertilised eggs collection

3.1.1 Total lipids

Results in this section are pooled data from eggs prior to fertilisation and in fertilised and non-fertilised eggs. There were no significant differences in total lipid (Table 27) extracted from Otter Ferry wild broodstock eggs before fertilisation and fertilised eggs. Lipid content of eggs from May spawners fed trial diets showed that eggs before fertilisation had a higher total lipid content than fertilised or non-fertilised eggs.

Table 27. Total lipid (mg /egg) in eggs before fertilisation, in fertilised eggs and in non-fertilised eggs. Means with different superscripts within the same rows are significantly different ($p<0.05$). Values are mean ± S.D., $n=20$

	Eggs before fertilisation	Fertilised eggs	Non-fertilised eggs
Otter Ferry Seafish wild broodstock	0.191±0.032	0.169±0.019	No data
May spawners before start of trial (2001)	0.174±0.026[a]	0.148±0.019[b]	0.158±0.016[b]

3.1.2 Lipid class

Ten lipid classes were identified (Figure 37) in Atlantic halibut eggs. Polar lipid classes predominated, with PC being the major lipid class. In terms of neutral lipids, TAG is the main lipid class in halibut eggs. In 2001 PI percentage in Otter Ferry wild broodstock fertilised eggs (Figure 38) was significantly higher than eggs before fertilisation.
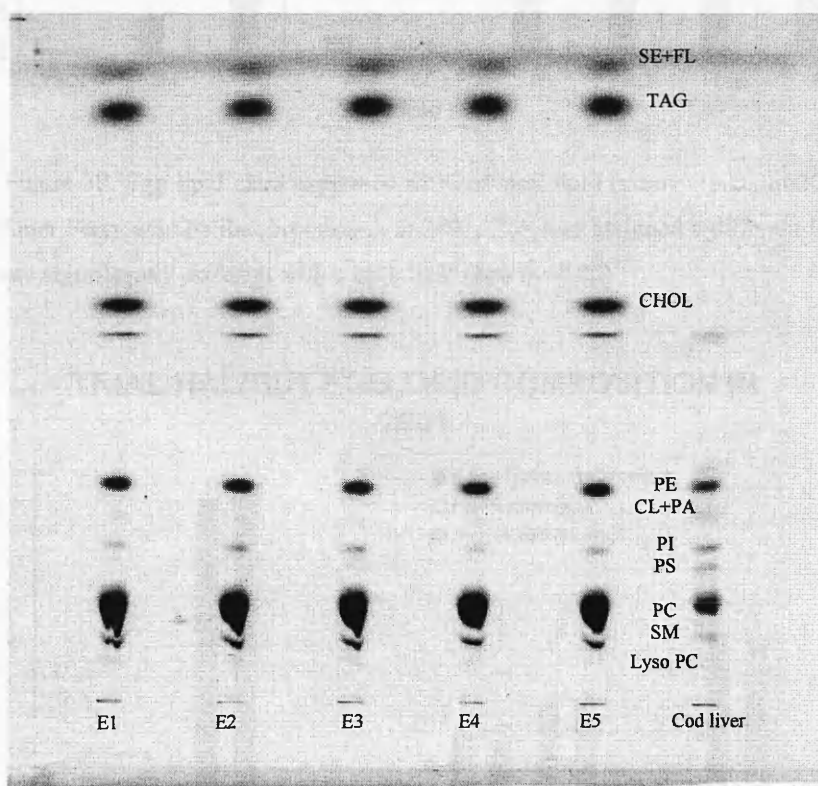
Figure 37 Atlantic halibut eggs (E=> 5 batches n=10) high-performance thin layer chromatography separation. Cod liver was used as a reference. Ten lipid classes were identified: LysoPC: Lysophosphatidylcholine. SM: Sphingomyeline. PC: Phosphatidylcholine. PS: Phosphatidylserine. PI: Phosphatidylinositol. CL+PG: Cardiolipid + Phosphoglycerol. PE: Phosphatidylethanolamine. Chol: Cholesterol. TAG: Triacylglycerol. SE+FL: Sterol+ solvent front line.

WILD BROODSTOCK EGG LIPID COMPOSITION IN 2001
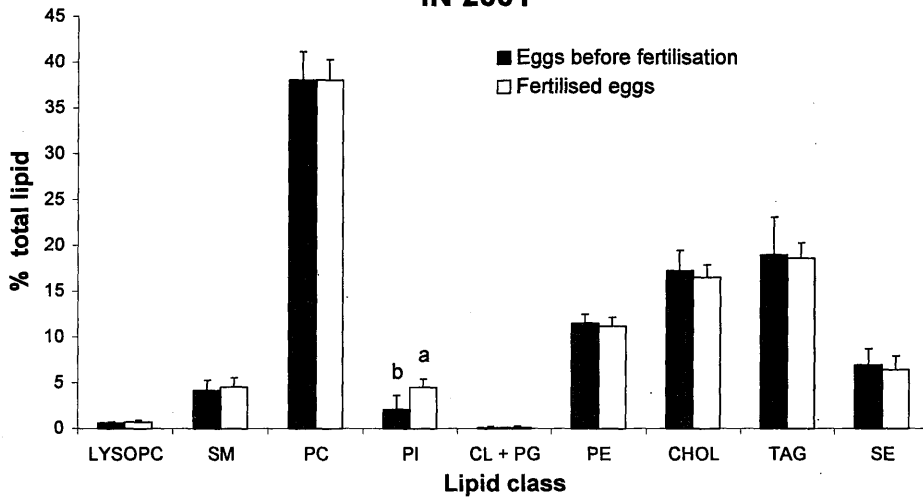
Figure 38. Egg lipid class expressed as % of total lipid (mean ± S.D., n=20) of Otter Ferry wild halibut broodstock in 2001. Columns assigned a different letter are significantly different within each lipid class (p<0.05).

TRIAL HALIBUT EGG LIPID COMPOSITION IN 2001
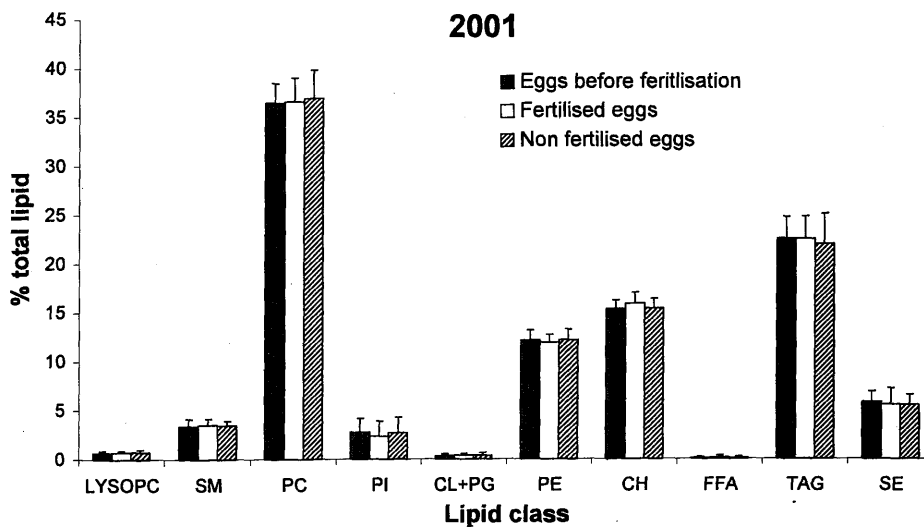
Figure 39. Egg lipid class expressed as % of total lipid (mean ± S.D., n=20) of Otter Ferry trial halibut broodstock in 2001 (before the start of the trial diets). There was no significant difference within each lipid class between the three egg stages.

There was no significant difference within the nine other lipid classes when comparing Otter Ferry wild broodstock eggs before and after fertilisation. Moreover, there was no significant difference observed in trial diet eggs (Figure 39) in 2001 when comparing either eggs before fertilisation to fertilised eggs or non-fertilised eggs. There were no significant differences between lipid class composition of wild broodstock and trial diet eggs in 2001. PC and TAG were respectively $37.4 \pm 2.5\%$ and $20.6 \pm 2.7\%$ of total egg lipids

3.1.3 Fatty acid compositions

The fatty acid composition of total lipid extracted from wild halibut broodstock eggs was dominated by PUFA, predominantly of the (n-3) series. The major PUFA and most abundant fatty acids in halibut eggs were DHA ($22:6n$-3) and EPA ($20:5n$-3). The next most abundant fatty acids were palmitic acid ($16:0$), stearic acid ($18:0$) and oleic acid ($18:1$). There was no significant difference in fatty acid composition when comparing eggs before fertilisation with fertilised egg spawned by wild broodstock in 2001 (Table 28) except $18:3n$-3. Moreover, eggs before fertilisation, fertilised and non-fertilised eggs spawned in May by trial diet broodstock in 2001 (Table 29) showed no significant differences among their fatty acid concentrations except $18:2n$-6 and $18:3n$-3.

Table 28. Fatty acid composition of wild Otter Ferry halibut broodstock eggs (µg FA/mg total lipid)

Fatty acid	Eggs before fertilisation	Fertilised eggs
14:0	13.41±1.73	13.05±1.51
16:0	109.93±8.82	106.64±8.11
18:0	25.96±2.46	25.50±2.87
16:1n-9	5.41±1.28	5.29±0.82
16:1n-7	16.09±2.37	15.75±2.37
18:1n-9	47.31±4.06	44.45±1.71
18:1n-7	14.55±1.08	13.83±0.81
20:1(n-9)	14.59±2.06	13.52±1.14
22:1(n-11)	5.97±0.98	5.22±0.50
18:2n-6	8.16±0.66	7.87±0.57
18:3n-6	0.46±0.25	0.31±0.25
20:4n-6 (ARA)	6.61±1.34	7.52±0.70
18:3n-3	1.85±0.17	1.77±0.24
18:4n-3	2.02±0.32	1.84±0.23
20:3n-3	0.87±0.10	0.72±0.25
20:4n-3	2.69±0.98	2.70±0.26
20:5n-3 (EPA)	67.54±7.31	62.79±5.14
22:5n-3	9.24±0.79	8.50±0.50
22:6n-3 (DHA)	159.19±14.54	151.66±11.11
Total	537.32±36.33	513.07±26.62
Σ Sat	152.21±11.39	148.13±10.20
Σ MSat	115.19±9.90	108.39±5.01
Σ PUFA	268.66±21.58	255.46±14.50
Σ n-3	243.67±20.81	230.31±13.27
Σ n-6	18.85±1.74	19.35±1.29
DHA: EPA	2.37±0.24	2.43±0.26
EPA: ARA	10.51±1.85	8.37±0.48
n-3:n-6	13±1.39	11.92±0.65

Values are means ± S.D., $n=20$. No significant difference observed between eggs before fertilisation and fertilised eggs fatty acid composition.

Table 29. Halibut broodstock trial May spawner's eggs fatty acid composition in 2001 (µg fatty acid / mg total lipid)

Fatty acid	Eggs before fertilisation	Fertilised eggs	Non fertilised eggs
14:0	12.26±2.46	13.00±2.76	13.31±2.65
16:0	110.99±13.08	115.17±10.72	118.81±9.74
18:0	19.60±5.35	20.36±2.72	21.37±2.13
16:1n-9	4.47±1.17	4.78±0.84	4.83±1.08
16:1n-7	19.07±3.02	20.15±2.36	20.82±2.65
18:1n-9	43.23±9.39	45.40±4.43	47.48±5.56
18:1n-7	13.60±3.27	14.34±1.13	14.88±1.24
20:1(n-9)	11.61±2.01	11.87±1.48	12.23±1.53
22:1(n-11)	4.09±0.67	4.21±0.53	4.35±0.52
18:2n-6	8.52±1.03 [b]	9.30±0.87 [ab]	9.45±0.92 [a]
18:3n-6	0.29±0.16	0.29±0.16	0.25±0.19
20:4n-6 (ARA)	7.31±1.33	7.13±1.51	7.73±1.23
18:3n-3	1.95±0.28	2.15±0.15	2.19±0.34
18:4n-3	1.84±0.33	1.91±0.27	1.97±0.36
20:3n-3	0.78±0.25	0.84±0.11	0.80±0.25
20:4n-3	2.29±0.50	2.38±0.37	2.44±0.42
20:5n-3 (EPA)	61.85±11.00	64.70±8.49	67.54±7.55
22:5n-3	8.30±1.33	8.84±1.00	9.14±0.67
22:6n-3 (DHA)	162.44±29.63	172.23±21.73	180.08±20.87
Total	517.62±77.97	543.21±49.70	564.28±49.04
$\sum$ Sat	144.99±17.49	150.84±14.17	155.94±13.31
$\sum$ MSat	106.20±18.86	11.27±8.83	115.07±10.42
$\sum$ PUFA	265.77±45.63	280.32±32.76	292.42±29.54
$\sum$ n-3	239.96±42.42	253.53±30.79	264.70±28.37
$\sum$ n-6	19.90±2.84	20.55±2.31	21.37±1.68
DHA: EPA	2.63±0.18	2.67±0.18	2.67±0.21
EPA: ARA	8.57±1.20	9.37±1.79	8.96±1.77
$\sum$ n-3: $\sum$ n-6	12.04±1.16	12.39±1.26	12.44±1.48

Values are means ± S.D., n=20. Means with different superscripts within the same rows are significantly different ($p<0.05$)

3.2 Variation in halibut egg lipid composition between eggs of different origin

Significant differences were observed between eggs spawned by broodstock halibut of different origin (Table 30). Palmitic acid, stearic acid, oleic acid and arachidonic acid were significantly higher in Otter Ferry wild broodstock spawned in 2001 than in St Andrews wild broodstock eggs spawned in 2002, as well as DHA: EPA ratio and (*n*-6) total PUFA. Eicosaenoic acids (20:1's) were lower in Otter Ferry broodstock eggs.

Table 30. Fatty acid composition of wild halibut broodstock eggs from two different geographic egg sources (μg fatty acid / mg total lipid)

Fatty acid	St Andrews F1 eggs 2002	Otter Ferry F1 eggs 2001
14:0	10.08±1.58[b]	13.23±1.60[a]
16:0	102.14±2.37	108.28±8.44
18:0	6.02±0.88[b]	25.73±2.62[a]
16:1n-9	16.45±1.46[a]	5.35±1.05[b]
16:1n-7	18.93±1.47	15.92±2.32
18:1n-9	38.24±2.65[b]	45.88±3.37[a]
18:1n-7	14.47±0.98	14.19±1.00
20:1(n-9)	20.54±2.04[a]	14.06±1.71[b]
22:1(n-11)	8.45±1.05[a]	5.60±0.85[b]
18:2n-6	6.78±0.82[b]	8.02±0.62[a]
18:3n-6	0.33±0.15	0.39±0.26
20:4n-6 (ARA)	3.69±0.48[b]	7.07±1.14[a]
18:3n-3	1.71±0.18	1.81±0.21
18:4n-3	1.52±0.32	1.93±0.29
20:3n-3	0.64±0.10	0.80±0.20
20:4n-3	2.15±0.32	2.69±0.70
20:5n-3 (EPA)	64.56±11.26	65.16±6.63
22:5n-3	9.79±1.41[a]	8.87±0.75[b]
22:6n-3 (DHA)	129.51±19.68[b]	155.42±13.20[a]
Total	480.17±32.62	525.20±33.47
$\sum$ Sat	133.55±2.35	150.17±10.76
$\sum$ MSat	115.56±7.34	111.79±8.41
$\sum$ PUFA	230.03±32.89	262.06±19.17
$\sum$ n-3	210.51±31.75	236.99±18.35
$\sum$ n-6	13.81±1.43[b]	19.10±1.52[a]
DHA: EPA	2.02±0.20[b]	2.40±0.25[a]
EPA: ARA	17.48±2.38[a]	9.44±1.72[b]
$\sum$ n-3: $\sum$ n-6	15.26±2.00[a]	12.46±1.20[b]

Values are means ± S.D., n=12. Means with different superscripts within the same rows are significantly different ($p<0.05$)

EPA:ARA ratio (Figure 40) in St Andrews wild broodstock eggs spawned in 2002 was significantly higher than in the two others eggs sources, on the other hand DHA:EPA ratio was significantly lower in St Andrews eggs spawned in 2002 than in St Andrews eggs spawned in 1996 and in 2001 Otter Ferry eggs. Σn-3:Σn-6 ratio was significantly lower in 2001 Otter Ferry eggs than in 1996 and 2002 St Andrews eggs.
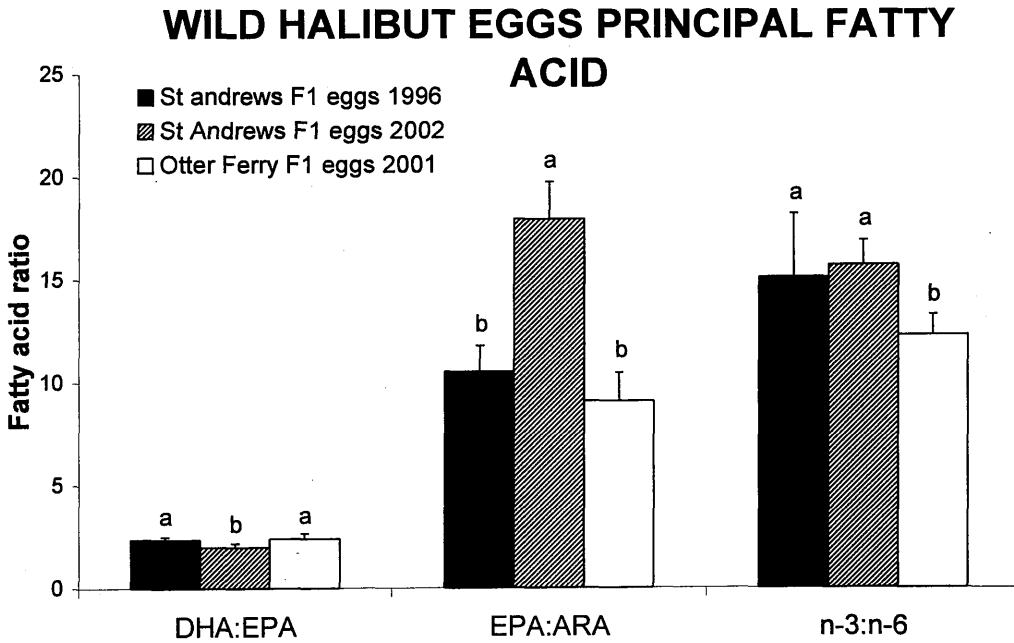
Figure 40. Principal fatty acid ratio (mean ± S.D., $n=12$) of wild halibut broodstock eggs from three different egg sources. Columns assigned a different letter are significantly different ($p<0.05$)

3.3 Effect of enriched ARA diet on halibut egg lipid quality

3.3.1 Total lipids

No significant diet effect was observed on total lipid content per egg spawned in May (Table 31) and July (Table 32) by halibut broodstock fed trial diets. Moreover, there were no significant differences between the incubated egg group and the non-incubated egg group in eggs spawned in May and in July. In 2003 total lipid was significantly higher in eggs produced in May by females fed the control, squid and 0.4% ARA diets. No significant difference in egg total lipid was observed in May spawners fed the 0.6% ARA diet through the three year project. In eggs spawned in July a significant increase in total lipid content was observed between 2002 and 2003. Total lipids in July spawned eggs were significantly higher than in May spawned eggs in 2002 and 2003.

Table 31. Total lipid (mg /egg) in Atlantic halibut eggs spawned in May according to their diet a through the three years of the project

	Egg quality	2001	2002	2003
Control diet	inc	0.152 ± 0.003^b	0.149 ± 0.014^b	0.188 ± 0.006^a
	ni	0.156 ± 0.010^b	0.150 ± 0.011^b	0.185 ± 0.013^a
Squid diet	inc	0.174 ± 0.013^{ab}	0.155 ± 0.009^b	0.190 ± 0.002^a
	ni	0.175 ± 0.015^{ab}	0.167 ± 0.008^b	0.195 ± 0.004^a
0.4% ARA diet	inc	0.151 ± 0.009^b	0.158 ± 0.006^b	0.198 ± 0.017^a
	ni	0.163 ± 0.018^{ab}	0.154 ± 0.009^b	0.184 ± 0.013^a
0.6% ARA diet	inc	0.177 ± 0.016	0.156 ± 0.025	0.172 ± 0.003
	ni	0.165 ± 0.011	0.166 ± 0.008	0.169 ± 0.009

Values are mean ± S.D., $n=12$ for each diet. Means with different superscripts within the same row are significantly different ($p<0.05$). No significant difference between incubated and non-incubated eggs was observed. No significant diet effect on total lipid was observed.

Table 32. Total lipid (mg /egg) in Atlantic halibut eggs spawned in July according to their diet in 2002 and 2003.

	Egg quality	2002	2003
Control diet	inc	0.176 ± 0.010^b	0.210 ± 0.009^a
	ni	0.198 ± 0.012	0.201 ± 0.011
Squid diet	inc	0.183 ± 0.032	0.202 ± 0.001
	ni	0.192 ± 0.014	0.202 ± 0.008
0.4% ARA diet	inc	0.176 ± 0.009^b	0.192 ± 0.004^a
	ni	0.193 ± 0.009	0.193 ± 0.041
0.6% ARA diet	inc	0.170 ± 0.011	0.193 ± 0.007
	ni	0.178 ± 0.013	0.189 ± 0.001

Values are mean ± S.D., $n=12$ for each diet. Means with different superscripts within the same row are significantly different ($p<0.05$) No significant difference between incubated and non-incubated eggs was observed. No significant diet effect on total lipid was observed

3.3.2 Lipid class compositions

In 2001 halibut eggs spawned by trial broodstock fed the 0.6% ARA diet were significantly different when comparing their lipid class composition (Figure 41) to eggs spawned by broodstock fed control, squid and 0.4% ARA diets. Overall, PC CH and SE were significantly lower and PE and PI were significantly higher. However, in 2002 (Figure 42) PC was lowest in eggs from females fed the 0.4% ARA diet, and in 2003 (Figure 43) PC distribution was reversed as the highest level was observed in eggs from females fed the 0.6% ARA diet and the lowest in eggs spawned by fish fed the 0.4% ARA diet. In terms of CH through the three year project and TAG, SE in 2003, these lipid classes were significantly lower in eggs from females fed the 0.6% ARA diet than in females fed control and squid diets and could be related to the lower lipid content observed in these eggs. Total phospholipid to CH ratio (Table 33) showed a significant decrease when comparing 2001 to 2003 for all diet treatements. Through the three spawning seasons, Σ PL: CH ratio in eggs spawned by females fed the 0.6% ARA diet was significantly higher than in eggs of females fed the other trial diets except in 2003 when compared to the control diet.
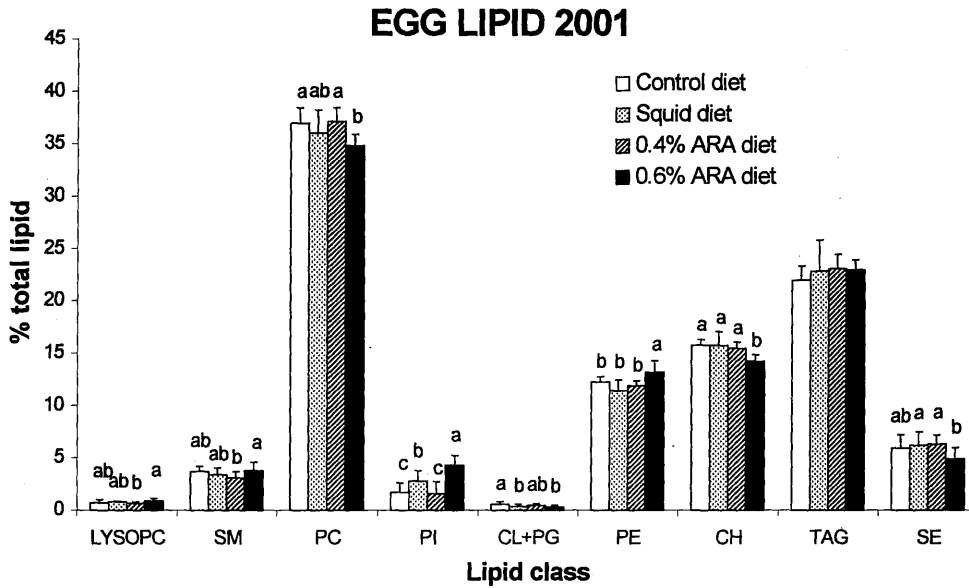
EGG LIPID 2001

Figure 41. Egg lipid class compositions (% of total lipid) in halibut broodstock fed trial diets in 2001. Columns assigned a different letter are significantly different within each lipid class ($p<0.05$).
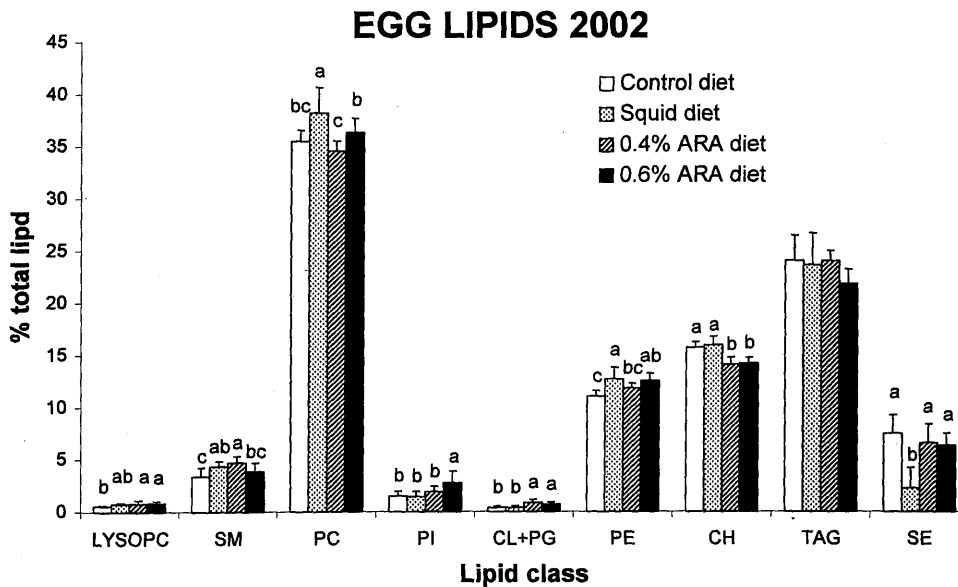
EGG LIPIDS 2002

Figure 42. Egg lipid class compositions (% of total lipid) in halibut broodstock fed trial diets in 2002. Columns assigned a different letter are significantly different within each lipid class ($p<0.05$).

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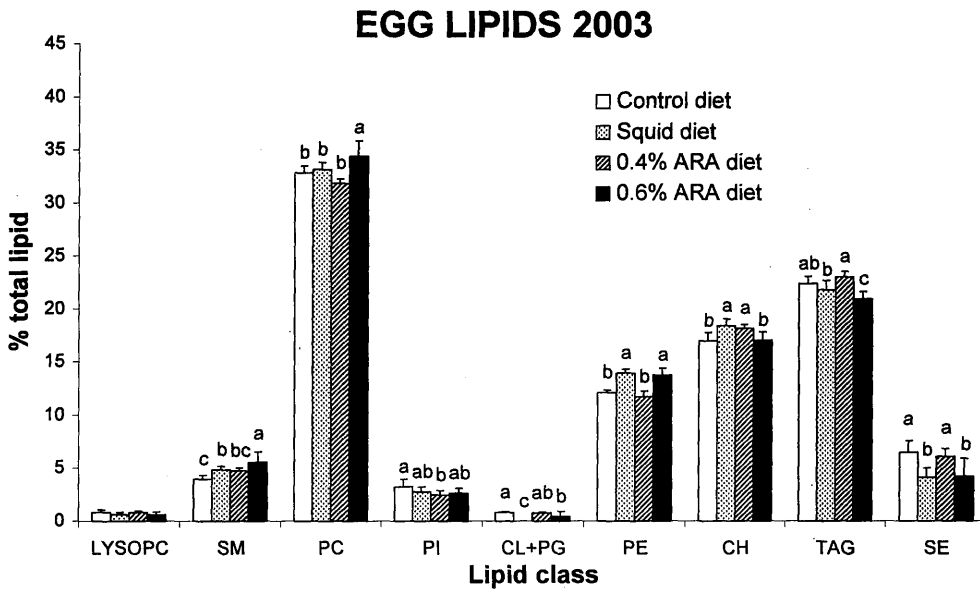
EGG LIPIDS 2003

Figure 43. Egg lipid class compositions (% of total lipid) in halibut broodstock fed trial diets in 2003. Columns assigned a different letter are significantly different within each lipid class ($p<0.05$).

Table 33. Σ phospholipid to cholesterol ratio (Σ PL: CHOL)

	2001	2002	2003
Control diet	3.27 ± 0.07 [b*]	3.09 ± 0.15 [c**]	2.89 ± 0.14 [ab***]
Squid diet	3.26 ± 0.41 [b*]	3.33 ± 0.15 [b*]	2.74 ± 0.10 [bc**]
0.4% ARA diet	3.32 ± 0.24 [b*]	3.49 ± 0.18 [b*]	2.57 ± 0.38 [c**]
0.6% ARA diet	3.75 ± 0.23 [a*]	3.70 ± 0.24 [a*]	3.02 ± 0.26 [a**]

Values are means ± S.D., $n=12$. Means with different superscripts within the same column are significantly different ($p<0.05$). Means[*]>means[**]>means[***] within the same row are significantly different ($p<0.05$)

3.3.3 Fatty acid compositions

A diet effect was observed in incubated eggs and non-incubated eggs in total fatty acid content (Figure 44), ARA (Figure 45), DHA/EPA ratio (Figure 46), EPA/ARA ratio (Figure 47) and $\Sigma n\text{-}3/\Sigma n\text{-}6$ ratio (Figure 48) through the three-years project.

Total fatty acid concentration decreased significantly between 2001 and 2003 for all treatments and means $\pm$ S.D. were respectively 565$\pm$54 and 491$\pm$46 µg fatty acid/mg lipid in these years (Figure 44). Significant diet effects on egg total fatty acid content were observed in both incubated and non-incubated eggs in 2001 and in non-incubated eggs in 2002 but there was no significant diet effect on egg total fatty acids in 2003. Eggs spawned by females fed the squid diet in 2001 showed the lowest level of total fatty acids and incubated eggs total fatty acid contents were lower than non-incubated egg total fatty acid content.

In 2001 there were no significant diet effects on ARA in eggs spawned by trial females and mean $\pm$ SD ARA concentration was 7.43 $\pm$ 1.26µg fatty acid/mg lipid. There were significant differences in ARA in eggs spawned by females fed the control and squid diets over the three spawning seasons. In 2002 females fed 0.6% ARA produced eggs with the highest ARA concentration; their non-incubated eggs were also higher in ARA than incubated eggs with respectively 24.03 $\pm$ 4.31 and 22.66$\pm$2.17 µg fatty acid/mg lipid. Eggs from females fed the 0.4% ARA diet were significantly lower in ARA

(18.08±1.79μg fatty acid/mg lipid) than for females fed the 0.6% ARA diet but significantly higher than eggs from females fed control and squid diets. A similar ARA concentration distribution was observed in eggs of females fed the 0.4% ARA diet in 2003. In 2003 egg ARA concentration was lower when spawned by females fed the 0.6% ARA diet than females fed the 0.4% ARA diet having, respectively, 42.66 ± 3.21 and 31.6±4.65μg fatty acid/mg lipid. As in 2002, halibut broodstock fed the 0.6% ARA diet produced eggs with significantly the highest ARA concentration. ARA concentration was significantly lower in non-incubated eggs (5.24 ± 0.87μg fatty acid/mg lipid) than in incubated eggs (6.44±0.61μg fatty acid/mg lipid) for females fed the squid diet (Figure 45).

The DHA/EPA ratio (Figure 46) was significantly higher in non-incubated eggs in 2001 and in both egg groups in 2002 and 2003 for females fed the 0.6% ARA diet. Non-incubated eggs showed a significantly lower DHA/EPA ratio than incubated eggs when produced by females fed the squid and 0.4% ARA diets in 2003. There were no significant diet effects on egg DHA/EPA ratio and EPA/ARA ratio in incubated eggs spawned in 2001.

The EPA/ARA ratio (Figure 47) was lower in eggs spawned by females fed the 0.6% ARA diet than in eggs spawned by females fed the 0.4% ARA diet, and was the highest, with no significant difference, in eggs spawned by females fed the control and squid diets in 2002 and 2003.
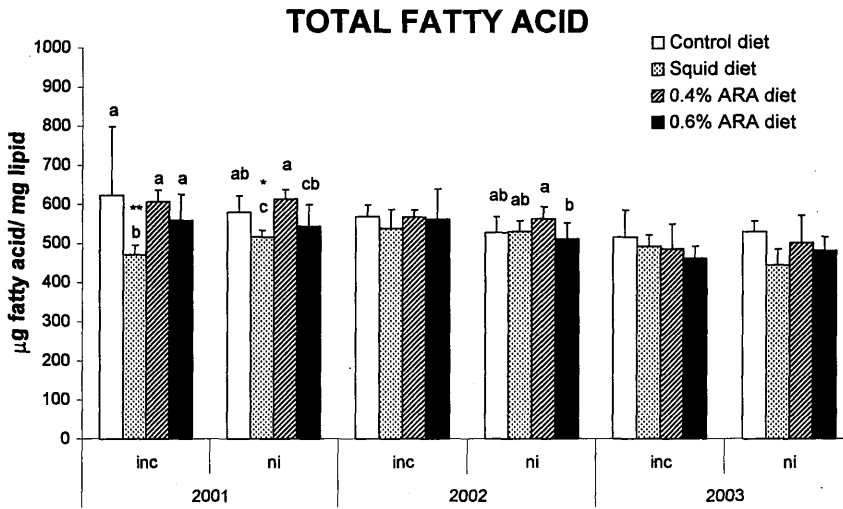
TOTAL FATTY ACID

Figure 44. Total fatty acid content in Atlantic halibut eggs (mean ± S.D., n=12) incubated (inc) or non-incubated (ni) and spawned by broodstock fed the trial diets over the three year project. Columns assigned a different letter are significantly different within the same egg group. * significantly higher than ** in the same year between incubated and non incubated eggs ($p<0.05$)
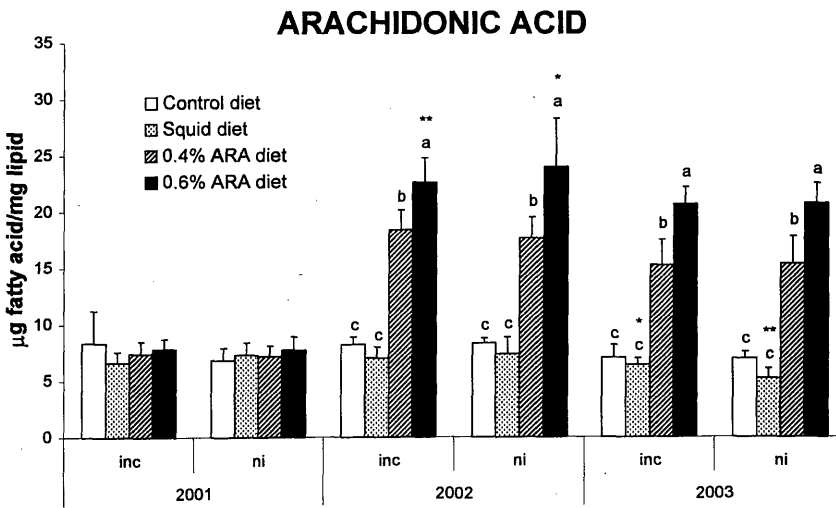
ARACHIDONIC ACID

Figure 45. Arachidonic acid concentration in Atlantic halibut eggs (mean ± S.D., n=12) incubated (inc) or non-incubated (ni) and spawned by broodstock fed the trial diets over the three year project. Columns assigned a different letter are significantly different within the same egg group. * significantly higher than ** in the same year between incubated and non incubated eggs ($p<0.05$)

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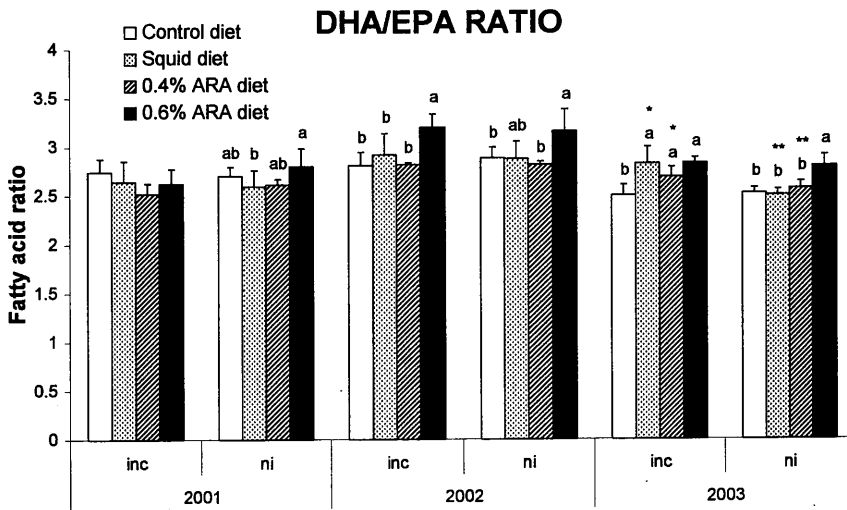
Figure 46. DHA/EPA ratio in Atlantic halibut eggs (mean ± S.D., n=12) incubated (inc) or non-incubated (ni) and spawned by broodstock fed the trial diets over the three year project. Columns assigned a different letter are significantly different within the same egg group. * significantly higher than ** in the same year between incubated and non incubated eggs ($p<0.05$)
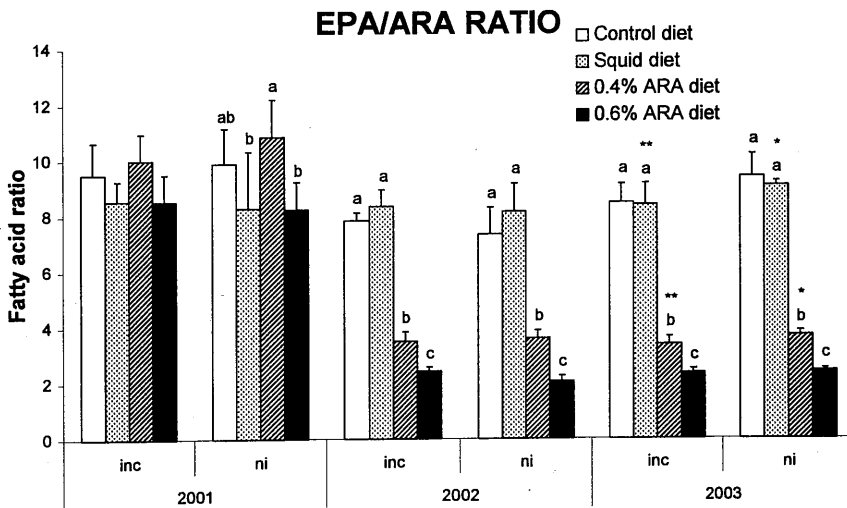
Figure 47. EPA/ARA ratio in Atlantic halibut eggs (mean ± S.D., n=12) incubated (inc) or non-incubated (ni) and spawned by broodstock fed the trial diets over the three year project. Columns assigned a different letter are significantly different within the same egg group. * significantly higher than ** in the same year between incubated and non incubated eggs ($p<0.05$)
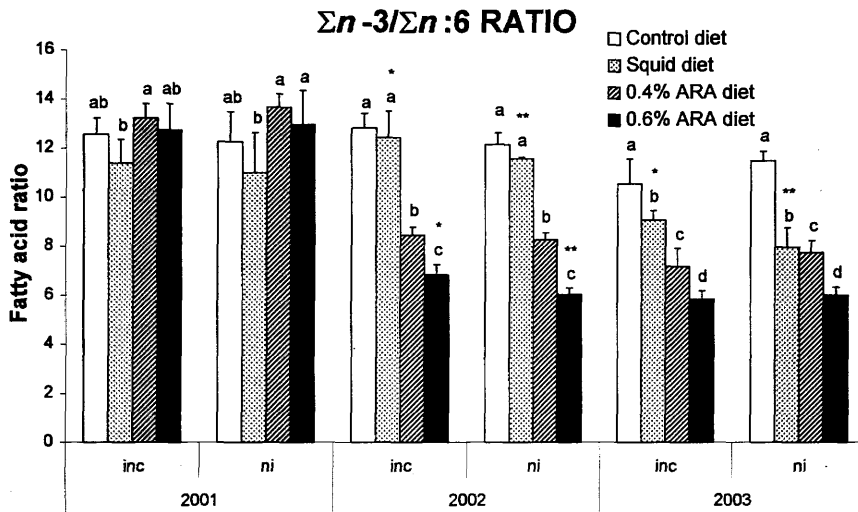
Figure 48. Σn-3/Σn-6 ratio in Atlantic halibut eggs (mean ± S.D.) incubated (inc) or non-incubated (ni) and spawned by broodstock fed the trial diets over the three year project. Columns assigned a different letter are significantly different within the same egg group. * significantly higher than ** in the same year between incubated and non incubated eggs ($p<0.05$)

Non-incubated eggs showed a significantly higher EPA/ARA ratio than incubated eggs when produced by broodstock fed the squid and 0.4% ARA diets in 2003.

In 2001, Σn-3/Σn-6 ratio (Figure 48) in incubated eggs from halibut fed the squid diet was significantly lower than in halibut fed the 0.4% ARA diet. Σn-3/Σn-6 ratio was also significantly lower in non incubated eggs from halibut fed the squid diet compare to halibut fed the 0.4% ARA diet and 0.6% ARA diet. In 2002 and 2003 significantly the lowest Σn-3/Σn-6 ratio was observed in eggs spawned by females fed the 0.6% ARA diet followed by eggs spawned by females fed the 0.4% ARA diet. There was no significant difference in Σn-

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$3/\Sigma n$-6 ratio between eggs spawned by halibut fed the control diet and eggs spawned by halibut fed the squid diet in 2002. In 2003 females fed the squid diet produced eggs with significantly lower Σn-3/Σn-6 ratios than when fed the control diet. Σn-3/Σn-6 ratio was significantly lower in non-incubated eggs than in incubated eggs spawned by females fed the squid and the 0.6% ARA diets in 2002 and spawned by females fed the squid diet in 2003.

4 Discussion

4.1 Halibut eggs before fertilisation, fertilised eggs and non-fertilised eggs

In order to study the baseline profile of halibut eggs in terms of lipid class and fatty acid composition, eggs at different stages in the fertilisation process were analysed. In the present study, eggs before fertilisation, fertilised eggs and non-fertilised eggs have shown no significant difference either in their lipid class composition or in their fatty acid composition. This is in accordance with observations by Parrish *et al.* (1993). However, total egg lipid content was significantly higher in eggs sampled before fertilisation than in fertilised or non-fertilised eggs. This can be explained by the fact that eggs before fertilisation were sampled directly in the ovarian fluid whilst fertilised and non-fertilised eggs were rinsed in UV treated seawater. The ovarian fluid was then highly diluted as well as its lipid content. Polar lipid classes predominated in halibut eggs with PC being the major class (37.41 ± 2.55%) and TAG (20.65 ±

2.69%) the main neutral lipid class. PC has long been known for its structural role in eggs, but has more recently been implicated as a major source of energy in the eggs and larvae of many fish species. PC is catabolised in halibut, plaice and turbot (Rainuzzo *et al.*, 1992) and in cod (Fraser *et al.*, 1988) during embryogenesis, prior to first-feeding. In terms of neutral lipid, analyses of lipid class composition of sea bass eggs revealed the presence of high levels of wax esters which provide an important source of energy in addition to, or instead of, TAG (Farndale *et al.*, 1999; Sargent *et al.*, 1999). Moreover high levels of triacylglycerol (TAG) were linked with the poorer quality eggs of sea bass (Navas *et al.*, 1997). Wax esters have also been observed in other marine fish species but seem to be absent or low in Atlantic halibut. TAG is generally regarded as the major energy reserve in fish eggs with long developmental times (Tocher & Sargent, 1984).

4.2 Halibut eggs from different origins and different year of collection

The values reported for Otter Ferry halibut egg lipid class composition correspond with published data (Table 34) for Atlantic halibut eggs (Falk-Petersen *et al.*, 1989, Bruce *et al.*, 1993). As for lipid class composition, the observed fatty acid profiles were similar to those recorded for halibut eggs collected from wild females at sea (Falk-Petersen *et al.* 1989) or from farmed broodstock (Parrish *et al.*, 1993; Bruce *et al.*, 1993).

Table 34: Lipid class composition (% of total lipid) of egg total lipid from different fish species.

	Sea bass	Atlantic Halibut		
	Bell, 1997	Falk-Petersen et al., 1989	Bruce et al., 1993	Otter Ferry 2001
Sphingomyelin (SM)	0.3	1.8	4.0	3.5
Phosphatidylcholine (PC)	10.9	62.2	43.2	36.7
Phosphatidylinositol (PI)	1	-	5.3	2.6
Phosphatidylethanolamine (PE)	3.1	6.7	14.6	12.1
Total polar lipid	15.8	70.8	67.4	56.1
Cholesterol (CH)	4.6	10.3	14.2	15.6
Triacylglycerol (TAG)	26.4	12.9	18.8	22.4
Sterol ester	49.7	-	-	5.68
Total neutral lipid	83.6	29.2	33	43.8

Halibut eggs, as for fish tissues in general, are naturally rich in highly unsaturated fatty acids (HUFA) of the n-3 series, DHA and EPA. In the present study it was interesting to observe significantly higher ARA content in Otter Ferry 2001 eggs than in 2002 Canadian eggs. 1996 Canadian eggs presented a lower EPA/ARA ratio when compared with 2002 Canadian eggs. In 1996 Canadian halibut broodstock was composed mainly of fish recently captured from the wild. The decrease in EPA, and more markedly in ARA, concentration in eggs spawned by St Andrews fish in 2002 could demonstrate a deficiency in essential EFA broodstock diet compared to their wild diet. In comparison EFA levels supplied in Otter Ferry broodstock diets seemed to be appropriate for the species. Similar fatty acid differences were observed

between wild and captive walleye (Czesny & Dabrowski 1998), striped bass (Gallagher *et al.*, 1998) and white sea bream (Cejas *et al.*, 2003) where the ARA percentage was higher in wild eggs than in eggs from captive fish whereas EPA showed the opposite trend, affecting the EPA/ARA ratio. The distinction between domesticated and wild fish based on egg fatty acids is already used to discriminate wild and cultured sturgeon caviar. By this method the conservation of wild stocks can be reinforced in parallel to the enhancement of stocks with fish produced by aquaculture (Czesny *et al.*, 2000).

4.3 Diet effect on halibut egg lipid quality

The main objective of the present study was to investigate the effect of increasing ARA levels in broodstock diets during three consecutive spawning seasons on lipid composition and quality in order to optimise halibut early life stages. Therefore eggs from halibut broodstock fed four different diets (control, squid, 0.4% ARA and 0.6% ARA diets) were analysed and their lipid composition determined. As no significant difference were observed in the lipid class profile and in the fatty acid profile of the different egg stages in the fertilisation process it was decided to sample only eggs before fertilisation in the present diet trial. To avoid lipid contamination from ovarian fluid it was decided to rinse the trial diet eggs, sampled before fertilisation, in UV treated seawater.

Total lipids in July spawned eggs were significantly higher than May spawned eggs, however, in terms of lipid class and fatty acid composition, no significant difference was found. Therefore, lipid class and fatty acid analyses used in the present study mainly concerned eggs spawned in May.

The fatty acid profile of the diets was very closely related to the levels in eggs subsequently produced by the broodstock. Halibut females fed ARA enriched diets presented a higher ARA content in their eggs suggesting a concentration and preferential deposition of this important EFA. Female halibut broodstock selected for the project were first time spawners in 2001 explaining the significant increase in egg total lipid through the three-spawning seasons. In 2002 and 2003 the same females could then be defined as repeat spawners. Improvement in reproductive performance and higher egg lipid content was observed in Atlantic halibut between first time spawners and repeat spawners thus allocating more lipids to egg production (Daniel *et al.*, 1993; Evans *et al.*, 1996). The increase in broodstock egg volume and total lipid content in 2002 and 2003 confirmed the previous observation of Evans *et al.* (1996). Thus, as suggested by Evans *et al.* (1996), higher lipid contents in repeat spawner eggs appears to be important for fertilisation success and egg viability in Atlantic halibut. In contrast, total lipid has been shown to be lower in viable eggs than in overripe eggs in turbot (Devauchelle *et al.*, 1988). The four different diets did not have any significant effect on egg total lipid over the three-year spawning season and no significant differences in egg total lipids between

incubated eggs and non-incubated eggs were observed. In terms of lipid class composition, Parrish *et al.* (1993) have shown a relationship between fertilisation rate and phospholipid level in captive halibut, the highest fertilisation rate corresponding to the lowest overall level of phospholipid. Otter Ferry halibut broodstock involved in the present project started to feed on the four formulated trial diets on the 1st May 2001, around the same time when May spawning broodstock began to spawn. Although for this reason no significant effects of the trial diets were expected regarding lipid class and fatty acid composition of 2001 eggs, significant differences were observed. Females fed the 0.6% ARA diet displayed the lowest production performances through the three-year project and their eggs showed significant differences in lipid class composition when compared to the three other dietary groups. In 2001 PC, CH and SE were significantly lower while lysoPC, SM, PI and PE were significantly higher in eggs from fish fed the 0.6% ARA diet. In 2002 and 2003 an increase was observed with significantly higher levels of PC in eggs from females fed the 0.6% ARA diet. However, CH percentage remained significantly lower in eggs from this group in 2003 and TAG became significantly lower in 2003 in these same eggs. No other clear pattern of egg lipid class composition was observed in relation trial diet. Previous studies in sturgeon have shown similar egg phospholipid contents among three different populations (species and location) indicating a more conservative and essential character of this lipid fraction compared to neutral lipids (Czesny *et al.*, 2000).

In halibut very similar egg lipid composition was shown regardless of origin, date of collection and egg viability. Cholesterol was the only lipid component showing a small but significant difference between egg categories (Bruce, 1998). In the present study the total phospholipids / cholesterol ratio varied significantly between trial diets and over the three spawning seasons. The significant decrease in ΣPL / CH ratio over the three spawning seasons may be related to the improvement in female reproductive performance and female first time spawners becoming repeat spawners. Furthermore broodstock fed the 0.6% ARA diet produced eggs with a significantly higher ΣPL / CH ratio over the three-year study. These results suggested a potential link between ΣPL / CH ratio and subsequent egg quality. As Parrish *et al.* (1993) suggested, eggs showing better fertilisation rates might be related to the low level of egg PL, in a way that PL content would be negatively correlated with fertilisation success.

In addition to the ΣPL / cholesterol ratio decrease, a decrease in total fatty acid content was observed over the three-year study. Significant differences were observed among the total fatty acid content in 2001. In addition, ARA contents were significantly higher and EPA content, EPA/ARA and Σn-3/Σn-6 ratios were significantly lower in eggs in 2002 and 2003 in females fed ARA enriched diets. The highest egg ARA content was observed in females fed the 0.6% ARA diet followed by eggs from females fed 0.4% ARA while both were significantly higher than in eggs from females fed control or squid diets. In contrast the EPA/ARA and Σn-3/Σn-6 ratio followed the opposite trend. The

effect of maternal diet with respect to n-3 / n-6 ratio was demonstrated in sturgeon, with cultured fish presenting a higher ratio than wild fish (Ceszny *et al.*, 2000). Evidence of increased ARA and of decreased EPA transfer, with subsequently lower EPA/ARA ratios, were previously observed in halibut eggs from females fed an ARA enhanced diet (Mazorra *et al.*, 2003). DHA/EPA ratios were significantly higher in eggs from females fed 0.6% ARA than in females fed the other trial diets during the three spawning seasons. However, there were no significant differences between DHA contents in the eggs, so the ratio differences are more likely due to decreased egg EPA content. In order to correlate egg fatty acid profile and egg quality, sampled eggs were separated into two groups, incubated eggs (viable eggs included in the fishfarm production with fertilisation rates > 35%) and non-incubated eggs (non viable eggs, fertilisation rate < 35%). ARA content was significantly higher in non-incubated eggs than in incubated eggs in females fed the 0.6% ARA diet in 2002 and this was the highest egg ARA content observed over the trial (49.8±3.1 μg/mg lipid). ARA content was significantly lower in non-incubated eggs than in incubated eggs of females fed the squid diet in 2003 and this was the lowest egg ARA content observed over the trial (11.0±1.6 μg/mg lipid). These maximal and minimal values could define the ARA thresholds for egg viability. Japanese flounder egg ARA content and egg quality parameters both decreased with increasing dietary *n-3* HUFA levels suggesting that a high level

of *n-3* HUFA in broodstock diet negatively affects egg quality in Japanese flounder (Furuita *et al.,* 2002).

In vitro studies have shown that free ARA induces seabass oocyte maturation in a dose- and time-dependent manner and enhanced gonadotropin, (GTH)-induced maturation, while EPA, DHA, and oleic acid were ineffective. Moreover, EPA and DHA exhibited a significant, dose-dependent, attenuation of GTH-induced maturation (Asturiano *et al.,* 2000; Sorbera *et al.,* 2001). This emphasises the importance of DHA/ EPA/ ARA ratio for female reproduction. Female halibut fed the squid and 0.4% ARA diets had DHA/EPA ratios significantly lower in non-incubated eggs than in incubated eggs and EPA/ARA ratios significantly higher in non-incubated eggs than in incubated eggs. The fatty acid composition of the PLs of salmon and cod eggs was dominated by (n-3) PUFA and in both cases the DHA/EPA ratio was approximately 2:1 (Tocher & Sargent, 1984; Wilson & Cowey 1985). In the present study DHA/EPA ratios were in the range of 2.5 to 3.2. The importance of balancing the three functional essential fatty acids, DHA, EPA and ARA, has been highlighted in fish nutrition (Sargent, 1995). Halibut egg fatty acid composition results could indicate the provision of an excessive level of ARA in the females fed the 0.6% ARA diet through the higher DHA/EPA ratio and lower EPA/ARA ratio in their eggs and their generally lower reproductive performances. Survival of walleye embryos was correlated with the concentration of PUFA suggesting that deficiency in n-3 fatty acids might be

associated with impaired development (Czesny & Dabrowski, 1998). Different fish species have different optimal fatty acid requirements. Fatty acid composition and quality of gilthead seabream eggs were affected when n-3 HUFAs were substituted in a broodstock diet by 18:1n-9 and 18:3n-3. The high negative correlation found between 18:3n-3 or 18:1n-9/HUFA ratio in polar lipids and egg fertility, suggests the importance of maintaining a high level of n-3 HUFA in egg membrane phospholipids to obtain high egg quality (Almansa et al., 1999). Higher quality eggs of European sea bass (Navas et al., 1997) and Asian sea bass (Nocillado et al., 2000) contained higher levels of total *n-3* fatty acids, including increased levels of both DHA and EPA. The small quantities of ARA in fish eggs are located almost exclusively in PI and a specific role for this lipid class and ARA in eicosanoid formation is indicated (Furuita et al., 2001). However, not all differences in broodstock performance can be due to diet lipid composition. In sea bass, general improvements in egg performance characteristics were seen between spawning seasons. The apparent overall improvements in egg survival and hatching between successive reproductive cycles may have been due to the age of the broodstock (Bruce et al., 1999). Changes in membrane lipid composition of halibut eggs might affect their physico-chemical properties and alter the supply of precursor fatty acids for eicosanoid production, thus influencing the process of fertilisation and the initiation of cell division (Bruce, 1998). No correlation was observed between the HUFA content of turbot eggs and any egg quality criteria

even though significant differences were observed between females fed trash fish (higher 18:3n-3, 20:4n-6, total lipid content and DHA/EPA ratio) and those fed with commercial pellets (Peleteiro *et al.*, 1995).

The higher reproductive performance of females fed a 0.4% ARA enriched commercial diet suggests that a ratio of 3.5:1 would be an optimum EPA/ARA ratio, which agrees with the data of Mazzora *et al.* (2003).

Chapter 7 Lipid dynamics in halibut early life stage

1 Introduction

One of the major difficulties with culture of early life stages of halibut is the long time periods required for egg incubation and maintenance of yolk-sac fry (Pittman *et al.,* 1990). Despite species differences, it is generally accepted that the quality of maternal nutrition has a direct influence on larval development throughout the period when the larvae are dependent upon their yolksac i.e. endogenous energy reserves. Study of the lipid dynamics in halibut early life stages could provide important indications to allow formulation of a satisfactory diet for first-feeding larvae.

1.1 Egg husbandry

In the wild water density and convective currents determine the vertical distribution of eggs. Seawater temperatures in halibut spawning zones generally vary between 4.5 and 7°C and salinities range from 33.9 to 35.0‰ (Haug *et al.,* 1982). After spawning fertilised eggs gradually move upwards in the water column. The vertical distribution of eggs is influenced by biological factors as well as the prevalent hydrography of the spawning ground. The eggs achieve a higher specific gravity in their latest developmental phase (Haug, 1990). Studies have shown that temperature during egg incubation affects rate

of development, growth and mortality as well as rate of protein synthesis and cell division at hatching (Bergh *et al.*, 1989; Pittman *et al.*, 1990). The optimal developmental temperature for halibut eggs is presumed to be 5°C. The lower and upper thermal tolerance limits for early life stages of Atlantic halibut would be 2 and 9°C respectively (Haug, 1990).

1.1.1 Egg incubation

Halibut eggs are usually incubated in cylindro-conical tanks and maintained in the water column using an upwelling water flow (Bergh *et al.*, 1989). 250 l fibreglass units are used with a split inlet valve on the bottom for introduction of new water and extraction of sedimented material and a sleeve filter for overflowing water at the top. The water flow is set at 3l/min and a maximum of 3l of fertilised eggs are incubated in one vessel. The husbandry routines are of great importance to hatching success. Use of a 'brine plug' should avoid any accumulation of dead or dying eggs as described by Jelmert & Rabben (1987). As eggs incubated in white light remained unhatched, a fitted lid on the top of the tank prevented light from reaching the developing eggs.

1.1.2 Egg disinfection

The surface of marine fish eggs constitutes a habitat well suited for many epibiotic bacteria and some of these are pathogenic. Intensive aquaculture systems imply monoculture of species at artificially high population densities, favouring bacterial development. The substantial amounts of bacterial activity

after 7 days of incubation were shown to be concentrated on the halibut egg surface and in the water shell surrounding each egg (Jelmert & Mangor-Jensen, 1987; Hansen & Olafsen, 1989). Great variation in quality between batches of halibut eggs and larvae are observed, and bacterial infections have been assumed to be a major cause of both egg mortality and occurrence of deformed larvae at early life stages (Bergh & Jelmert, 1990; Pittman *et al.*, 1990). Disinfection just prior to hatching of haddock eggs did not remove the bacterial flora but may have killed it and thereby reduced transfer to the emerging larvae (Morrison *et al.*, 1999). Several procedures are currently used to disinfect fish eggs to prevent transmission of diseases and therefore reduce problems with bacterial development in intensive egg incubation systems (Harboe *et al.*, 1994; Salvesen & Vadstein, 1995; Salvesen *et al.*, 1997). At Otter Ferry Seafish, 10ml of Kick-start 2® is added every three days to conical incubation vessels. Halibut eggs should not be transported immediately after closure of the blastopore, but much closer to hatching (Galloway *et al.*, 1999). Prior to moving to yolk sac conical tank, eggs at 69 dd are bathed for one minute in 30ml of Kick-start 2® thoroughly mixed with 10l of UV treated seawater. Kick-start 2® is an advanced biocidal formulation composed of 5% stabilised peroxyacetic acid and hydrogen peroxide mixture. Kick-start 2® is approved for use in the circulation cleaning and industrial sanitizing of equipment such as tanks, pipelines, evaporators, filters, aseptic equipment, and for sanitizing previously cleaned food contact surfaces of equipment. It can also be used for:

Kick-start 2® shows broad-spectrum efficacy against gram-negative and gram-positive bacteria, *E. coli* and many other organisms. Efficacy is unaffected at temperatures as low as 4.5°C, effective and stable at pH values between 5 and 9. Egg halibut disinfection by Kick-start 2® immersion is often preferred to glutaraldehyde from the standpoint of operator safety (Kristjannson, 1995).

1.1.3 Hatching

An annular hatching gland on the anterior yolk sac produces halibut-hatching enzyme (Helvik *et al.,* 1991). The darkness-generated signal ultimately causes release of hatching enzymes which digest the *zona radiata* so the larva may mechanically open the eggshell (Helvik & Pittman, 1990; Helvik & Walther, 1992). Halibut eggs raised in darkness hatched between days 14 and 16 after fertilisation at 6°C. Exposure to white light at a late egg stage and then return to darkness gives synchronised hatching within 90-120 minutes (Helvik & Walther, 1993).

1.2 Yolk sac larvae

When hatched at 6-7 mm standard length the larva is completely unpigmented and the head is confluent with the yolk sac (Rollefsen, 1934). There is no stomodeum and a faint ring of hatching glands can be seen on the anterior yolk sac reaching to behind the otoliths. There is a pair of external branchial pits connected to the oesophagus through which the larva may drink shortly after hatching. The gut is straight, the urinary bladder simple and the heart is a

simple tube. The yolk sac changes from an elliptical form to a pear-shaped form then reduces to a tubular form on the right side of intestine. Halibut larvae develop organs and pigmentation during the yolk-sac stage whereas other species complete it previously in the egg stage (Pittman, 1987). The behaviour of halibut larvae during the yolk-sac stage is limited to passive rising/sinking, resting and swimming. The buoyancy of halibut larvae is influenced both by the rearing salinity and by larval age (Lein *et al.*, 1997). Incubation of embryos at 8°C increased white muscle fibre hyperplasia compared to those incubated at 5°C (Galloway *et al.*, 1999). Moreover, development was slowest but total and relative protein synthesis highest in larvae raised at 3°C. Halibut larvae raised at 9°C quickly developed abnormalities, such as small hearts and livers, large peritoneal and pericardial spaces associated with sublethal stressors (Bergh, *et al.*, 1989; Lein *et al.*, 1997). During the yolk sac stage, halibut larvae double their length and increase their body weight more than six-fold (Blaxter *et al.*, 1983). Fish larvae expend their limited energy resources contained in the yolk sac with priority on the development of organs of importance for feeding and swimming. This is supported by the observation of axial musculature, which is the fastest growing tissue in the fish larval body (Galloway *et al.*, 1999). The developing embryos depend exclusively on endogenous yolk reserves to supply nutrients for energy and growth until they are able to actively first feed (Rønnestad *et al.*, 1995). Absorption of yolk is believed to occur by endocytotic uptake yolk mass, via the yolk syncytium, which encloses the

entire yolk mass after epiloby (Kjørsvik & Reiersen, 1992). In Atlantic halibut the liver is functional when 50% of the yolk sac mass has been absorbed. Based on histological observations it was concluded that the liver supersedes the role of the yolk syncytium as the major organ of yolk absorption at this stage (Kjørsvik & Reiersen, 1992). Nutrient movements between yolk and larvae in Atlantic halibut yolksac larvae have been studied. For example both ascorbic acid (AA) and α-tocopherol (α-TOH) function as potent antioxidants. Constant content of these nutrients (from hatching until first feeding) in Atlantic halibut larvae suggested that there was no significant loss or catabolism of these components during the yolksac stage. However, absorption from yolk to larvae of α-TOH is better correlated with uptake of protein than of lipids and 75% of AA was taken up in the first third of the yolksac stage (Rønnestad *et al.,* 1999). In halibut eggs it observed that energy metabolism is mainly lipid- or carbohydrate-based during the mid-development period but switches to free amino acids as hatching approaches (Finn *et al.,* 1991). Later in the yolksac stage of the Atlantic halibut, proteinic amino acid catabolism dominates (Rønnestad, 1992; Rønnestad *et al.,* 1998). There seemed to be some, but relatively minor, changes in the relative composition of lipids in the yolk throughout development, which are indicative of a non-selective endocytotic bulk uptake of lipids from the yolk (Rønnestad *et al.,* 1995). The amounts of lipids as well as the lipid classes catabolised vary among species and other sources of energy, such as carbohydrates and proteins, are also used

(Rainuzzo *et al.*, 1997). Differences were observed in activities of digestive enzymes. Trypsin, amylase, lipase and alkaline phosphatase showed a general pattern of increased activities from 161 to 276 dd. Trypsin activities reached their highest values at 230 dd, whereas lipase and alkaline phosphatase peaked at 276 dd. The pattern of continuous increase in lipase activity through the yolksac period in Atlantic halibut suggests that larvae are prepared to feed on lipid-rich zooplankton by 276 dd (Gawlicka *et al.*, 2000). This high lipase activity in the later stages of yolksac resorption coincides with the period of increased use of lipids for energy by halibut larvae (Rainuzzo *et al.*, 1992; Rønnestad *et al.*, 1995).

1.3 Overall aims

Study the effect of ARA enriched broodstock diet on Atlantic halibut early life stages (egg before fertilisation, egg at disinfection, and yolksac larvae just before first feeding) through:

➤ Survival at different stages in 2002 and 2003

➤ Total lipid and lipid class dynamics.

➤ Fatty acid and main PUFA ratio dynamics

2 Materials and Methods
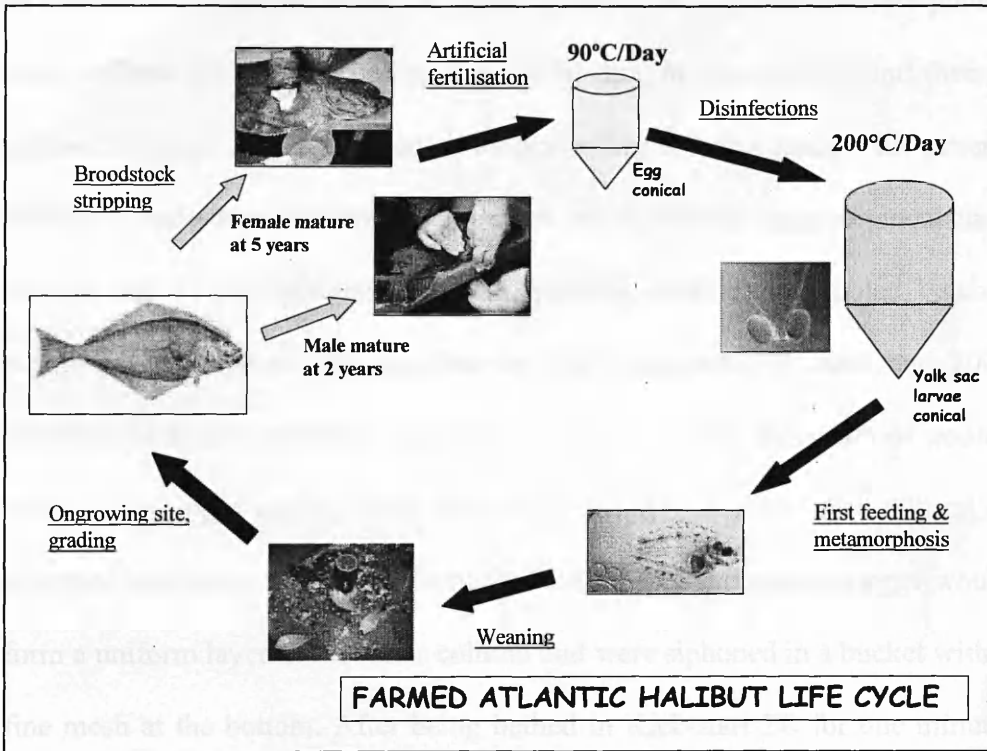
Figure 49. Farmed Atlantic halibut life cycle

The farmed Atlantic halibut life cycle followed at Otter Ferry SeaFish Ltd. is shown in Figure 49. Eggs and milt are collected by stripping the halibut broodstock. Artificial fertilisation is carried out by mixing eggs and milt in UV treated seawater. Egg batches with fertilisation rates above 35% are incubated in conicals with upwelling 5°C UV treated seawater. Then eggs are disinfected and moved to a yolk sac conical at 69 dd. Hatching occurs 90 dd post fertilisation and at 200 dd yolk sac resorption is completed and larvae are ready to be moved to a first feeding tank.

2.1 Halibut survival through first life stages

In order to study the diet effect on halibut production, egg batches were pooled when volume allowed, according to the trial diet, in egg conicals and then in yolksac conicals. Since this had to be performed in accordance with general fishfarm production standards (minimum of 21 batches eggs in incubation conical and 11 of disinfected eggs in hatching conical) no pooled batches according to the diet were possible in 2001. However, in 2002 and 2003 increases in trial broodstock egg production allowed the formation of pooled conicals with eggs coming from broodstock fed the same trial diet. 400 ml of saturated salt water was added from the bottom of the conical so eggs would form a uniform layer in the water column and were siphoned in a bucket with a fine mesh at the bottom. After being bathed in Kick-start 2® for one minute, eggs were moved to a graduated sieve and total volume was recorded before placing the eggs in a yolksac conical. Hatching rate was the egg volume obtained at disinfection (69 dd), divided by egg volume total placed in the incubated conical. At 200 dd a light was placed at the top of the hatching conical, yolksac larvae being phototrophic they came to the surface where they were removed carefully with a jug to be transferred to the first feeding tank. Yolksac larval survival was estimated according to the number of yolksac larvae collected at the surface of the conical.

2.1.1 Eggs at disinfection sampling

Prior to bathing in the Kick-start 2®, when eggs were coming from a pooled egg sample of the same trial diet broodstock, eggs were collected with a plastic pipette and placed in petri dishes. Under a microscope 30 developed eggs were sampled and placed in 7ml glass vials containing 5ml of chloroform: methanol (2:1, v:v) + 0.01% BHT in duplicate and kept in a freezer until further biochemical analyses. Ten batches per diet were obtained in 2002 and four batches per diet in 2003 (Table 35). Lipid extraction, lipid class analyses and fatty acid composition were carried out as indicated in chapter 2 sections 2.3, 2.4 and 2.6 respectively.

Table 35. Halibut early life stages samples analysed in 2002 and 2003

	Egg before fertilisation	Egg at disinfection	Yolksac larvae prior to first feeding
Survival	Fertilised eggs	Hatched eggs	Yolksac larvae
2002	12 x 10 eggs for each diet	10 x 30 eggs for each diet	6 x 20 larvae for each diet
2003	12 x 10 eggs for each diet	4 x 30 eggs for each diet	2 x 20 larvae for each diet

2.1.2 Yolksac larvae

When transfering yolksac larvae from conical to first feeding tank at 200 dd after fertilisation, 20 larvae were sampled and placed in 7ml glass vials containing 5ml of chloroform: methanol (2:1, v:v) + 0.01% BHT, in duplicate,

and kept in a freezer untill further biochemical analyses. Six batches per diet were obtained in 2002 and two batches per diet in 2003 (Table 35). Lipid extraction, lipid class analyses and fatty acid composition were carried out as indicated in chapter 2 sections 2.3, 2.4 and 2.6 respectively.

2.2 Statistical analyses

Atlantic halibut eggs at disinfection and yolksac larval survival, total lipid, lipid class and fatty acid composition were pooled for statistical analyses using SPSS for windows version 11.5. Data were tested for normality using the method of Kolmogorov and Smirnov. Once validated, data were analysed by ANOVA and the *post-hoc* means comparison was performed by Tukey-s test when comparing eggs before fertilisation, eggs at disinfection, yolksac larvae and broodstock trial diet effects (Puri, 1996). Data were analysed by Leven's test for equality variance and variances T-test when comparing 2002 to 2003 survival (Puri, 1996). The significance level was set as $p<0.05$. All results are given as mean ± standard deviation (S.D.).

3 Results

3.1 Halibut first life stages survival

No significant diet effect was observed in 2002 and 2003 on first life stages survival (Figure 50) from eggs produced by halibut broodstock fed the trial diets, apart from females fed the 0.6% ARA diet which produced yolksac larvae with significantly higher survival rates in 2002 than in 2003. Fertilisation rate was slightly but not significantly higher in eggs from females fed the 0.4% ARA diet in 2002. When comparing mean cumulative survival (Figure 51) from spawned eggs to yolksac larvae between 2002 and 2003, no significant difference was observed.

FIRST LIFE STAGES SURVIVAL
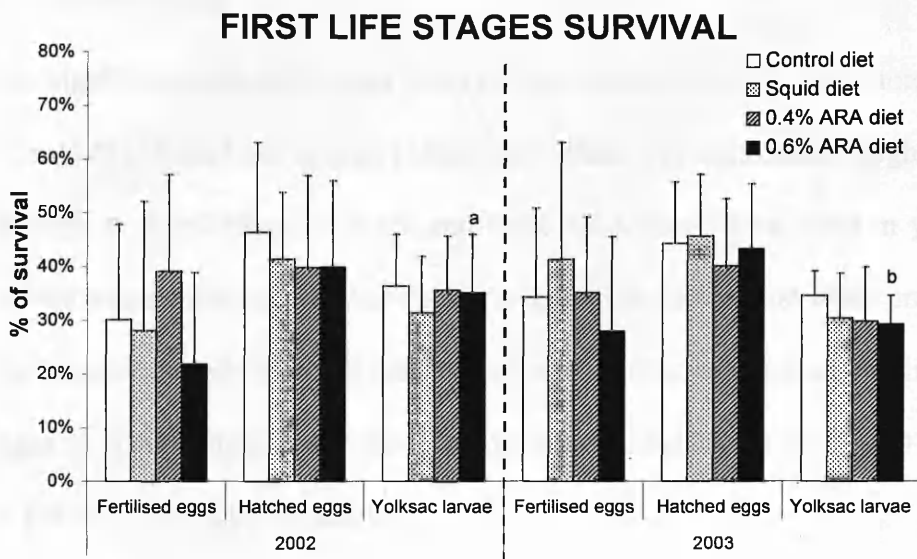
Figure 50. Halibut first life stages survival according to the trial broodstock diets in 2002 and 2003. Means± S.D expressed as % of survival; n=25 and n=27 in 2002 and 2003 respectively. Columns assigned a different letter are significantly different within the same diet between 2002 and 2003.

EARLY LIFE STAGES CUMULATIVE SURVIVAL
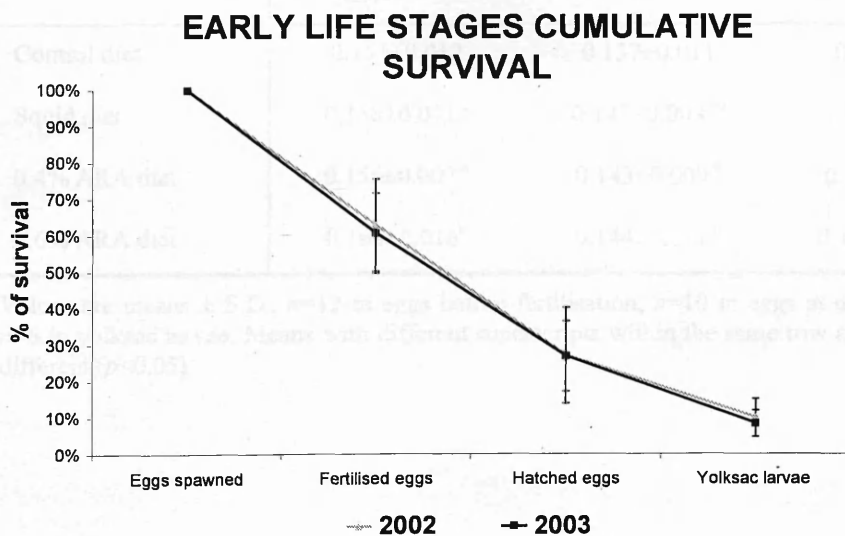
Figure 51. Mean cumulate survival of experimental halibut early life stages. Means± S.D expressed as % of survival; n=100 and n=108 in 2002 and 2003 respectively.

3.2 Total lipid

No significant diet effect was observed on halibut first life stage total lipid (Table 36). Total lipid in eggs before fertilisation was significantly higher than in eggs at disinfection for 0.4% and 0.6% ARA diets. Total lipid in yolksac larvae was significantly higher than in eggs before fertilisation when produced by broodstock fed the squid diet. Mean total lipid in eggs before fertilisation, eggs at disinfection and yolksac larvae was 0.157±0.012, 0.143±0.010 and 0.148±0.013 mg/egg respectively.

Table 36. Total lipid in eggs (mg/egg) and Yolksac larvae (mg/yolksac larvae) according to the broodstock trial diets.

	Egg before fertilisation	Egg at disinfection	Yolksac larvae
Control diet	0.151±0.012	0.137±0.011	0.143±0.012
Squid diet	0.158±0.011 [a]	0.147±0.009 [ab]	0.140±0.007 [b]
0.4% ARA diet	0.156±0.007 [a]	0.143±0.009 [b]	0.148±0.013 [ab]
0.6% ARA diet	0.163±0.016 [a]	0.144±0.011 [b]	0.160±0.0013 [ab]

Values are means ± S.D., $n=12$ in eggs before fertilisation, $n=10$ in eggs at disinfection and $n=6$ in yolksac larvae. Means with different superscripts within the same row are significantly different ($p<0.05$)

3.3 Lipid class composition

Eggs before fertilisation (Figure 52) produced by females fed the 0.6% ARA diet had a significantly higher PC and lower TAG content than produced by females fed the other trial diets. At disinfection eggs followed the same lipid class (Figure 53) distribution to that of eggs before fertilisation. PC dominated the polar lipid composition and TAG the neutral lipid composition. No significant diet effect was observed at disinfection life stages.

In contrast, yolksac larvae (Figure 54) produced by broodstock fed the 0.6% ARA diet had significantly the highest PC content compared to the other trial diets. When fed the squid diet, broodstock produced yolksac larvae with a significantly higher CH content and lower SE content.

To compare lipid class composition of halibut between early life stages (Figure 55), means of the four diet treatments and individual (/eggs or /yolksaclarvae) units was used. A significant decrease in PC was observed with values starting at 36.22±2.03% in eggs before fertilisation then decreasing to 33.51±2.02% in eggs at disinfection and finishing at 27.90±2.99% in yolksac larvae. In contrast, PS, PI, CL+PG and SE contents were significantly higher in yolksac larvae than in egg stages. PE was significantly lower and TAG was significantly higher in eggs at disinfection than in eggs before fertilisation and in yolksac larvae. It seems that PS, CL+PG and SE significantly increased between disinfection and yolksac larvae while TAG significantly decreased.

EGGS BEFORE FERTILISATION
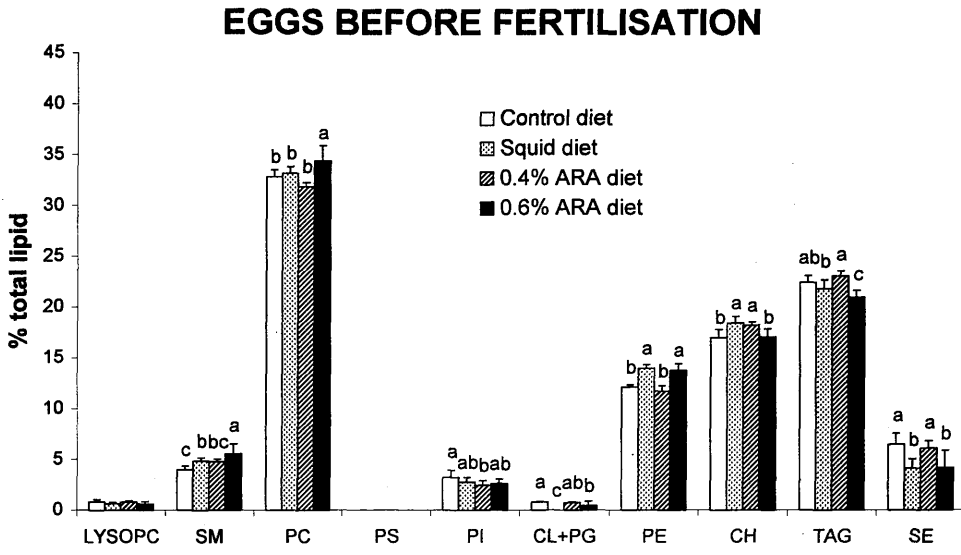
Figure 52. Lipid class composition of eggs before fertilisation expressed as % of total lipid from halibut broodstock fed trial diets. Columns assigned a different letter are significantly different within each lipid class ($p<0.05$).

EGGS AT DISINFECTION
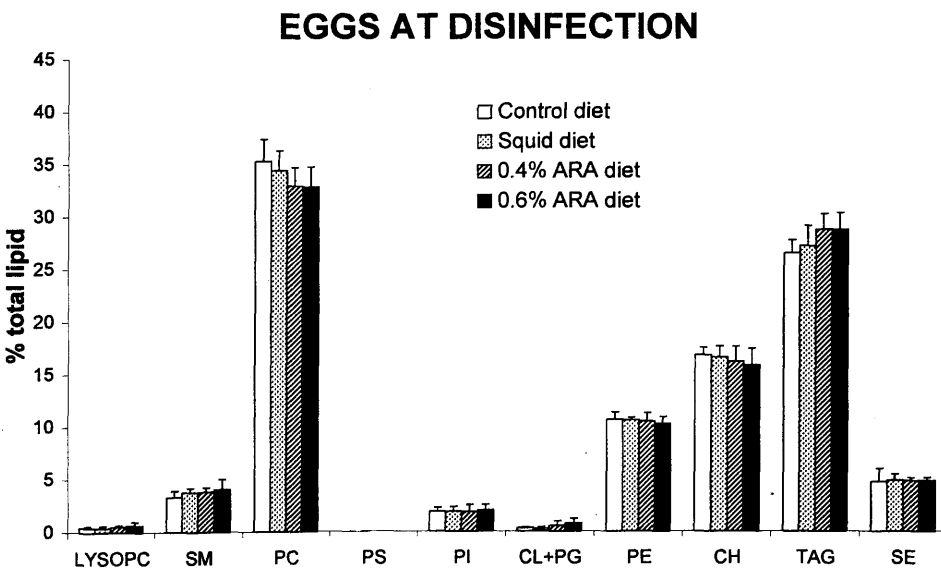
Figure 53. Lipid class composition of eggs at disinfection (% of total lipid) in halibut broodstock fed trial diets. No significant effect of broodstock diet was observed.
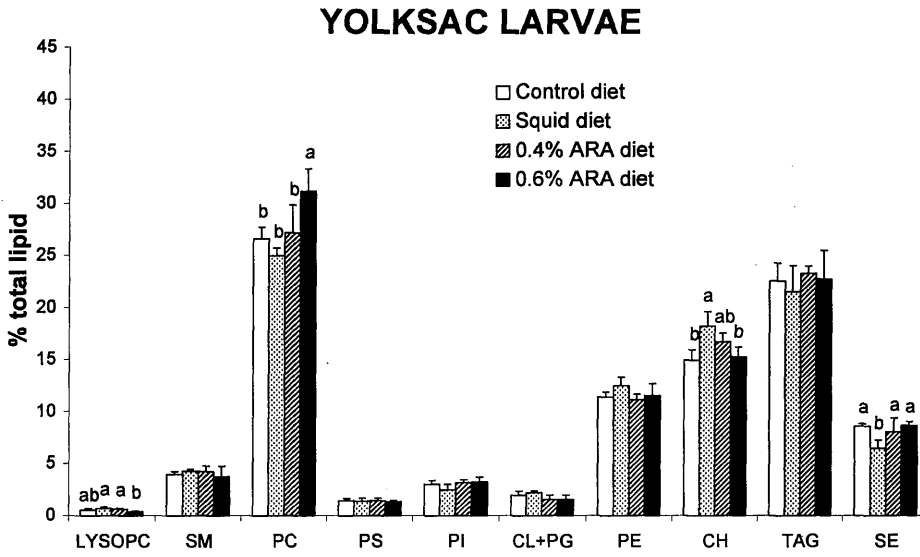
YOLKSAC LARVAE

Figure 54. Yolksac larvae lipid class composition (% of total lipid) in halibut broodstock fed trial diets. Columns assigned a different letter are significantly different within each lipid class ($p<0.05$).

LIPID CLASS DYNAMICS IN HALIBUT FIRST LIFE STAGES
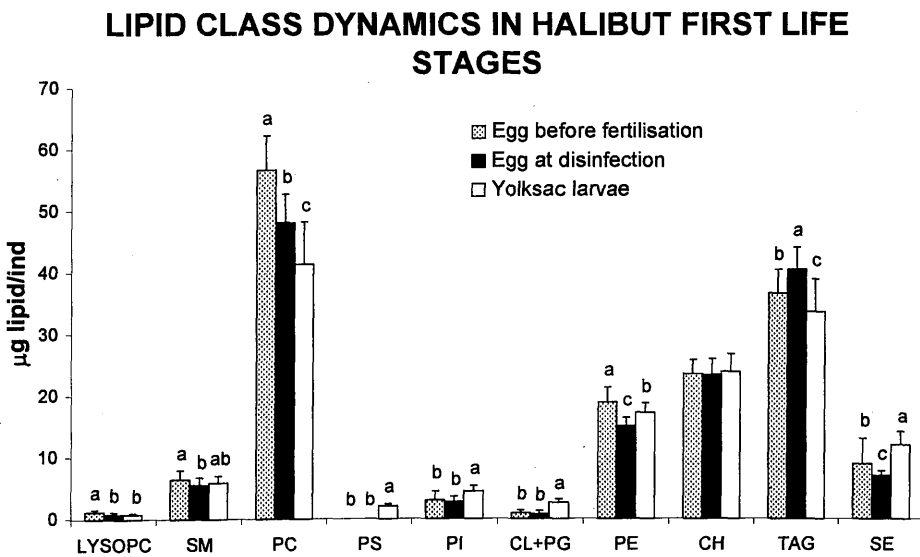
Figure 55. First life stages lipid class composition expressed as µg lipid/ind. Columns assigned a different letter are significantly different within each lipid class ($p<0.05$)

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The total phospholipid to cholesterol ratio (Table 37) was highest in eggs before fertilisation from broodstock fed the 0.6% ARA diet and lowest in eggs from broodstock fed the control diet. No significant diet effect was observed in eggs at disinfection on PL: CHOL ratio. In contrast, significantly the highest ratio was observed in yolksac larvae produced by females fed the 0.6% ARA diet. The ratio decreased significantly from eggs before fertilisation to yolksac larvae, except when females were fed the control diet.

Table 37. Σ phospholipid to cholesterol ratio (Σ PL: CHOL) in halibut first life stages

	Egg before fertilisation	Egg at disinfection	Yolksac larvae
Control diet	3.09±0.15 [c]	2.88±0.29	2.89±0.26 [ab]
Squid diet	3.33±0.15 [b*]	2.86±0.20 [**]	2.31±0.14 [c***]
0.4% ARA diet	3.49±0.18 [b*]	2.85±0.24 [**]	2.62±0.27 [bc**]
0.6% ARA diet	3.70±0.24 [a*]	2.91±0.26 [**]	3.14±0.39 [a**]

Values are means ± S.D., n=12 for eggs before fertilisation, n=10 for eggs at disinfection, n=6 for yolksac larvae. Means with different superscripts within the same column are significantly different ($p<0.05$). * Means within the same row are significantly different ($p<0.05$)

In contrast PC: PE ratio (Table 38) was significantly higher in eggs before fertilisation produced by broodstock fed control diet than all other diets. In yolksac larvae, it was significantly higher in ARA enriched diets. The overall production was characterised by a significantly higher PC: PE ratio in eggs at disinfection than in eggs before fertilisation and significantly the lowest ratio was observed in yolksac larvae.

Table 38. PC: PE ratio in halibut first life stages

	Egg before fertilisation	Egg at disinfection	Yolksac larvae
Control diet	3.21±0.13 [a]	3.31±0.07	2.33±0.14 [b]
Squid diet	3.01±0.17 [b]	3.25±0.22	2.00±0.09 [c]
0.4% ARA diet	2.92±0.12 [b]	3.17±0.38	2.45±0.29 [ab]
0.6% ARA diet	2.89±0.16 [b]	3.23±0.36	2.70±0.10 [a]
Total	3.01±0.19 [**]	3.22±0.31 [*]	2.40±0.30 [***]

Values are means ± S.D., $n=12$ for eggs before fertilisation, $n=10$ for eggs at disinfection, $n=6$ for yolksac larvae. Means with different superscripts within the same column are significantly different ($p<0.05$). * Means within the same row are significantly different ($p<0.05$)

3.4 Fatty acid dynamics

In the present study, to compare the fatty acid composition of total lipid from the three halibut early life stages (eggs before fertilisation, eggs at disinfection and yolksac larvae) (Table 39) means of the four diet treatments and individual (/eggs or /yolksaclarvae) units were used. Palmitic acid (16:0), oleic acid (18:1n-9), EPA (20:5n-3) and DHA (22:6n-3) were dominant. Stearic acid (18:0) was the only fatty acid to show significantly increasing amounts from eggs before fertilisation to yolksac larvae. Gadoleic acid (20:1n-9) decreased significantly from eggs before fertilisation to eggs at disinfection but then a significant synthesis was observed from eggs at disinfection to yolksac larvae. All other fatty acid contents decreased significantly between eggs before fertilisation to yolksac larvae except 18:3n-6 and ARA.

Yolksac larvae had lower total fatty acid contents (Figure 56) than eggs before fertilisation in all diets except 0.6% ARA and eggs at disinfection for control diet. Differences were significant only for eggs from females fed control or 0.4% ARA diets. Broodstock fed the control diet produced yolksac larvae with lower total fatty acid contents than broodstock fed the 0.6% ARA diet.

Females fed the 0.6% ARA diet produced eggs before fertilisation and eggs at disinfection with a significantly higher ARA concentration (Figure 57) followed by females fed the 0.4% ARA diet with ARA concentration in these eggs being significantly higher than when produced by females fed control and squid diets. ARA concentration was significantly lower in yolksac larvae than

in eggs before fertilisation and eggs at disinfection apart from eggs from females fed the squid diet.

No other significant differences were observed in ARA concentration of yolksac larvae produced by females fed ARA enriched diets, however, the ARA concentration was still significantly higher than when for eggs from females fed the control or squid diets.

Females fed the 0.6% ARA diet produced eggs before fertilisation and eggs at disinfection with a significantly higher DHA/EPA ratio (Figure 58) than females fed the squid or 0.4% ARA diets, but no significance difference was observed in their yolksac larvae. In contrast, females fed the control diet produced yolksac larvae with a significantly lower DHA/EPA ratio than females fed the 0.6% ARA diet but no significant difference was observed between their eggs at disinfection.

No significant EPA/ARA ratio differences were observed between the 3 stages for control and squid diets (Figure 59). For ARA enriched diets significant differences were shown with lower EPA/ARA ratio in yolksac larvae for the 0.4% ARA diet and in eggs before fertilisation in 0.6% ARA diets.

Table 39. Fatty acid composition in halibut early life stages (μg FA/ind) when produced by halibut broodstock trial May spawners in 2002. Values are means of all diet groups ± S.D. Means with different superscripts within the same rows are significantly different ($p<0.05$)

Fatty acid	Eggs before fertilisation	Eggs at disinfection	Yolk sac larvae
14:0	1.83±0.16[a]	1.67±0.16[b]	1.34±0.18[c]
16:0	17.39±1.11[a]	16.32±1.34[b]	15.67±1.15[b]
18:0	3.16±0.34[b]	3.03±0.37[b]	3.57±0.35[a]
16:1n-9	1.01±0.13[a]	0.99±0.10[a]	0.78±0.07[b]
16:1n-7	2.92±0.39[a]	2.67±0.29[b]	2.19±0.20[c]
18:1n-9	7.40±0.84[a]	6.66±0.64[b]	5.90±1.19[b]
18:1n-7	2.27±0.19[a]	2.05±0.16[b]	1.88±0.47[b]
20:1n-9	1.88±0.17[a]	1.65±0.30[b]	1.89±0.39[a]
22:1n-11	0.67±0.09[a]	0.59±0.08[b]	0.70±0.33[a]
18:2n-6	1.24±0.17[a]	1.12±0.15[b]	0.92±0.24[c]
18:3n-6	0.08±0.03	0.07±0.03	0.09±0.08
20:4n-6 (ARA)	2.24±1.19	2.04±0.31	1.92±0.94
18:3n-3	0.35±0.05[a]	0.31±0.04[b]	0.22±0.03[c]
18:4n-3	0.29±0.05[a]	0.26±0.04[b]	0.15±0.02[c]
20:3n-3	0.12±0.02[a]	0.12±0.02[b]	0.10±0.02[b]
20:4n-3	0.38±0.06[a]	0.33±0.05[b]	0.22±0.04[c]
20:5n-3 (EPA)	9.27±1.01[a]	8.24±1.10[b]	7.18±1.62[c]
22:5n-3	1.34±0.14[a]	1.17±0.16[b]	0.96±0.23[c]
22:6n-3 (DHA)	27.11±2.46[a]	23.88±3.11[b]	20.76±5.33[c]
Total	85.33±6.41[a]	77.05±7.53[b]	70.19±8.66[b]
Σ Sat	22.81±1.46[a]	21.44±1.82[b]	20.99±1.64[b]
Σ MSat	17.98±1.63[a]	16.10±1.39[b]	14.80±2.20[b]
Σ PUFA	44.42±4.08[a]	39.40±5.01[b]	34.28±7.99[c]
Σ n-3	38.94±3.56[a]	34.39±4.44[b]	29.65±7.24[c]
Σ n-6	4.26±1.27	3.83±0.97	3.55±0.94
DHA: EPA	2.94±0.20	2.90±0.16	2.87±0.22
EPA: ARA	5.44±2.66	4.92±2.34	4.72±2.32
Σ n-3: Σ n-6	9.88±2.69	9.40±2.16	8.56±1.88
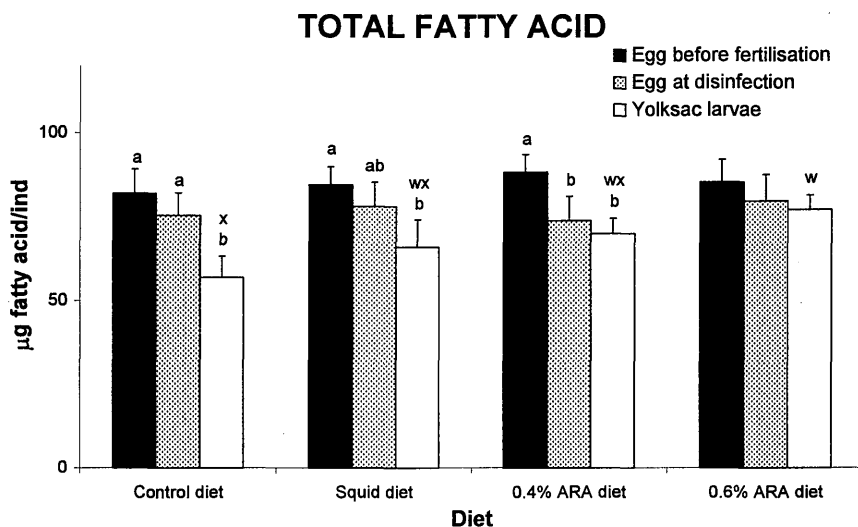
TOTAL FATTY ACID

Figure 56. Total fatty acid content in Atlantic halibut early life stages produced by broodstock fed the trial diets (Mean ± S.D., n=12). Columns assigned a letter a to c are significantly different within the same diet. Columns assigned w to z are significantly different within the same life stages ($p<0.05$)
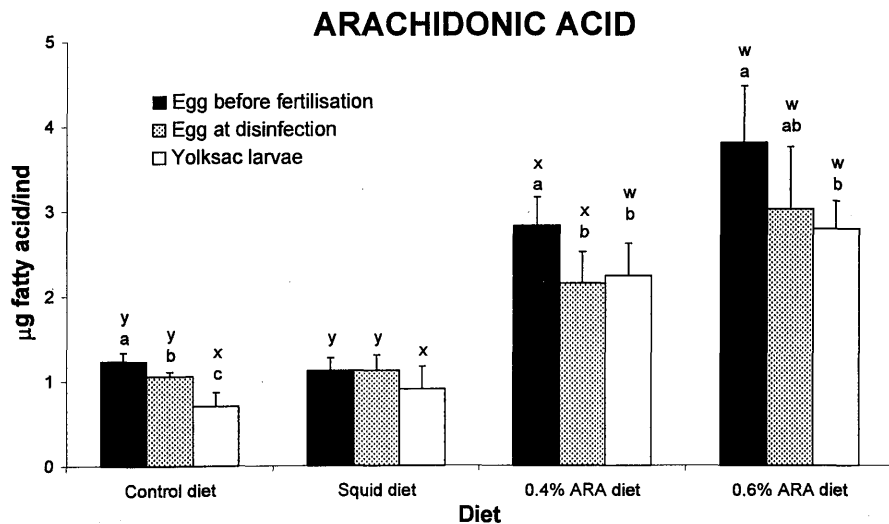
ARACHIDONIC ACID

Figure 57. Arachidonic acid concentration in Atlantic halibut early life stages produced by broodstock fed the trial diets (Mean ± S.D., n=12). Columns assigned a letter a to c are significantly different within the same diet. Columns assigned w to z are significantly different within the same life stages ($p<0.05$)
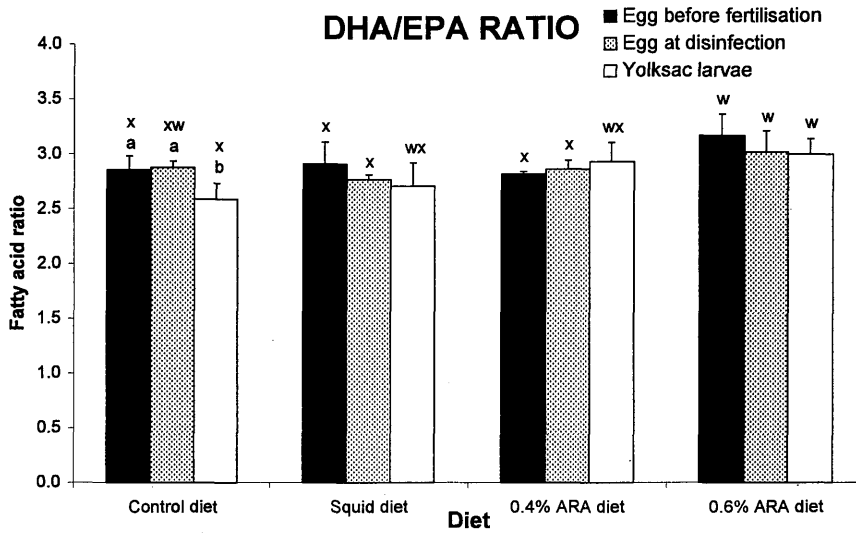
Figure 58. DHA/EPA ratio in Atlantic halibut early life stages produced by broodstock fed the trial diets (Mean ± S.D., n=12). Columns assigned a letter a to c are significantly different within the same diet. Columns assigned w to z are significantly different within the same life stages ($p<0.05$)
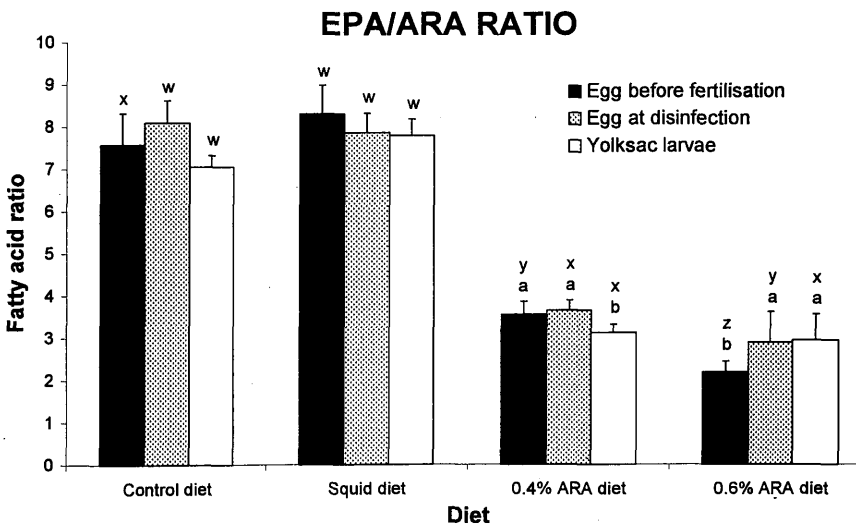
Figure 59. EPA/ARA ratio in Atlantic halibut early life stages produced by broodstock fed the trial diets (Mean ± S.D., n=12). Columns assigned a letter a to c are significantly different within the same diet. Columns assigned w to z are significantly different within the same life stages ($p<0.05$)

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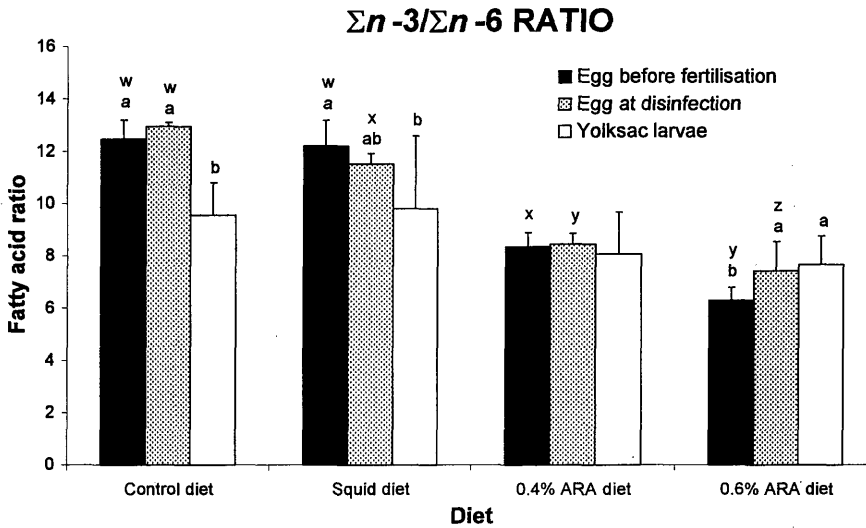
Figure 60. Σn-3/Σn-6 ratio in Atlantic halibut early life stages produced by broodstock fed the trial diets (Mean ± S.D., n=12). Columns assigned a letter a to c are significantly different within the same diet. Columns assigned w to z are significantly different within the same life stages ($p<0.05$)

EPA/ARA ratio was strongly affected by diet as ARA enriched diets displayed significantly lower (3-4 fold) ratios compared to control and squid diets. No EPA/ARA significant differences were observed between stages for control and squid diets. A similar trend was observed in females fed ARA enriched diets in their n-3/n-6 ratio (Figure 60). However, n-3/n-6 ratio in yolksac was significantly lower than in eggs before fertilisation and eggs at disinfection when produced by females fed control or squid diet.

4 Discussion

4.1 Farmed halibut early life stages survival

Survivals to specific developmental stages and final production of fry have been used in aquaculture as measures of egg quality. Haddock egg batches with low hatching rates (<5%) generated larvae that died sooner than progeny originating from eggs with high hatch rates (>85%) (Trippel *et al.*, 1999). Striped bass broodstock diets supplemented with arachidonic acid rich oil were shown to have significant beneficial effects on larval hatching rate (Harel *et al.*, 2002). A significantly higher ARA level in turbot eggs showed a high correlation with hatching percentage (Lavens *et al.*, 1999). All parameters used to assess egg quality as hatching rate or larval survival were highest in Japanese flounder fed a 0.6% ARA diet but a higher level of ARA (1.2%) negatively affected both egg and larval quality (Furuita *et al.*, 2003). In the present study, only egg batches with fertilisation rates over 35% were incubated and this early selection had direct consequences on the results. First, the number of conicals with eggs produced by females fed the same trial diet was low. Second, lower egg quality batches were not incubated. Third, most egg batches were mixed not only with eggs from females fed different trial diets but also with eggs from fishfarm broodstock. All these parameters had a higher effect on hatching rates and yolksac survival than the four different diets fed to the trial broodstock.

4.2 Lipid dynamics in early life stages

Lipids are major sources of metabolic energy throughout the embryonic developmental stages in fish (Sargent, 1995). A net decline in total lipid between eggs before fertilisation and after hatching suggests that they are utilised both as substrates for energy and as precursors for biomembranes and biologically active molecules through embryogenesis (Rønnestad et al., 1995 & 1998). In the present study about 9% of the total lipid was catabolised until the presumed onset of hatching. There seemed to be only small quantitative changes in the lipid content in yolksac larvae from hatching to just before first feeding. This supports the notions that free amino acids are the only substrates catabolised for energy metabolism during this stage (Rønnestad et al., 1995). PC dominated the lipid pool of newly spawned and hatched halibut and this is consistent with other reports on this species (Falk-Petersen et al., 1989; Rønnestad et al., 1995). Lipid utilisation patterns in Senegal sole during early developmental stages favoured neutral lipids, particularly triacylglycerol and sterol ester fractions. Fertilised egg and yolksac larvae are richer in neutral lipids and a decrease during development is observed. In contrast, a significant increase occurred in proportions of phospholipids, mainly due to significant increases in minor classes such as PS, PI and CL, whereas major phospholipid classes such as PE remained constant during development. A significant increase of PS, PI and CL+PG in trial halibut eggs content was observed, as well as a decrease in TAG from hatching eggs to yolksac larvae, while the PC

proportion significantly decreased between fertilised egg to yolksac larvae prior first feeding. Evidence of PC being used for energy and synthesis of new membranes confirmed previous observations made in Atlantic halibut. Approaching first feeding selective catabolism of PC and a net synthesis of PE in halibut yolksac larvae were observed, resulting in a shift in the lipid class composition in the developing larvae compared with that of the yolk (Rønnestad et al., 1995). In the present study the PC: PE ratio was significantly higher in eggs before fertilisation than in yolksac larvae and the highest value was observed in eggs at disinfection produced by females fed the control diet (3.31±0.07). Thereafter the PC: PE ratio decreased significantly in yolk sac larvae. A significant increase in the PC: PE ratio and cholesterol content would produce a decrease in membrane fluidity during embryonic development, coinciding with the predicted upward movement of larvae in the water column (Evans et al., 1996). Although PC: PE ratio decrease was previously observed in halibut first life stages, the ratio in the present study was lower than the ratio observed in an earlier study (4.6-5.3) by Rønnestad et al., (1995). The decline of this ratio could be due to the net catabolism of PC when the PE proportion remained constant. A constant PE concentration in developing yolksac larvae of Atlantic halibut and a preferential retention of DHA observed by Rønnestad et al. (1995) may support the high biological value of these lipids, especially because they are known to be particularly associated with the development of neural tissue such as the brain and retina (Sargent et al., 1995). After hatching

and up to the stage before first feeding, catabolism of phospholipid, particularly PC has been demonstrated in larvae of halibut, plaice and cod, but not in turbot, whereas PE tended to be synthesized in all four species (Rainuzzo *et al.*, 1992).

4.3 Fatty acid dynamics in early life stages

From fertilisation to yolksac larvae prior to first feeding (200 dd) stages, a marked decrease in (n-3) PUFA and an increase of 18:0 (stearic acid) was observed. During early development in fish, phospholipase A_2 (PLA_2) regulates membrane lipid modifications, which relate to changes in environmental conditions and provision of fatty acids required for metabolic energy substrates and prostaglandin biosynthesis. PLA_2 activity was undetectable at fertilisation but reached 230 pmol/mg/h in 10-d embryos and increased by a further 120% at hatching (Evans *et al.*, 1998). Net consumption of (n-3) PUFA in PC following fertilisation and during early embryonic development has previously been observed in Atlantic halibut (Falk-Petersen *et al.*, 1989; Evans *et al.*, 1998). The consumption of PC was mainly related to the reduction in DHA observed in this species. The fatty acids released from phospholipid were mainly used as energy substrates by the growing halibut larvae; DHA was quantitatively one of the most important fatty acid fuels in energy production (Rønnestad *et al.*, 1995). In Murray cod and trout cod, freshwater carnivores, the amount of ARA in total lipid increased during the transformation from egg to yolk-sac-resorbed larvae and they had a tendency to retain PUFA. DHA was

conserved in early ontogeny as well as the DHA: EPA ratio (Gunasekera *et al.,* 1999). DHA plays an important role in perch and was spared by starved larvae to the detriment of the other fatty acids and despite the extreme conditions of fasting, the n-3/n-6 ratio of starved larvae remained unchanged (Abi-ayad *et al.,* 2000). In contrast, catabolism of DHA was observed in the present study from eggs before fertilisation to yolk sac larvae. Similar catabolism of DHA was observed in sea bass eggs (Navas *et al.,* 1997). This confirms the idea that significant differences occur in the fatty acid profile in relation to early ontogeny in freshwater and marine fish species. A higher hatching rate of seabass eggs was associated with higher DHA: EPA and ARA: EPA ratios (Navas *et al.,* 2001). EPA content in Japanese flounder eggs is more sensitive to change of dietary *n-3* HUFA than DHA, and increase of the EPA levels in eggs, due to an increase of EPA level in broodstock diet, depressed the egg ARA level (Furuita *et al.,* 2002).

ARA was significantly higher in eggs before fertilisation and eggs at disinfection when produced by female halibut fed the ARA enriched diets. ARA decrease from eggs before fertilisation to yolksac larvae was significant. Interactions between *n-6* and *n-3* fatty acids are well established in animal nutrition. It is well established that *n-3* fatty acids are preferred for conversion to long chain unsaturated products and therefore have an inhibitory effect on n-6 fatty acid bioconversion. In the present study, the main effect of the broodstock diet on egg fatty acid composition was observed on *n-3*: *n-6* ratio

and the effect on *n*-3 PUFA compositions, especially EPA, in relation to the level of ARA available to halibut larvae. When fed the 0.6% ARA diet females produced eggs before fertilisation with significantly lower *n*-3: *n*-6 ratios than those produced by females fed the other trial diets. The *n*-3: *n*-6 ratio increased significantly in 0.6% ARA diet eggs at disinfection probably due to the use of *n*-6 (ARA) for energy. In comparison a significant decrease in *n*-3: *n*-6 ratio was observed from eggs before fertilisation to yolksac larvae in females fed control and squid diets, due to catabolism of *n*-3 HUFA used for energy in preference to *n*-6 HUFA. Broodstock seemed to use first the surplus fatty acid that is not essential for biological function then as this fatty acid becomes limiting, their preference for energy use will switch to other fatty acids. In this situation decreased *n*-3: n-6 ratio might be due to a higher utilisation of EPA compared to ARA in females fed control and squid diets. Increase of *n*-3: *n*-6 ratios in females fed the 0.6% ARA diet reflects the availability of ARA compared to EPA. However, when produced by females fed the 0.4% ARA diet, no significant changes were observed in *n*-3: *n*-6 ratio in first life stages, perhaps indicating the correct balance of ARA in these halibut larvae. The 0.4% ARA diet effect on DHA: EPA: ARA ratio in early life stages might be used as reference for the development of artemia enrichment fulfilling Atlantic halibut first feeding larvae EFA requirement.

Chapter 8 General discussion and conclusions

1.1 Halibut broodstock husbandry and nutrition

Improvements in our understanding of the appropriate culture conditions and management procedures were one of the main objectives of the project. This was essential to reliably produce the numbers of eggs and fry required by grow-out farms. All hatchery protocols have been improved and standardised over the three-year project. It was also decided to monitor production from individual females with the help of pit tagging and external tagging in order to obtain accurate results and to initiate the setting up of a breeding program. Optimised stripping frequency, stripping protocol and artificial fertilisation protocol were defined during the project and are now used in Otter Ferry SeaFish Ltd. general production.

The manipulation of the annual photoperiod cycle had some clear consequences for the spawning activity of the females and the results of the present study. Female May spawners showed a positive adaptation to artificial photoperiod. Spawning season windows were such that it was possible to define the starting spawning day for each individual female within two or three days of accuracy. However, female July spawners did not properly adapt to artificial photoperiod such that through the three years of the experiment none of the females spawned in the same period.

1.2 Arachidonic acid

The project also focused on the importance of the diet for halibut broodstock and the effect of supplemented ARA. The importance of ARA in fish nutrition has tended to be overlooked in preference to EPA and DHA, probably due to the predominance of the latter two HUFA in fish tissues. The essential dietary requirement in many marine fish for *n*-3 HUFA such as EPA and DHA has been well established over a number of years (Sargent *et al.*, 1995, 1999a and b 2001). However, Castell *et al.* (1994) and Estévez *et al.* (1997) demonstrated that dietary ARA promotes growth of juvenile turbot and Japanese flounder respectively. Bessonart *et al.* (1999) and Koven *et al.* (2001) found a positive dietary ARA effect on juvenile gilthead seabream growth and survival. More recently, and of more interest for the present project, Bruce *et al.* (2000) and Mazzora *et al.* (2003) showed that reproductive performance of European seabass and Atlantic halibut broodstock, respectively, improved by being fed diet containing both DHA and ARA. Furuita *et al.* (2003) also observed improvement in Japanese flounder egg hatching rates when broodstock were fed a 0.6% ARA diet although a negative effect was observed with a higher (1.2%) ARA diet. In the present project ARA has been used as an Atlantic halibut broodstock enhancer and several aspects of halibut reproduction were studied in order to define the effect of ARA. A positive effect was observed on weight variation when Atlantic halibut broodstock were fed ARA enriched diets. Moreover female growth observed over the three year project indicated

that feeding frequency, and general husbandry protocols were close to optimum for the well being of these fish. The diet was designed according to previous knowledge on halibut broodstock nutritional requirements and special attention was given to the choice of wheat gluten as a binder. Arising from the project results, Skretting has now commercialised a halibut broodstock diet, Vitalis® containing 60% protein, 20% lipid with 0.4% ARA and 10% wheat gluten.

1.3 Effect of ARA enriched diet on female reproductive performance

Bromage *et al.* (2001) and Mazzora *et al.* (2003) observed a significant improvement in fertilisation rates in Atlantic halibut broodstock fed a diet enriched in ARA. In the present study, halibut broodstock females fed the 0.4% ARA diet produced a higher volume of eggs in 2002 and showed a higher fecundity than broodstock fed other experimental diets. However, when females were fed the 0.6% ARA diet, a generally negative effect on egg production was observed including a two-week delay in the start of their spawning season. It appears that excess ARA in the diet could have a negative effect on halibut female reproductive performance.

1.3.1 Biochemical egg quality assessment in Atlantic halibut

The potential of an egg to produce viable fry can be determined by biochemical parameters such as lipid content and composition. Specifically, egg lipids have several critical roles in the development of fish embryos. No significant

difference was observed either in the lipid class composition or in the fatty acid composition between eggs before fertilisation, fertilised and non-fertilised eggs in the present study. Improvement in reproductive performance was correlated with higher egg lipid content in trial broodstock from 2001 to 2003. Polar lipid classes predominated in halibut eggs, with PC being the major class. Halibut eggs, as for fish tissues in general, are rich in HUFA of the *n*-3 series, especially DHA and EPA.

1.3.2 Halibut eggs from different geographic and time origins

When comparing halibut eggs spawned by Canadian broodstock in 1996 and in 2002, a significantly higher fatty acid concentration was observed in eggs spawned in 1996. This could demonstrate a deficiency in EFA in the 2002 Canadian broodstock diet compared to their wild diet. By comparison, EFA supplied in Otter Ferry broodstock diet seemed to be in appropriate proportions for the species.

1.3.3 ARA supplemented diet effects on halibut egg lipid quality

A general improvement in experimental females' reproductive performance was observed through the three-year project and correlated with a significant increase in their egg total lipid except for eggs from females fed the 0.6% ARA diet. Females fed the 0.6% ARA diet presented significant differences in their lipid class composition from 2001 to 2003. While PC percentage was significantly lower in 2001, the profile changed in 2002 to finally inverse in

2003 with PC being significantly higher than in eggs produced by females fed the other experimental diets. As expected, halibut females fed the ARA enriched diets presented a proportionally higher ARA content in their eggs than females fed control or squid diets, suggesting a concentration and preferential deposition of this important EFA. In 2003, the difference in ARA content between incubated eggs and non-incubated eggs, with respect to the experimental diets, might define ARA thresholds for egg viability. Eggs produced by females fed the 0.6% ARA diet highlighted the importance of balancing the three functional essential fatty acids, DHA, EPA and ARA in the broodstock diet. Significantly lower EPA/ARA and n-3: n-6 ratio as well as significantly higher DHA/EPA ratio was correlated with poor quality eggs.

1.3.4 Lipid dynamics in halibut eggs before fertilisation to yolksac larvae

Study of the dynamics in halibut early life stages could provide important indications to allow formulation of an optimal diet for first-feeding larvae. The lipid utilisation pattern in Atlantic halibut in early developmental stages was characterised by a significant catabolism of PC and TAG and a significant relative increase of PS, PI and CL+PG in halibut from hatching eggs to yolksac larvae. Evidence of PC being used for energy and synthesis of new membranes confirmed previous observations made in Atlantic halibut (Rønnestad *et al.*, 1995). A decline was observed in PC:PE ratio mainly due to PC catabolism while PE remained constant from eggs at disinfection to yolksac larvae. This

may support the high biological value of PE particularly associated with the development of neural tissue such as brain and retina (Tocher & Harvie, 1988). The consumption of PC might be related to the catabolism of DHA observed in the present study between eggs before fertilisation to yolksac larvae.

When produced by female halibut fed ARA enriched diets, the three stages studied had a significantly higher ARA concentration than for eggs from females fed control or squid diets. When produced by females fed the 0.4% ARA diet, no significant changes were observed in *n*-3: *n*-6 ratio between eggs before fertilisation to yolksac larvae but a significant increase and decrease, in this ratio, was observed in females fed the 0.6% ARA diet and females fed control or squid diets respectively. These results indicate that the best balance of ARA was found in halibut larvae produced by females fed the 0.4% ARA diet and similar ARA concentrations might be beneficial in Artemia enrichments in order to fulfil the requirement in first feeding larvae.

1.4 Dietary ARA effects on halibut broodstock reproductive performance in males

Males with a spermatocrit close to 50% reflect the best quality for use by fish farmers as this milt can be stripped easily from the male, mixed easily during the artificial fertilisation process and show high spermatozoid density. Atlantic halibut males fed the 0.4% ARA diet in 2003 had the longest milt production

season and their spermatocrits were consistently in the 50% range. In 2002 by injecting Ovaplant® in Atlantic halibut males, the duration of good quality and expressible milt available was successfully extended within 7 days post-implant, compared to control fish that showed signs of drying up and high spermatocrit values.

A linear relationship was established between spermatocrit and total lipid in milt. Lipid class distribution in halibut males was characteristic of marine species spawning in cold deep water. PE, PC and cholesterol dominance in milt lipids may protect sperm from osmotic and cold stress. A significantly higher percentage of cholesterol in males fed the control diet may reflect lower milt quality. Indeed, males fed the control diet had a shorter milt production period and milt was thicker making stripping difficult. Moreover, a higher pressure had to be applied to the fish to express the milt, which resulted in urine contamination. This was not observed in males fed the 0.4% ARA diet, and a longer period of milt expression could be linked to a higher level of PI and PS and an optimum ARA concentration in milt.

When fed ARA enriched diets, halibut milt showed a proportional elevation of ARA concentration. A rapid and selective uptake of ARA into tissues, especially cells related to reproduction, has been established. A significantly lower level of EPA was observed in males fed the 0.6% ARA diet but not in males fed the 0.4% ARA diet and the unbalanced EPA: ARA ratio could explain the lower quality of milt produced by males fed 0.6% ARA. When

studying the relationship between PI and ARA in halibut milt, the curve suggested the establishment of an optimum milt ARA concentration of around 51.4 µg ARA/mg lipid. This corresponds to the observed halibut male milt ARA concentration range when fed with the 0.4% ARA diet.

The role of PGs on gonadal steroidogenesis has been well established. Results from the present study confirmed the positive effect of PGs in gonadal steroidogenesis. Consequently, males fed the 0.4% ARA diet produced the highest quality milt with a significantly higher concentration of $PGF_{2\alpha}$.

Overall conclusions

➢ Photoperiod and temperature must be monitored carefully to obtain correct adaptation of halibut broodstock to required spawning season

➢ Fishfarm stripping frequency, stripping protocol and artificial fertilisation protocol have been optimised

➢ A positive effect was observed on weight gain when halibut broodstock were fed the ARA enriched diets.

➢ The 0.6% ARA diet had a generally negative effect on egg production and quality.

➢ The 0.4% ARA diet had a positive effect on halibut broodstock reproductive performance:

• Females showed higher egg volume and higher fecundity in 2002 and produced early life stages with the optimal DHA: EPA: ARA ratio balance

• Males had the longest milt production period and their spermatocrit were consistently in the 50% range, characteristic of milt quality looked-for by hatchery managers.

➢ Skretting has now commercialised a halibut broodstock diet, Vitalis®.

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