A STUDY OF BIOCHEMICAL COMPOSITION IN CAPTIVE ATLANTIC HALIBUT (Hippoglossus hippoglossus) EGGS AND LARVAE

PEIHONG ZHU

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A study of biochemical composition in captive Atlantic halibut (*Hippoglossus hippoglossus*) eggs and larvae

By

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Abstract

In this study, lipid classes, fatty acids, free amino acids (FAA) and protein content in captive Atlantic halibut (*Hippoglossus hippoglossus*) eggs were measured to investigate the relationship between biochemical composition of the eggs and egg quality, the effect of the physiological condition of broodstock females on egg biochemical composition, and changes in the biochemical composition of eggs and larvae in both the embryonic and early larval stages.

High neutral lipid (NL) level and low (n-6) and (n-3) polyunsaturated fatty acid (PUFA) levels seem to be associated with low egg quality. For eggs with a fertilization success $\geq$ 75%, there were significantly lower amounts of total NL and sterol (ST) per egg ($P<0.01$, $P<0.05$, respectively) compared to eggs with a fertilization success < 75%. For eggs that produce larvae with survival time $\geq$ 15.9 days, the amount of triacylglycerol (TAG) was significantly lower than in eggs that produced larvae with survival times < 15.9 days ($P<0.01$). For eggs with a fertilization success $\geq$ 75%, the proportion of (n-6) PUFA in TAG ($P<0.01$), the proportion of 20:5 (n-3) in phosphatidylethanolamine (PE) ($P<0.05$), and 18:2 (n-6) ($P<0.05$) in the total lipid per egg, were significantly higher than for eggs with fertilization success < 75%.

The average dry weight of eggs followed a pattern similar to that of the average food intake of the broodstock female, while the average egg lipid content did not change. The spawning rhythm of the females also affected egg composition. The ash free dry weight and total protein of the eggs decreased as the spawning season proceeded ($P<0.05$). In TAG, the levels of 18:0 and 20:4 (n-6) per egg, and in PE, the level of (n-6)

PUFA per egg decreased significantly over the spawning season ($P>0.05$). The maturity of the spawners affected egg quality and egg lipid composition as well. Repeat spawners produced eggs with significantly higher fertilization success than first-time spawners ($P<0.05$). The total neutral lipid (TNL) and ST were significantly lower for eggs from repeat spawners than for eggs from first-time spawners ($P<0.01$, $P<0.05$, respectively).

Total FAA decreased continuously ($p<0.01$) over the embryonic and larval stages investigated. TAG decreased significantly for the larval stages ($P<0.05$). When hatching occurred, there was a significant increase in all lipid classes except ST. It is suggested that FAA may be the source of carbon skeletons for lipid synthesis at hatching.

Thus, this study has shown that lipid and fatty acid composition in eggs from captive Atlantic halibut is associated with egg quality; however, FAA are the biochemical components that are most utilized during embryonic and early larval stages. At the end of embryonic stage and early larval stage, these amino acids appear to be directed towards lipid synthesis and then towards protein synthesis.

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Table of Contents

List of Tables

List of Figures

List of abbreviations

AA Arachidonic acid

AEC S-2-aminoethyl-L-cysteine

AFDW Ash free dry weight

ALC Alcohol

AMPL Acetone mobile polar lipids

ANOVA Analysis of variance

BSA Bovine serum albumin

BW Body weight

DHA Docosahexaenoic acid

DPH Days post hatch

DW Dry weight

EAA Essential amino acids

EFA Essential fatty acids

EPA Eicosapentaenoic acid

FAA Free amino acids

FAME Fatty acid methyl esters

FFA Free fatty acids

FID Flame ionization detector

GC	Gas chromatography
NEAA	Nonessential amino acids
NL	Neutral lipids
MUFA	Monounsaturated fatty acids
PAA	Protein bound amino acids
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Polar lipids
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
SAT	Saturated fatty acids
SE	Sterol ester
SM	Sphingomyelin
ST	Sterol
TAG	Triacylglycerol
TL	Total lipid
TLC	Thin layer chromatography
TNL	Total neutral lipid
TPL	Total polar lipid
VLDL	Very low density lipoprotein

Chapter 1. Introduction

1.1. Background

Reports of the benefits of seafood rich diets in reducing the risk of cardiovascular disease, rheumatism, and diabetes have led to marked changes in our eating habits (Castell, 1988). The demand for seafood in the market is increasing (Ahmed & Anderson, 1994), but the fishery resources are severely depleted, mostly as a result of overexploitation. According to Banabe (1994), since the 1970's, fishing world wide has removed 90 million tonnes of the 100 million tonnes of seafood that could be supplied by the world's oceans. Thus, aquaculture has been accorded a new importance as a method of producing seafood. Moreover, since both finfish and shellfish are subjected to contamination in their natural habitat, aquaculture can reduce the risk of contamination since the fish are raised in a more controllable environment (Ahmed & Anderson, 1994). As far as economic aspects are concerned, compared to livestock farming, the investment needed for aquaculture, especially marine aquaculture, is much lower (Arrignon, 1982).

Canada commands an enormous diversity of freshwater and saltwater aquatic resources. Canada is also the largest exporter of fish product to USA, one of the largest and fastest growing seafood markets in the world. Manci (1990) estimated that job opportunities in the aquaculture industry could increase from the 1988 level of 1,500 full-time positions to over 5,000 by the year 2000, not including job creation in associated industries. Aquaculture is especially important to the economy of Newfoundland, an economy traditionally very

much dependent on the fishery, which is now heavily burdened by unemployment due to the disappearance of fishery resources. Research aimed at a better understanding of the potentially profitable species, and ultimately at improvement of aquaculture techniques, is now very active in Canada due to increasing government funding.

Atlantic halibut (*Hippoglossus hippoglossus*) has been the focus of a vast amount of research during the past decade because of its high potential as a candidate for aquaculture. This species belongs to the family Pleuronectidae. It is the largest of the flatfish: it can grow to a length of 2.5 meters and a weight exceeding 300 kilograms (Zwanenberg, 1986). The area off Newfoundland coast is a good habitat for Atlantic halibut.

Atlantic halibut commands the highest price of all flatfish. The European market sells about 4,000 tonnes of frozen halibut annually, and it has been estimated that the demand for fresh halibut on the European market could reach 20,000 tonnes over the next 20 years. U.S. market trends are expected to be similar (Brown & Keough, 1994). High fishing intensity caused by a high demand in the market, has resulted in a significant and continuous decrease of the stock in its natural habitat since the 1960s (Haug, 1990). Therefore, the high market value of this species and its low supply from the commercial fishery make aquaculture of Atlantic halibut both necessary and potentially profitable. Moreover, its good survival after metamorphosis, fast growth, and late maturity in captivity promises commercial feasibility.

1.2. Biochemical compositions in eggs and yolk-sac larvae of marine fish

The major obstacle for Atlantic halibut aquaculture so far has been the mass mortality

which occurs during the yolk-sac and the first feeding stages (Lonning *et al.*, 1982; Ingram, 1987). This is due to the fact that the larvae hatch at a very premature stage with a poorly developed muscle system, and mouth and eyes that are not functional (Blaxter *et al.*, 1983; Helvik & Whalther, 1993). Halibut larvae are unable to ingest exogenous food until about 35 days after hatching depending on rearing temperature. The whole yolk-sac stage lasts about 250 degree-days (i.e., rearing temperature in °C times the number of days), one of the longest of all marine fish species. Providing appropriate feed to the first feeding larvae will greatly improve their viability. An understanding of biochemical changes during the embryonic and yolk-sac larval stage, will be of great help in improving the production of high quality food for first feeding larvae, and in reducing the mortality of larvae at this crucial stage.

Another difficulty associated with halibut aquaculture is that efficient methods for determination of egg and larval quality are not available. During the endogenous feeding stage (i.e. the embryonic and pre-feeding larval stage), survival and development are solely dependent on nutrients stored in the yolk. Therefore, the biochemical composition may be a significant determinant of egg quality for this species. The formation of the yolk is completed in the ovary, and its biochemical composition is closely related to the dietary and physiological conditions of the broodstock. In order to produce high quality eggs, it is also important to investigate the relationship between the biochemical composition of eggs and the condition of broodstock.

1.2.1. Lipids and fatty acids

Lipids and fatty acids are involved in energy production, membrane formation and synthesis of biochemically active substances such as some steroid hormones and prostaglandins. Extensive research has been directed to elucidate the importance of lipids and fatty acids during embryonic and larval development of marine fish.

Polyunsaturated fatty acids (PUFA), especially 22:6(n-3) (docosahexaenoic acid, DHA) and 20:5(n-3) (eicosapentaenoic acid, EPA), are abundant in marine fish eggs, and are actively involved in the modulation of membrane fluidity. (n-3)PUFA as well as (n-6)PUFA, predominantly 20:4(n-6) (arachidonic acid, AA), are the precursors for eicosanoid synthesis. Due to the limited activities of elongase and desaturase in marine fish, (n-3) and (n-6)PUFA especially 20:5(n-3), 22:6(n-3), and 20:4(n-6) cannot be synthesized *in vivo* from 18:3 (n-3) (α-linolenic acid) or 18:2(n-6) (linoleic acid) as in mammals. Therefore, when the issue of lipid metabolism in marine fish is addressed, emphasis is placed on 20:5(n-3), 22:6(n-3), and 20:4(n-6), fatty acids that are defined as essential fatty acids (EFA) for marine fish, meaning that they must be supplied in the diet.

Dietary deficiencies of EFA in broodstock fish may adversely affect the viability of eggs and larvae. However, due to the complexity of lipid metabolism, and its susceptibility to biological and environmental factors, the quantitative relationship between essential fatty acids and eggs and larval quality (fertilization success, hatchability, and larval survival) in marine species is rather obscure (Bruce *et al.*, 1993; Peleteiro *et al.*, 1995; Watanabe *et al.*, 1985; Wiegand *et al.*, 1991). Thus lipids and fatty acids have not been recommended for use

as an index to determine the quality of eggs and larvae (Kjorsvik *et al.*, 1990). However, this does not eliminate the importance of lipids especially (n-3) and (n-6) PUFA in the development of fish embryos and larvae.

1.2.1.1. Lipid and fatty acid composition of marine fish eggs

The lipid class and fatty acid compositions of total lipid from Atlantic halibut eggs were first reported in 1986 by Falk-Peterson and co-workers. The total lipid accounted for 12% of egg dry weight. Approximately 71% of total lipid was polar lipid and 29% was neutral lipid. Phosphatidylcholine (PC) was by far the largest component (62% of total lipid), followed by triacylglycerol (TAG) (13%), and sterol (ST) (10%). Phosphatidylethanolamine (PE), another major phospholipid, accounted for about 7% of the total lipid.

In the polar lipid (PL), (n-3) PUFA were the major fatty acids (44% of weight), with 31.5% of 22:6(n-3) and 10.9% of 20:5(n-3). There were 27.8% of saturated fatty acids and 18.9% of monoenoic fatty acids in the polar lipids. In neutral lipids (NL), there was as much as 55.8% monoenes, while saturates accounted for 21%, and (n-3)PUFA for 16.7%. As in the PL, 22:6(n-3) (10.9%) and 20:5(n-3) (3.5%) were the two major PUFA in NL. Another important PUFA, 20:4(n-6) (2.7%) was found mainly in the PL.

These lipid class and fatty acid profiles of Atlantic halibut eggs agree well with values obtained with eggs of other marine species as documented by Tocher & Sargent (1984), especially those eggs without oil globules (i.e. cod, haddock, whiting, and saithe). All these species produce eggs with 10-15% of dry weight as lipid. PL and NL make up about

70% and 30% of total lipid (TL) respectively, with PC and PE being the major PL, and TAG and ST being the major NL. About 45% of fatty acids present in PL are (n-3) PUFA, while in NL, monoenes are the major component, ranging from 30-40%.

Considering the large amounts of vitellogenin taken up by the growing oocytes, it seems reasonable to assume that lipids bound to vitellogenin contribute significantly to the TL composition of the eggs (Silversand & Haux, 1995), especially for the species like Atlantic halibut that do not have any oil globules in the eggs. The ratio of phospholipid to TL is similar in halibut vitellogenin and in egg yolk (Norberg, 1995). The characteristically high levels of (n-3) PUFA in egg yolk are also present in vitellogenin. About 50% of the total fatty acid in vitellogenin are 22:6(n-3) and 20:5(n-3), and this high level is fairly conservative in vitellogenin from different species (Silversand & Haux, 1995). Although vitellogenin is the major vehicle for lipid allocation to the oocytes, lipids can also be transported by other plasma lipoproteins such as VLDL (Babin & Vernier, 1989; Wallaert & Babin, 1994).

In female northern pike, during the time of ovary recrudescence, the weight percentage of (n-3)PUFA, especially 22:6(n-3), declined significantly in liver, muscle and adipopancreatic tissue as well as in ovary neutral lipid, while the weight percentage of (n-3)PUFA in ovary PL increased significantly (Schwalme *et al.*, 1993). This suggests a specific selection of (n-3)PUFA during the process of vitellogenesis and emphasizes the importance of these fatty acids in the development of the fish embryos.

1.2.1.2. Biological condition of the broodstock and lipid and fatty acid composition of eggs

Although there are reports indicating the uniformity of lipid and fatty acid profiles in marine fish eggs, there are also reports suggesting that the lipid and fatty acid composition in marine fish eggs is highly variable, even among eggs produced by individual females of the same species kept under the same environmental conditions and fed with identical diets (Peleteiro *et al.*, 1995). This high variability extends to eggs produced by a single female in one spawning season (Kjorsvik *et al.*, 1990; Parrish *et al.* 1994a).

According to Wiegand (1996), three sources of lipid can be incorporated into the egg yolk during vitellogenesis (i.e., production of egg yolk): dietary lipid ingested by the broodstock during vitellogenesis, lipid reserves that are stored prior to vitellogenesis and *de novo* synthesis of lipid in the liver or ovary of the broodstock. The latter two sources are apparently closely related to dietary lipid ingestion. Dietary and stored lipid may be modified in the liver prior to incorporation into vitellogenin. Among the constituents of broodstock diets, lipids are the chemical components that affect the composition of eggs most (Watanabe *et al.*, 1985).

A direct effect of broodstock diets on fatty acid composition in eggs has been found in gilthead sea bream (*Sparus aurata*) (Mourente & Odriozola, 1990). The eggs produced by broodstock fed a diet with lower (n-3) PUFA contained a lower weight percentage of (n-3) PUFA in both TAG and PC, but had higher levels of 20:4 (n-6) and monoenes compared with eggs from the broodstock taking a (n-3)PUFA rich diet. The saturated fatty acid levels changed only slightly. The fatty acid composition in TAG was affected to a larger extent than

that in PC. The 20:5(n-3) level in both lipid classes was more consistant than was the 22:6(n-3) level. Lipid class composition was unaffected (Mourente & Odriozola, 1990).

Administration of a (n-3)PUFA deficient diet to trout for three months prior to spawning, resulted in a decrease in (n-3)PUFA levels in adipose tissue, total lipoproteins, vitellogenin, lipovitellin (which contains yolk lipid), and the oil globule. The level of total (n-6)PUFA increased, but in contrast to gilthead sea bream, 22:6(n-3) was little affected (Leger *et al.*, 1981).

Wiegand (1996) concluded that there is a strong selection pressure to maintain the proportions of (n-3)PUFA, especially 22:6(n-3), in yolk PL within a relatively narrow range. However, the effect of a long-term (n-3)PUFA deficieny in diet may be severe and detrimental to both the wellness of the broodstock and the development of embryos and larvae.

As shown by the two studies mentioned above, the decline of (n-3)PUFA is often accompanied by an increase of (n-6)PUFA. In mammals, it is accepted that (n-3)PUFA, principally 20:5(n-3), have a physiological role in modulating the formation of eicosanoids from 20:4(n-6) by competing with the enzymes which convert 20:4(n-6) to eicosanoids (Sargent, 1995). A similar modulation mechanism apparently exists in fish; the adverse effect of (n-3)PUFA deficient diets may not result directly from less (n-3)PUFA in the diet, but from a lower ratio of 20:5(n-3)/20:4(n-6). The elevated (n-6)PUFA level during embryogenesis and larval development is likely to increase the susceptibility of the resulting larvae to external stressors (Sargent, 1995).

Besides diet, other biological conditions may also affect the composition of lipid in

egg. Some species (e.g., halibut, turbot) fast during spawning, that is, they stop feeding during spawning. McEvoy *et al.* (1993) found that in captive turbot, late-season eggs contained less long chain (n-3)PUFA, such as 22:6(n-3), 22:5(n-3), and 20:5(n-3), in phospholipids than in eggs produced early in the season. They relate this to the cessation of feeding during spawning. However, this reduced (n-3)PUFA level may result from the change of lipid composition in the ovary with the development of this tissue (Wiegand & Idler, 1985), which in turn may be regulated by hormone levels.

In halibut, the levels of estradiol-17β, testosterone, and vitellogenin fluctuate in females during the spawning season along with ovulation of successive batches of eggs. (Methven *et al.*, 1992). Lipid and fatty acid composition of eggs varied among batches spawned by the same female in the same spawning season (Parrish *et al.*, 1994a). However, whether the fluctuation of hormone and vitellogenin will affect the biochemical compositions of the eggs in different batches is unknown. Srivastava and Brown (1993) reported that testosterone-treated females produced eggs with less lipid, carbohydrate, and protein than those produced by the control group. Further investigations in this area are needed. Variation in lipid in eggs can also be found among eggs produce by females of different age (Kim, 1974; Kuznetsov & Khalitov, 1978; Evans *et al.*, 1996).

Timing of ovulation for Atlantic halibut is crucial for the viability of the eggs produced. Deferred stripping of eggs after ovulation will lead to overripening eggs (Norberg *et al.*, 1991), but knowledge of changes in biochemical composition during the process of overripening is poor. Devauchelle *et al.* (1988) found overripe eggs of turbot contained more lipid than viable eggs.

1.2.1.3. Metabolism of lipids and fatty acids in embryos and larvae

In marine fish embryos and larvae at the endogenous feeding stage, yolk lipid is mobilized for development, since endogenous synthesis of lipid is limited. Lipid is catabolized to produce energy, and to release (n-3) and (n-6)PUFA for synthesis of biologically active eicosanoids. The PUFA moiety in phospholipids may also be essential in maintaining membrane fluidity under low environmental temperatures.

It is established that in addition to free amino acids, lipids are also catabolized as an energy substrate. Different species use different strategies as to which lipid class is the preferred energy substrate and as to the timing of lipid catabolism. In species with oil globules, such as turbot (Finn & Fyhn, 1995) and gilthead sea bream (Ronnestad *et al.*, 1994), lipid is used mainly after hatching, while in species without an oil globule such as cod, lipid is catabolized following the completion of epiboly, i.e. when the syncytium layer completely covers the yolk-sac (Finn *et al.*, 1995b).

In the gilthead sea bream, TAG is more abundant than PC in the egg due to the presence of an oil globule, and it is catabolized more than PC (Ronnestad *et al.*, 1994). The absolute amount of all fatty acids decreased, and saturates and monoenes were utilized to a greater extent than PUFA. No transfer of PUFA between PC and TAG occurred (Ronnestad *et al.*, 1994). In cod embryos and larval stages, PC is the predominant lipid component and it is used more than TAG. The relative fatty acid composition in PC changed little, but in TAG, there was a greater depletion of the monoenes compared to PUFA and saturates. No mass transfer of PUFA from PC to TAG was detected (Fraser *et al.*, 1988; Finn *et al.*, 1995b).

Preferential catabolism of PC over TAG was also reported in embryonic and early larval development of Atlantic herring (Tocher *et al.*, 1985b). The levels of (n-3) and (n-6)PUFA increased in Pacific halibut (Whyte *et al.*, 1993), Atlantic halibut (Falk-Petersen *et al.*, 1989) and Atlantic herring (Tocher *et al.*, 1985a) during embryonic and larval development, implying a sparing mechanism of these PUFAs for non-energetic functions. Generally, in species without oil globules, PC is the predominant lipid class to be catabolized in embryonic and yolk-sac larval stages. While there may be net depletion of PUFA, the relative content of these fatty acids is maintained or even increased.

As mentioned above, it is generally believed that lipogenesis in embryonic and larval stages is limited, but Terner *et al.* (1968) discovered a lipid synthesis capacity in trout embryos incubated with 1-^{14}C-acetate. Cetta and Capuzzo (1982) also reported an increase of lipid mass in winter flounder embryos. Therefore, it is reasonable to view the aforementioned decrease in lipid as a net outcome of catabolism and synthesis.

The metabolic pattern of lipids in embryonic and larval fish can be affected by various environmental factors. Fraser *et al.* (1987) reported that when pre-feeding herring larvae were released into a large enclosure containing live prey, resembling the condition in the wild, larval TAG content initially decreased more rapidly than in those larvae held in an enclosure without any prey. The later is the normal situation for pre-feeding larvae in culture. The difference in TAG catabolism is presumably because of greater activity of larvae living in an environment containing live prey. The temperature at which different species normally develop could play an important role in lipid utilization in the embryo and larva. It is known that piokilotherms increase the ratio of polyunsaturated to saturated fatty acids in membrane

lipids in response to lowered ambient temperature. Since changes in environmental temperature are large enough to perturb structures such as cell membranes (reviewed by Bell *et al.*, 1986; Williams & Hazel, 1995), it would be reasonable to suggest that lipid metabolism and other biochemical processes of early developmental stages may be affected by rearing temperature. Buddington *et al.* (1993) suggested that in white sturgeon, the capacity for homeoviscous adaptation was acquired after hatching, and the embryo is incapable of adjusting PUFA composition properly to adapt to the temperature change in the environment. In addition to temperature, pH and salinity may also influence PUFA composition (Bell *et al.*, 1986; Hazel & Williams, 1990).

1.2.1.4. Functions of lipids and fatty acids

PC is the main component in the lipid membrane bilayer matrix, and it is the largest lipid component (comprising about 60% of total lipid) in eggs of most marine species investigated, especially in eggs without oil globules. Although PC was generally regarded as a source of metabolic energy for developing fish eggs and larvae, the preference for PC catabolism is probably related to other roles, such as the release of essential PUFA (20:4(n-6), 20:5(n-3), and 22:6(n-3)), phosphorus and choline (Fraser *et al.*, 1988). Phosphorus is an essential component in nucleic acids and ATP. Choline may be involved in neural transmitter synthesis (Tocher *et al.*, 1985b). Both phosphorus and choline are important nutrients in fish meal (Ogino *et al.*, 1979; Millikin, 1982). As a component of lipoproteins, another role of PC may be associated with the transfer of NL from the yolk sac to the developing embryo (Fraser

et al., 1988).

PE is the second largest phospholipid component in Atlantic halibut and cod eggs. PE is a lipid class rich in PUFA (Bell, 1989). The proportion of PE in goldfish intestine and trout liver increased at low environment temperatures (Miller *et al.*, 1976; Hazel, 1979), suggesting a role for this lipid class in homeoviscous adaptation. PE is the only lipid classes containing plasmalogens in fish eggs (Ranuzzo *et al.*, 1992). In bovine brain, PE contains about 50% plasmalogens, suggesting an important role of PE in the neural system (Bell, 1989). Ranuzzo *et al.* (1992) found very small amount of plasmologens in PE in cod eggs, Bell (1989) reported that PE plasmalogens were not present in eggs of cod, presumably because the brain is not present at this early embryonic stage.

Sphingomyelin (SM) is a major component of mammalian membranes. Its level increases with age at the expense of PC. This process appears in all vertebrates and is likely to cause increased membrane microviscosity. SM and its derivatives are also involved in cellular signal transduction (Kolesnick, 1991). The function of SM in fish has rarely been studied.

TAG is considered to be a reserve lipid since cellular membranes contain only very small amounts. However, TAG probably plays a double role, storing large amounts of saturated fatty acids for energy purpose and serving as a temporary reservoir of physiologically important PUFA.

Cholesterol seems to be the major sterol in fish, it is the precursor for some hormones, and it has been suggested to form a complex with phospholipids which is why it is an important component of biological membranes (Ranuzzo *et al.*, 1992).

Almost all fatty acids in fish eggs and larvae are presented as fatty acyl moieties of phospholipid and NL, and only very small amounts of free fatty acids are found. Monoenes are suggested to be a preferred substrate for catabolism by some authors (Wiegand, 1996). The functions of (n-3) and (n-6)PUFA could be as energy sources, as precursors for enzymatic oxidation to produce eicosanoids and prostaglandins or in phospholipids as structural components (Ackman & Kean-Howie, 1995).

The di-22:6(n-3) species in cod retina phospholipids are strikingly high (30% in PC, 75% in PE, and 60% in PS), suggesting a role of 22:6(n-3) in the normal function of eyes (Bell & Dick, 1991). Recently Bell *et al.* (1995b) found that a dietary deficiency in 22:6(n-3) impaired vision at low light intensities in juvenile herring (*Clupea harengus*). The role of 20:5(n-3) is to modulate the formation of eicosanoids from 20:4(n-6). A high (n-6)/(n-3) ratio is thought to have an adverse effect on embryo and larval development (Sargent, 1995). Although 20:4(n-6) is only present in very small amounts, the oxidation derivatives of this fatty acid are very active in modulating various biochemical process such as reproduction, inflammatory reactions, stress reactions, water transport, and osmoregulation etc.

There are reports that 22:6(n-3) and 20:5(n-3) enriched diets have improved larval survival under normal conditions (Watanabe, 1991, 1993; Mourente *et al.*, 1993) and under stressed conditions as well (Kanazawa, 1995). However, excessive (n-3) PUFA may cause negative effects on larva (Fernandez-Palacios *et al.*, 1995; Rodriguez, 1994) and mature fish (Ackman & Kean-Howie, 1995; Watanabe, 1982). Recent advances in EFA research emphasize the ratio of 22:6(n-3)/20:5(n-3) and (n-3)/(n-6), rather than absolute amounts of these fatty acids (Cure *et al.*, 1995; Abi-ayad *et al.*, 1995; Bell *et al.*, 1995a; Sargent, 1995).

1.2.2. Amino acids

Free amino acids (FAA) and protein bound amino acids (PAA) are abundant in marine teleost eggs and larvae. The majority of the research on their roles as osmolytes and energy substrates in early developmental stages of marine teleost has been carried out in Norway during the past decade.

1.2.2.1. Abundance of FAA in marine fish eggs

Atlantic halibut eggs contain 2300 nmol/egg of FAA (Finn *et al.*, 1991; Ronnestad 1992), and thus have the largest FAA pool among all the species examined. In mackerel (*Scomber scombrus*) eggs, only 30 nmol/egg was detected. Although the absolute amount of FAA is highly variable among different species, after being adjusted for egg volume, the resulting range of concentration is narrowed to 150~170 mM (Ronnestad, 1992). This high FAA concentration compared to that in terrestrial vertebrates, and its stability in different species of pelagic teleost eggs suggests a role for FAA in osmoregulation in the hyperosmotic marine environment. The FAA pool represents 20% to 50% of the total amino acids (FAA+PAA) in pelagic fish eggs at spawning (Ronnestad, 1992; Ronnestad *et al.*, 1993). The FAA pool also comprises a large portion of the total dry weight of newly spawned eggs. FAA make up almost half of the dry weight of the haddock (*Melanogrammus aeglefinus*) egg cells (Riis-vestergaard, 1987) and 18% of the dry weight of Atlantic halibut eggs (Finn *et al.*, 1991) and 50% of the yolk constituents in marine fish larvae are in the form of FAA (Fyhn,

1989). Most of the FAA pool are almost depleted when the larvae first start feeding.

In marine teleost fish, the spectrum of FAA in the egg is very similar among species examined by Ronnestad (1992), since the FAA pool originates from the breaking down of a same yolk protein, phosvitin, which has a nearly constant composition of amino acids. However, as development proceeds, variation may occur since the FAA pool may be utilized differently in difference species.

1.2.2.2. FAA and protein as important energy substrates in developing embryos and larvae

In adult fish, protein (i.e. amino acids) plays a pivotal role in energy production (Mommsen & Walsh, 1992). As reviewed by van Waarde (1983), with respect to their primary sources of energy, fish appear to be quite different from mammals. In mammals, carbohydrate is the major immediate substrate for energy production, and excessive carbohydrate is converted to NL; while in fish such as carp, dietary amino acids are not only used directly for energy production, they are also the major precursors for synthesis of lipid and carbohydrate. During routine activity, the contribution of protein catabolism to energy production is usually over 40%, and basal aerobic metabolism seems to be almost completely covered by protein catabolism. Carbohydrate is poorly utilized in fish, and seems to be used mostly under anaerobic conditions. Under natural conditions, most fish live on a protein-rich carnivorous diet, and they are able to use ingested amino acids predominantly as an energy source. One should not be surprised by this distinctive metabolic trait, since amino acids have easy access to the tricarboxylic acid cycle following deamination to α-keto acids (Fyhn,

1990). From there they can either be catabolized to produce energy, or anabolized to synthesize fatty acid or glucose. Fish eggs and yolk-sac larvae seem to have patterns similar to those in adult fish for energy production, especially in those species without visible oil globules in the yolk (Ronnestad, 1992; Finn *et al.*, 1995a, 1995c). One of the differences is that a significant amount of the amino acids catabolized is from the FAA pool rather than from PAA.

FAA are consistently depleted during the egg and larval stage till the end of yolk resorption (Holleland & Fyhn, 1986; Mangor-Jensen & Fyhn, 1987; Fyhn, 1989; Ronnestad *et al.*, 1993; Finn *et al.*, 1995a, 1995c), both for species with and without oil globules. The majorities of FAA reside in the yolk, and need to be transferred to the embryo and larval body compartment before they can be used. In species without oil globules (cod, halibut, and lemon sole : Ronnestad, 1992; Finn *et al.*, 1995a) the resorption of the FAA pool did not occur until after the completion of epiboly when the syncytium layer is formed. The area of the yolk syncytium layer reaches its maximum and becomes equal to the yolk surface area at completion of epiboly. This tissue contains abundant mitochondria and endoplasmic reticula. Therefore, it is believed to be the main site of metabolism during the early embryonic stage. The rate of yolk resorption is a function of the surface area (Hemming & Buddington, 1988).

In cod eggs, before epiboly is completed, the energy may come from the catabolism of carbohydrate (Finn *et al.* 1995a). No carbohydrate data for Atlantic halibut eggs has been reported. Whyte *et al.* (1993) reported a continuous increase in total carbohydrate content in Pacific halibut (*Hippoglossus stenolepis*) which is closely related to the Atlantic halibut. From after epiboly to hatch, FAA usually contributes more than 60% of the substrates used to

fuel metabolism. After hatch, with the depletion of FAA, protein is catabolized to produce energy, still making amino acid (FAA+PAA) the dominant energy substrate (Fyhn & Serigstad, 1987; Finn *et al.*, 1991; 1995a; 1995c). By measuring the total NH_3 production (NH_3/NH_4^+ in egg and larva plus NH_3/NH_4^+ excreted to ambient environment) and O_2 consumption, these authors conclude that FAA and PAA are involved in aerobic energy metabolism, while anaerobic metabolism, if present in the egg and larval stage, is not important.

In cod eggs that have just been spawned, essential amino acids (EAA) comprised about 55% of the total FAA pool, nonessential amino acids (NEAA) comprised 45% (Finn *et al.*, 1995a). With NEAA being used more rapidly than EAA, the composition changed such that EAA% increased and NEAA% decreased during egg and early larval stages (first two days post hatch) in cod (Finn *et al.*, 1995a). By using the calculated value from Finn's data (1991) and Ronnestad's data (1992), a similar trend was found in halibut eggs and early larval stages. The trend was reversed as first feeding approached, with EAA being used more rapidly than NEAA (Ronnestad, 1992; Finn *et al.*, 1995a).

Individual amino acids in the FAA pool decreased, with two exceptions: taurine remained constant through all developmental stages studied, while phosphoserine increased (Ronnestad, 1992). The timing and extent of the decrease varies with different amino acids and different species. Generally speaking, the most abundant amino acids are the ones most used.

Aside from energy production, a large portion of FAA is directed to protein synthesis (Ronnestad *et al.*, 1993). FAA and PAA should not be viewed as two completely separated

amino acid groups and dynamic exchange between these two amino acid pools can not be ruled out. It is probably more appropriate to see protein as the storage form of FAA (Finn *et al.*, 1995a).

The use of FAA as precursors for gluconeogenesis and lipogenesis in the egg and larval stages has hardly been studied. Finn *et al.* (1995a) reported an increase of glycogen as well as glucose after epiboly in cod eggs, and attributes the increase to gluconeogenesis from FAA. The significance of this metabolic pathway was not discussed. In his reviews (1983, 1988), van Waarde included some reports on coupling of amino acid catabolism to fatty acid chain elongation in perch embryos and adult carp under anoxic condition, and synthesis of neutral lipid from injected glutamate.

1.2.2.3. The role of FAA in osmolality and buoyancy regulation

The FAA pool serves an osmotic function as it is being built up, i.e., even before the egg is released. Significant amounts of FAA in the eggs occur at the final maturation stage of oocytes, shortly before ovulation (Thorsen *et al.*, 1996). Phosphoprotein in the oocyte is extensively hydrolyzed, resulting in a dramatic expansion of the FAA pool (Craik & Harvey, 1984). The vitelline membrane becomes permeable to salt and water concurrently. This increase in osmotically active molecules drives a massive uptake of water and K^+ from the extracelluar tissue. The vitelline membrane becomes almost impermeable to water and solutes upon spawning and fertilization (Potts & Eddy, 1973). The mechanism behind this permeability change is still unknown. The hydration process produces eggs with a volume

three to five times larger than the originating vitellogenic oocyte and a water content of 90-94% (Thorsen *et al.*, 1996; Craik & Harvey, 1987). The water reservoir in the eggs is the only source of water needed for metabolism in the embryo before drinking is initiated, and more importantly, it enables embryos to cope with the small but inevitable osmotic water loss after spawning (Riis-Vestergaard, 1987). Buoyancy is determined primarily by this high water content (Craik & Harvey, 1987).

In newly spawned eggs, FAA makes up about 50% of the osmolality in the eggs of herring, marine and brackish water cod and Atlantic halibut (Holleland & Fyhn, 1986; Thorsen *et al.*, 1996; Riis-Vestergaard, 1982). In an osmotic stress environment such as seawater, high intracellular ionic strength is needed. High concentrations of FAA (except basic amino acids arginine and lysine) in marine fish embryos are more suitable to build this high ionic strength than inorganic ions, because FAA will not perturb the function of macromolecules such as enzymes. Also since most of amino acids are zwitterionic at physiological pH's,transmembrane potential is maintained (Yancey *et al.*, 1982).

As stated above, after activation of the development of the embryo by fertilization, the FAA pool is continuously depleted during the egg and larval stage until the end of yolk resorption. The depleted FAA are catabolized as substrates that produce the energy needed for development, maintenance, and homeostasis (Fyhn, 1989), or polymerized into body protein. Smaller amounts of FAA are also used as precursors for the synthesis of nucleic acids, glycogen, lipid, and some biologically active substances. By participating in these metabolic processes, FAA are depleted and the total amount of osmolyte is decreased to counteract the continuously decreasing yolk volume and water loss (Riis-Vestergaard, 1982;

Ronnestad *et al.*, 1993). Despite the water loss, the yolk osmolality of halibut eggs and larvae become even lower when development proceeds, largely because of the sharply decreased concentration of FAA after hatch.

The major nitrogenous end product of FAA metabolism, NH_3/NH_4^+, accumulates in the yolk at the egg stage and decreases after hatching occurrs (Finn *et al.*, 1991; Fyhn & Serigstad, 1987). Before hatching, the excessive inorganic osmolyte K^+ that has accumulated before spawning decreases (Riis-Vestergaard, 1982; Thorsen *et al.*, 1996). The accumulated NH_4^+ is believed to substitute for this heavier ion, so that the density of the eggs is decreased to increase the buoyancy (Thorsen *et al.*, 1996). The change of buoyancy may be important for the developing egg and larva to "choose" a suitable depth in the water column where there is the proper salinity, light conditions, temperature, oxygen concentration, and for feeding larvae, edible food. The role of FAA and NH_3/NH_4^+ as osmolality and buoyant substances is especially important for the early developmental stages, when the organs responsible for osmolality and buoyancy regulation are not yet functioning.

Although there are reports of marine embryos taking up small molecule nutrients from the environment, due to the low permeability of the vitelline membrane, those nutrients taken up are quantitatively unimportant (Ronnestad, 1992). Thus one still can make the statement that the yolk is the sole nutrient resource for the embryos and pre-feeding larvae. For those species, such as Atlantic halibut, which have a prolonged pre-feeding stage, efficient utilization of the limited nutrient is crucial. FAA is a less efficient energy substrate compared to lipid: The combustion value is 21.00kJ/g for FAA, and 35.56kJ/g for lipid (Finn *et al.*, 1995b). However, its importance in osmolality and buoyancy regulation may override

its comparatively low efficiency in energy production.

1.2.2.4. End products of amino acid metabolism: Ammonia and urea

In adult fish, ammonia is the principal end product of nitrogenous metabolism, although urea may comprise as much as 20-46% of total nitrogenous output (Mommsen & Walsh, 1992; Sayer & Davenport, 1987; Wright *et al.*, 1995). In fish, ammonia mainly comes from the catabolism of protein and nucleic acids. Production of ammonia requires little metabolic expenditure, and the end product un-ionized NH_3 is a small lipophilic molecule that can diffuse easily across lipid membranes. As an ion, NH_4^+ is also substantially permeable. NH_3 is highly toxic if accumulated in the cell. However, in fish eggs and larvae, although NH_3 accumulated before hatching, NH_3 is trapped in the yolk that has pH<5 (Ronnestad, 1992). In mammals, the ornithine-urea cycle is the main route for ureogenesis, while according to Mommsen & Walsh (1992), urea production in adult fish is through uricolysis following turnover of nucleic acids. Although this process has considerable metabolic cost, the product, urea is a substance with no toxicity to the living organism.

Read (1968) found ornithine carbamoyltransferase and arginase activity in early embryos of the dogfish (*Squalus suckleyi*) and the skate (*Raja binoculata*), suggesting that a functional ornithine-urea cycle occurred in the early embryo stages of these two elasmobranch species. Depeche *et al.* (1978) detected $^{14}CO_2$ incorporation of urea in rainbow trout (*S. gairdneri*) embryos at a late stage. In a recent study Wright *et al.* (1995) found the expression of carbamoyl phosphate synthetase and ornithine transcarbamylase (two enzymes

in ornithine-urea cycle) activities in rainbow trout (*Oncorhynchus mykiss*) after hatch, and these continued to increase until 17 days after the onset of first feeding. Adult liver enzyme activity of this species was several-fold lower.

As discussed above, the NH_3 content of halibut eggs and larvae and its excretion to ambient water is well documented, but the production of urea has not been studied in embryonic and larval stages of this species. Finn *et al.* (1995c) found that in halibut larvae after 14 days post hatch, the NH_3 value calculated from FAA catabolism is slightly higher than that actually detected; they attributed this discrepancy to the formation of urea in liver, since the liver is fully developed at this stage. In embryos, urea was not taken into consideration (Ronnestad, 1992). Enzyme and isotope studies are needed so that definite conclusions on this issue in halibut larvae can be drawn.

1.3. Summary

It can be concluded from the above studies that both lipid (including fatty acids) and amino acids (FAA and PAA) are extensively catabolized as energy substrates during embryonic and pre-feeding larval stages of marine fish. The pattern of the catabolism may differ among different species, but generally speaking, the most abundant component is usually the one that ends up being used the most. In addition to energy production, both lipids and amino acids are also actively involved in biochemical and biophysical processes. Free amino acids are important factors in osmoregulation. (n-3)PUFA are involved in the maintenance of membrane stability at low environmental temperatures. (n-6)PUFA are

preferred precursors for the synthesis of eicosanoids which are important signal transduction chemicals in biological system. These biochemical components are important for the normal functioning of developing embryos and larvae, but the correlation between these biochemical compositions and the viability of eggs and larvae is poorly defined. The effect of physiological condition of broodstock fish (age, ovulation rhythm, hormone level etc.) on the biochemical composition of eggs has been studied less than dietary factors.

1.4. Objectives:

1. To investigate the correlation between the quality of Atlantic halibut eggs and biochemical composition.
2. To investigate physiological condition of broodstock females that may affect the biochemical composition of eggs.
3. To investigate the changes in biochemical composition in different embryonic and larval stages.

Chapter 2. Materials and Methods

2.1. Animals

2.1.1. Broodstock

All Atlantic halibut eggs were obtained from captive broodstock. The halibut were kept in covered tanks with a diameter of 5 m and depth of 1.25 m. The temperature in the tank was kept between 5-10°C. The tanks were filled with a continuous flow of seawater (70L/min) from Logy Bay, Newfoundland. The fish were fed herring, mackerel, squid, artificial pellets and vitamin supplements twice a week except during the spawning season (Daniel *et al.*, 1993).

Spawning of the fish was predicted by monitoring the level of estradiol-17β and vitellogenin in blood (Methven *et al.*, 1992). Eggs and sperm were obtained by gentle stripping of the fish. The eggs are stripped within 6 hours of ovulation (release of eggs into abdomen) (Brown & Keough, 1994).

2.1.2. Determination of fertilization success

To observe the fertilization of the eggs, 3 ml of eggs with ovarian fluid were mixed with 10 μl of milt, and seawater was added immediately. Fertilization was carried out immediately after stripping of the eggs. The motility of the sperm was observed under the

microscope before fertilization. Fertilization success was determined by counting the number of eggs that had successfully completed two divisions (4-cell stage) or three divisions (8-cell stage). To ensure the stability of the quality of the sperm, the milt used in fertilization was a mixture of the milt from two males.

2.1.3. Rearing of larvae

Fertilized eggs were kept in 200 L upwelling incubators with a flow rate of 0.4-0.75 l/min. The temperature was maintained at ~5°C and the salinity at 34.6 ‰. All incubators were kept in the dark. About 1000 newly hatched larvae were transferred to 5 petri-dishes (15 cm of diameter) containing filtered seawater with penicillin-G (60 mg/L) and streptomycin sulfate (100 mg/L). The water was changed and dead larvae were removed every two days. All petri-dishes were kept in a dark cold room with a temperature of 5°C. All larvae used in this study were feeding endogenously. In 1991- 1993, viability of the larvae was determined by the length of time (days) from just hatch to the time when 100% mortality of the larvae occurred. In 1995, viability was determined when 95% mortality occurred.

2.2. Analyses of biochemical components

2.2.1. Storage of samples

Eggs or larvae for lipid and fatty acid analysis were rinsed and dried on soft tissue

paper, stored in 10 ml vials containing 2 ml ice cold $CHCl_3$. The vials were filled with N_2 gas, sealed with Teflon tape and stored at -20°C until analysis.

Samples for protein and free amino acid analysis were put into cryovials and stored in a -20°C freezer.

2.2.2. Lipid extraction

Total lipid was extracted by the method of Bligh and Dyer (1959). A predetermined amount of ketone (3-hexadecanone) was added into each sample as an internal standard. The eggs or larvae were ground up using a glass rod, the rod was rinsed thoroughly with methanol and chloroform (1:1 v/v) after grinding, and 1 ml of distilled water was added. The vial was then filled with N_2 gas, sealed with Teflon tape and sonicated in an ice water bath for 3 minutes. The vials were left in a -20°C freezer overnight, allowing the water and chloroform layers to separate. The chloroform layer was transferred to a clean vial, filled with N_2 gas and stored at -20°C until analysis.

2.2.3. Lipid class analyses

Lipid class analyses was carried out using an Iatroscan Mark V TLC/FID analyzer (Iatron Laboratories, Tokyo, Japan) (Parrish, 1987). The lipid extract was applied to silica gel coated Chromarods-SIII (RSS Inc., CA, USA) and developed in four solvent systems. After being focused twice in acetone, and conditioned in a humidity chamber for 5 minutes, the

rods were put into the first development solvent, hexane : diethyl ether : formic acid (99:1:0.05, v/v/v). A double development was used to separate the first group of NL: hydrocarbon, sterol ester and ketone. After development, the rods were partially scanned to the position after the ketone peak. The second group of NL were separated in the second solvent system, hexane : diethyl ether : formic acid (80:20:1, v/v/v). The rods were developed in this solvent system for 40 minutes, and the peaks of TAG, free fatty acids (FFA), alcohol (ALC) and ST were scanned. The peak of acetone mobile polar lipid (AMPL) was acquired by developing in the third solvent, acetone, twice, for 12 and 10 minutes, respectively. To separate the phospholipids PE, PC, and SM, the rods were focused in the last solvent, chloroform : methanol : water (70:35:3.5, v/v/v), conditioned in a humidity chamber and developed twice in this solvent for 35 and 30 minutes. The chromatograms of the four scans were combined, and the area of each peak was calculated using TSCAN DATA software (RSS Inc., CA, USA). The peak of each component was identified by comparison with the chromatogram of standards acquired concurrently with the samples. The relation between the weight of the lipid component on Chromarods and the area of the corresponding peak obtained was calibrated by applying known amount of standards on the Chromarod and carrying out the development under the same conditions as described above. All the organic solvents used were from EM Science (Gibbstown, NJ, USA).

2.2.4. Fatty acid analyses

The fatty acid compositions of three lipid classes PE, PC, and TAG were analyzed on

a Hewlett Packard 5890 series II gas chromatography with a flame ionization detector (FID) (Hewlett Packard, Palo Alto, CA, USA).

PE, PC and TAG were separated on silica-gel 60 plates (Fisher, Fair Lawn, NJ, USA). Lipid extracts containing ~2 mg of lipid were blown dry under N_2 gas and dissolved in 20 μl of chloroform : methanol (2:1, v/v) mixture. All of the sample was applied to the silica-gel plate, dried and put into a development tank. PL were separated in chloroform : methanol : acetic acid : formic acid : water (70:30:12:4:2, v/v/v/v). NL were separated in hexane : ethyl ether : acetic acid (85:15:2, v/v/v). Standard TAG (triolein), PC (dipalmitolphosphatidylcoline) and PE (DL-α-phosphatidylethanolamine, dipalmitoyl) were also spotted on the plate to indicate the position of each lipid class. The spots representing TAG, PC and PE were scraped off into transmethylation vials, and hexane was added to extract the lipid from the silica gel powder.

Fatty acid methyl esters (FAME) were prepared by the official method of the A.O.C.S. (the American Oil Chemists' Society, 1989, Ce1b-89). 0.5 N NaOH in methanol was added to the transmethylation vial containing TAG, PC or PE. The vial was then filled with N_2 gas, sealed and heated at 100°C for 7 minutes. After the vial was cooled to 30 - 40°C, 14% BF_3/Methanol (Pierce, Rockford, IL, USA) was added, the vial was filled with N_2 gas and heated again at 100°C for 5 minutes. FAME were extracted with hexane. The hexane layer was taken out and dried under N_2 gas. Extracted FAME were dissolved in 10 μl of carbon disulfur (Fisher, Fair Lawn, NJ, USA), 0.2 μl of the solution was injected in to the GC. The column used was a 30 m Supelcowax-10 with a internal diameter of 0.53 mm (Supelco Inc., Bellefonte, PA, USA). The oven, injector and detector temperatures were set at 205, 225

and 225°C, respectively. Helium was used as the carrier gas (5 ml/min).

The peaks on the chromatogram were identified by comparison with a marine oil FAME standard PUFA-1 (25 mg/ml) (Matreya Inc., Pleasant Gap, PA, USA). The weight percentage of each fatty acid was calculated according to percentage of peak area using Hewlett Packard Chem Station software.

All organic solvents used were from EM Science, Gibbstown, NJ, USA; and all lipid class standards were from Sigma Chemical Co., St. Louis, MO, USA.

2.2.5. Protein assay

Total protein was analyzed by the method of Lowry (1951). BSA (1 mg/ml) was used as a standard. The eggs or larvae were homogenized in 0.5 N NaOH. The standard was dissolved in 0.5 N NaOH. Both the standard and the homogenate were heated at 80°C for 30 minutes. Measurements were carried out on a LKB Biochem NOVASPEC 4049 spectrophotometer at 660 nm.

2.2.6. Free amino acid and NH_3 analyses

The method for FAA analyses is adopted from Gorden & Cornect (1987). Halibut eggs and larvae were homogenized in H_2O. A known amount of AEC (S-2-amino ethyl-L-cysteine in HCl, Beckman, Fullerton, CA, USA) was added at this stage as internal standard. The homogenate was deproteinized using 0.4 M perchloric acid. The sample was left at room

temperature for 20 minutes. The mixture was then centrifuged at 12,000 g for 10 minutes. The supernatant was separated, the pH was adjusted to 2.2 with 3N KOH, and mixed with equal volume of lithium citrate buffer with 0.3M Li^+, pH 2.2 (Beckman, Fullerton, CA, USA).

The contents of free amino acids and the level of NH_3 was analyzed on a Beckman 121MB amino acid analyzer, using a Benson D-X8.25 cation exchange resin with a bed size of 200 X 2.8 mm and a single-column-three-buffer method. Quantitation of the results was achieved using a Hewlett Packard Computing Integrator Model 3395 A (see Gorden & Cornect, 1987 for details).

2.2.7. Dry weight, ash content, and ash free dry weight

For measurement of egg dry weight, 10 eggs randomly selected from each batch of eggs spawned were dried overnight in a forced-air oven at 105 °C. Ash content was determined by charring the eggs in a weighed crucible over a Bunsen burner and then heating in a muffle furnace at 550 °C until the ash had a white appearance (Srivastava *et al.*, 1993). Ash free dry weight was calculated from subtracting ash weight from dry weight.

2.3. Statistics

Statistical analyses were performed using Minitab release 10Xtra (Minitab Inc., State College, PA, USA). The normality of data distribution was tested using the Anderson-Darling

test. Significant differences were tested by single factor analysis of variance (ANOVA), Duncan multiple comparison test, or by Student-t test. Probability levels of 5% and 1% were taken as significant levels.

Chapter 3. Results

3.1. Biochemical composition of unfertilized Atlantic halibut eggs

3.1.1. General characteristics

The average diameter of unfertilized Atlantic halibut eggs was about 3 mm, therefore, the calculated volume of the egg was about 14 μl. The average dry weight (DW) of the unfertilized egg was about 1.52 mg (Table 1). Ash constituted 9% of the DW. The volume of the egg was significantly correlated with egg DW ($n = 51$, $r^2 = 0.437$, $P<0.01$) and egg TL ($n = 51$, $r^2 = 0.256$, $P<0.01$).

The sum of lipid, protein, and FAA made up about 88% of DW and 96% of the ash free dry weight (AFDW). Protein was the most abundant biochemical component in unfertilized eggs, making up about 60% of DW. The weight of FAA was a calculated value obtained by multiplying the average molar mass of total FAA per egg by an average molecular weight of 130 Dalton (Finn *et al.*, 1995). The resulting weight of FAA was about one fourth of the total amino acid pool (FAA+PAA). The total lipid accounted for about 10% of DW.

3.1.2. Lipid classes and fatty acids

PL and NL constituted 76.7% and 22.9% of the total lipid respectively (Table 2). PC

Table 1. Biochemical contents of unfertilized Atlantic halibut eggs. Eggs were from two brood stocks (DW: dry weight; AFDW: ash free dry weight; FAA: free amino acids; S.D.: standard deviation).

	spawning season	sample size	mean (μg/egg)	S.D.	DW%
DW	1993~1995	n=23	1522.6	120.8	
AFDW	1993~1995	n=22	1393.7	117.3	91.5
Ash	1993~1995	n=22	139.9	50.0	9.2
Lipid	1993~1995	n=25	154.4	27.9	10.1
FAA	1995	n=10	287.3	43.2	18.9
Protein	1994~1995	n=12	898.3	94.5	59.0

Table 2. Lipid classes of unfertilized Atlantic halibut eggs. Data are average of 25 batches of eggs of two brood stocks spawned from 1993 to 1995 seasons, and for each batch, a sample of 15 eggs are selected. (TAG: triacylglycerol; ST: sterol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; NL: neutral lipids; PL: polar lipids; Wt: weight; S.D.: standard deviation).

	mean (μg/egg)	S.D.	mean (Wt%)	S.D.
TAG	18.5	2.7	12.2	1.3
ST	13.9	2.1	9.3	1.6
PE	14.8	4.8	9.8	2.4
PC	91.0	19.7	60.2	4.1
SM	9.4	1.6	6.5	1.2
Σ NL	34.7	4.9	22.9	2.9
Σ PL	118.8	23.8	76.7	2.1
Total	154.4	28.0		

was by far the largest component among all the lipid classes present in unfertilized eggs, accounting for 60% of total lipid. TAG was the largest NL and the second largest of all lipid classes (12% of total lipid). The amounts of ST and PE were about the same (9% of total lipid). SM was about 6.5% of total lipid. In addition to the lipid classes mentioned above, hydrocarbon and AMPL could also be detected in some samples. The amounts of these two quantitatively unimportant lipid classes were about 0.2 and 2.5 μg/egg respectively.

The fatty acid compositions (Wt%) of PC, PE and TAG were determined separately. Seventeen batches of eggs produced during 1994 and 1995 spawning seasons were used. Although the fatty acid profiles of the three lipid classes were different (Figure 1), they shared some similarities: 16:0, 18:1(n-9) and EPA+DHA were the most abundant fatty acids in each of the saturates, monoenes and polyunsaturates categories. In PC, saturates were the major components (56% of PC fatty acids), the levels of PUFA (21% of PC fatty acids) and monoenes (17% of PC fatty acids) were similar. In PE fatty acids, total saturates were slightly higher than monoenes, but their levels were very close (38% and 33% respectively). Polyenes were 23% of PE fatty acids. Among TAG fatty acids, the levels of monoenes, saturates, and polyenes were about 48%, 35%, and 13% respectively. The (n-3)/(n-6) ratios in PC, PE and TAG were 10.5, 14.6, and 4.5 respectively. PE had the highest percentage of (n-3) PUFA (about 20%) among the three lipid classes (Figure 1).

3.1.3. Free amino acids

Total free amino acids were about 2300 nmol/egg (Table 3). Using the calculated egg

Figure 1. Fatty acids profiles of PC, PE and TAG in Atlantic halibut eggs.

Each bar represents the average ± S.D. of 17 batches of eggs produced by two females during the 1994 and 1995 spawning seasons. For each batch, triplicates samples of 15 eggs were collected randomly.

PC: phosphatidylcholine; PE: phosphatidylethanolamine; TAG: triacylglycerol; SAT: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA polyunsaturated fatty acids; S.D: standard deviation.

Figure 1.

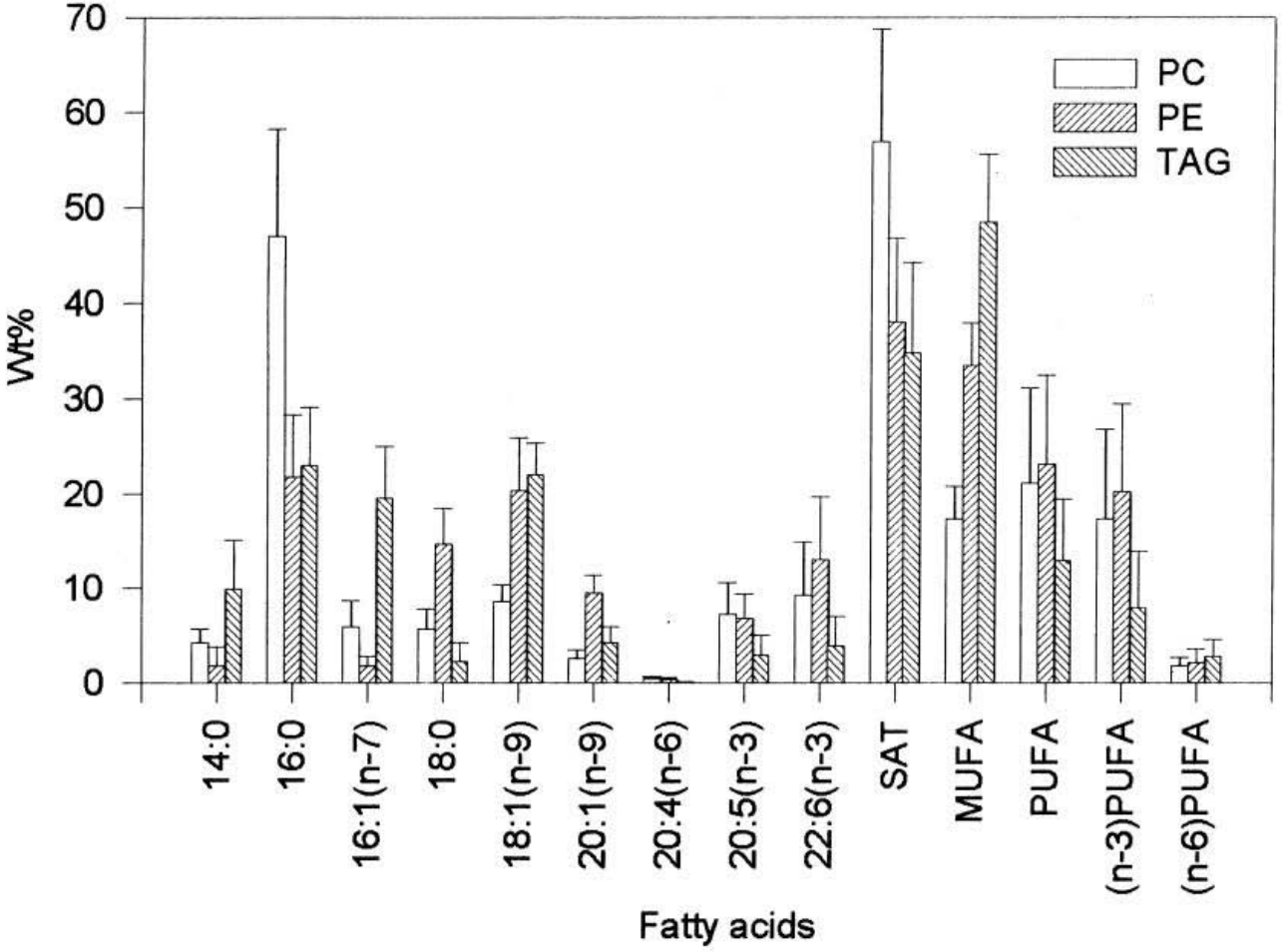

70
60
50
40
30
20
10
0
Wt%
PC
PE
TAG
14:0
16:0
16:1(n-7)
18:0
18:1(n-9)
20:1(n-9)
20:4(n-6)
20:5(n-3)
22:6(n-3)
SAT
MUFA
PUFA
(n-3)PUFA
(n-6)PUFA
Fatty acids

Table 3. Free amino acids in unfertilized Atlantic Halibut eggs. Data are means of 10 batches of eggs produced by two brood stocks in 1995 spawning season, for each batch, a sample of 15 or 30 eggs are randomly selected. (EAA: essential amino acids; NEAA: nonessential amino acids; FAA: free amino acids; S.D.: standard deviation).

	nmol/egg		mol%	
	mean	S.D.	mean	S.D.
EAA				
Threonine	95.13	14.50	4.15	0.16
Valine	162.02	23.88	7.06	0.23
Methionine	60.22	7.82	2.63	0.11
Isoleucine	120.56	18.49	5.26	0.25
Leucine	237.23	35.62	10.34	0.37
Tyrosine	51.60	9.26	2.25	0.10
Phenylalanine	52.12	8.04	2.27	0.09
Lysine	197.17	29.83	8.59	0.26
Histidine	40.86	6.65	1.78	0.14
Arginine	94.54	15.05	4.12	0.15
NEAA				
Cysteic acid & Phosphoserine	7.10	1.17	0.31	0.02
Taurine	67.17	10.81	2.93	0.11
Aspartic acid	81.42	15.31	3.55	0.30
Serine	269.15	42.17	11.73	0.48
Asparagine	97.88	16.82	4.27	0.49
Glutamic acid	144.25	21.33	6.29	0.20
Glutamine	135.52	19.85	5.91	0.37
Proline	65.79	11.95	2.87	0.26
Glycine	79.21	12.23	3.45	0.14
Alanine	221.59	34.53	9.66	0.41
α-aminobutyric acid	0.61	0.16	0.03	0.01
Cysteine	3.04	1.19	0.13	0.05
Tryptophan	10.33	2.49	0.45	0.06
Σ EAA	1111.45	166.42	48.45	1.36
Σ NEAA	1182.70	179.62	51.55	1.65
Σ FAA	2294.14	345.77	--	--
NH_3	44.92	13.19	--	--

volume (14 μl), the concentration of FAA was about 160 mM. FAA were distributed almost equally between essential amino acids (EAA) and nonessential amino acids (NEAA), although the amount of the latter was slightly higher. Leucine, lysine, valine, and isoleucine (in descending amounts) comprised 65% of essential free amino acids. Serine, alanine, glutamine, and glutamic acid (in descending amounts) constituted about 70% of the nonessential free amino acids. In unfertilized eggs, NH_3 was a comparatively minor component accounting for 45 nmol/egg. Urea was not detectable.

3.2. Egg biochemical composition in relation to fertilization success and larval viability.

For Atlantic halibut eggs, a fertilization success $\geq$ 75% usually indicates a high egg quality, while a percentage lower than 75% indicates poor egg quality (Dr. J. A. Brown, Ocean Science Centre, Memorial University of Newfoundland, personal communication). To find quantitative differences in the biochemical components of better eggs and of poorer eggs, the biochemical contents of the unfertilized eggs that gave higher fertilization success ($\geq$75%) was compared with those that gave lower fertilization success (<75%).

Larval viability was determined by the survival time of unfed larvae, i.e., the length of time between hatching and 100% mortality. The average survival time was 15.9 days. Lower larval viability was defined as a survival time shorter than 15.9 days, while a survival time $\geq$ 15.9 days was defined as higher viability. The biochemical compositions of unfertilized eggs that subsequently fertilized and produced higher and lower viability larvae were compared. Lipid classes data was obtained from eggs produced from the 1991 to 1995 spawning

seasons, and fatty acid data were collected from eggs produced in the 1994 and 1995 spawning seasons.

In order to accentuate differences between "better" and "poorer" eggs, the same data sets were also examined statistically after removing the middle third of the data set when ordered according to fertilization success or survival time. However, the comparison of lipid class and fatty acid data between the upper 1/3 and lower 1/3 showed similar results, as did the comparison between the upper 1/2 and lower 1/2 of the data sets.

3.2.1. Lipid classes and fatty acids

As shown in Figure 2, the eggs with a higher fertilization success had significantly lower ST (16.77 ± 5.20 μg/egg *versus* 20.31 ± 5.78 μg/egg, $P<0.05$) and NL (40.00 ± 10.00 μg/egg *versus* 49.10 ± 12.80 μg/egg, $P<0.01$) than those with lower fertilization success rates.

Among TAG fatty acids (Figure 3a) in "better" eggs, there was a significantly higher level of Σ(n-6) PUFA ($P<0.05$), and a significantly lower level of 16:1(n-7) ($P<0.05$) compared to "poorer" eggs. Of the PE fatty acids, 20:5 (n-3) was significantly higher ($P<0.05$) in "better" eggs than in "poorer" eggs (Figure 3b), while 14:0 was significantly lower in the former than in the latter ($P<0.05$). PC fatty acid profiles between "better" and "poorer" eggs were not significantly different ($P>0.05$). The molar mass of individual fatty acids in total lipid was calculated by adding the molar mass of each fatty acid in PC, PE and TAG. "Better" eggs had a significantly higher molar mass of 18:2(n-6) (nmol) in total lipid, and a significantly lower 14:0 than "poorer" eggs (Figure 4).

Figure 2. Comparison of lipid classes in Atlantic halibut eggs with fertilization success above and below 75% (n = 19, n = 33, respectively).

Each bar represents the average $\pm$ S.D. of measurements on "n" batches of eggs. For each batch, triplicate samples of 15 eggs were selected randomly.

TAG: triacylglycerol; ST: sterol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; TNL: total neutral lipid; TPL; total polar lipid; TL; total lipid; S.D.: standard deviation.

* $P<0.05$, ** $P<0.01$.

Figure 2.

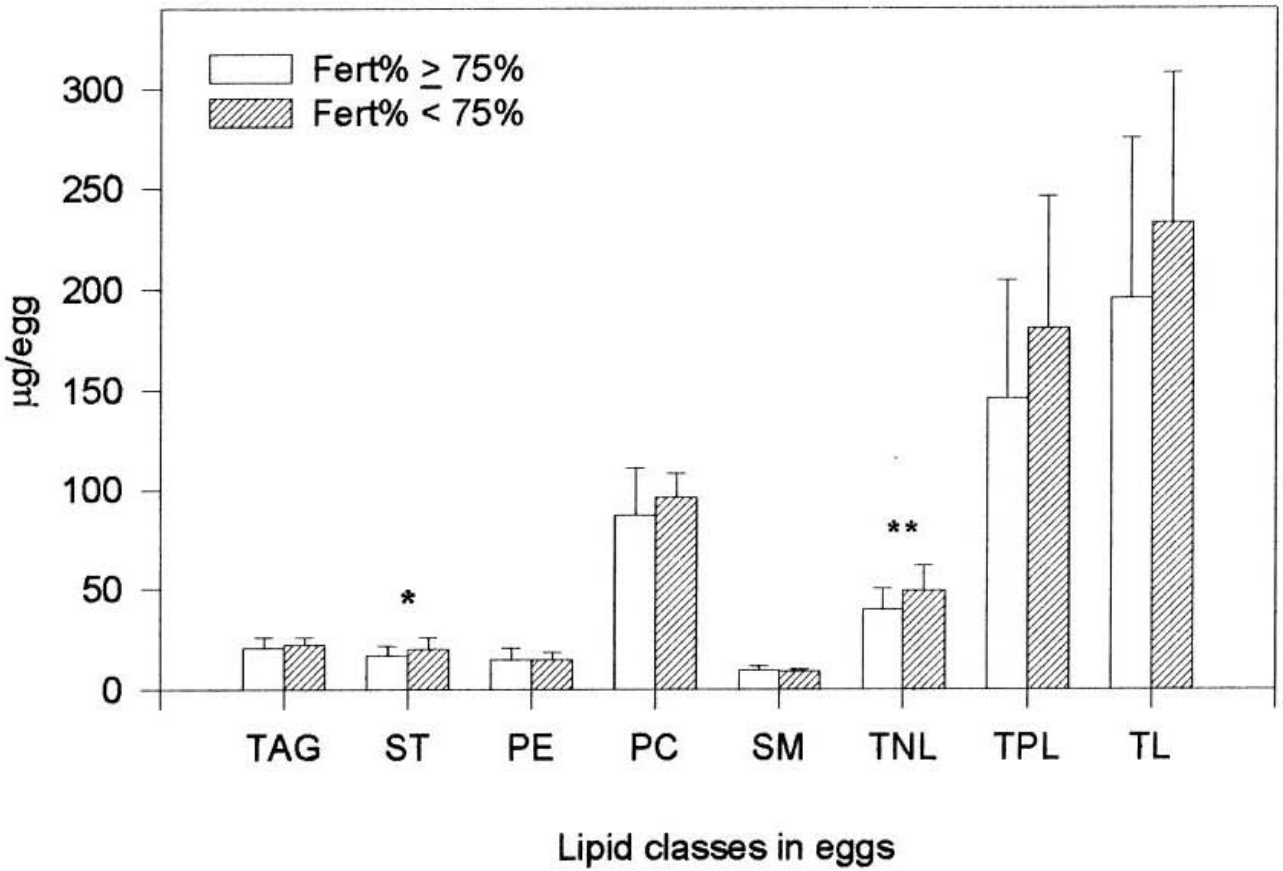

Fert% ≥ 75%
Fert% < 75%
300
250
200
150
100
50
0
µg/egg
*
**
TAG
ST
PE
PC
SM
TNL
TPL
TL
Lipid classes in eggs

Figure 3. Comparison of TAG and PE fatty acids in Atlantic halibut eggs with fertilization success above and below 75%.

(a) Comparison of fatty acids in TAG (n=11, n=6, respectively), (b) Comparison of fatty acids in PE (n=11, n=6, respectively). Each bar represents the average ± S.D. of "n" batches of eggs. For each batch, triplicate samples of 15 eggs were collected randomly. TAG: triacylglycerol; PE: phosphatidylethanolamine; Fert: fertilization; SAT: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; S.D.: standard deviation.

* $P<0.05$, ** $P<0.01$.

Figure 3.

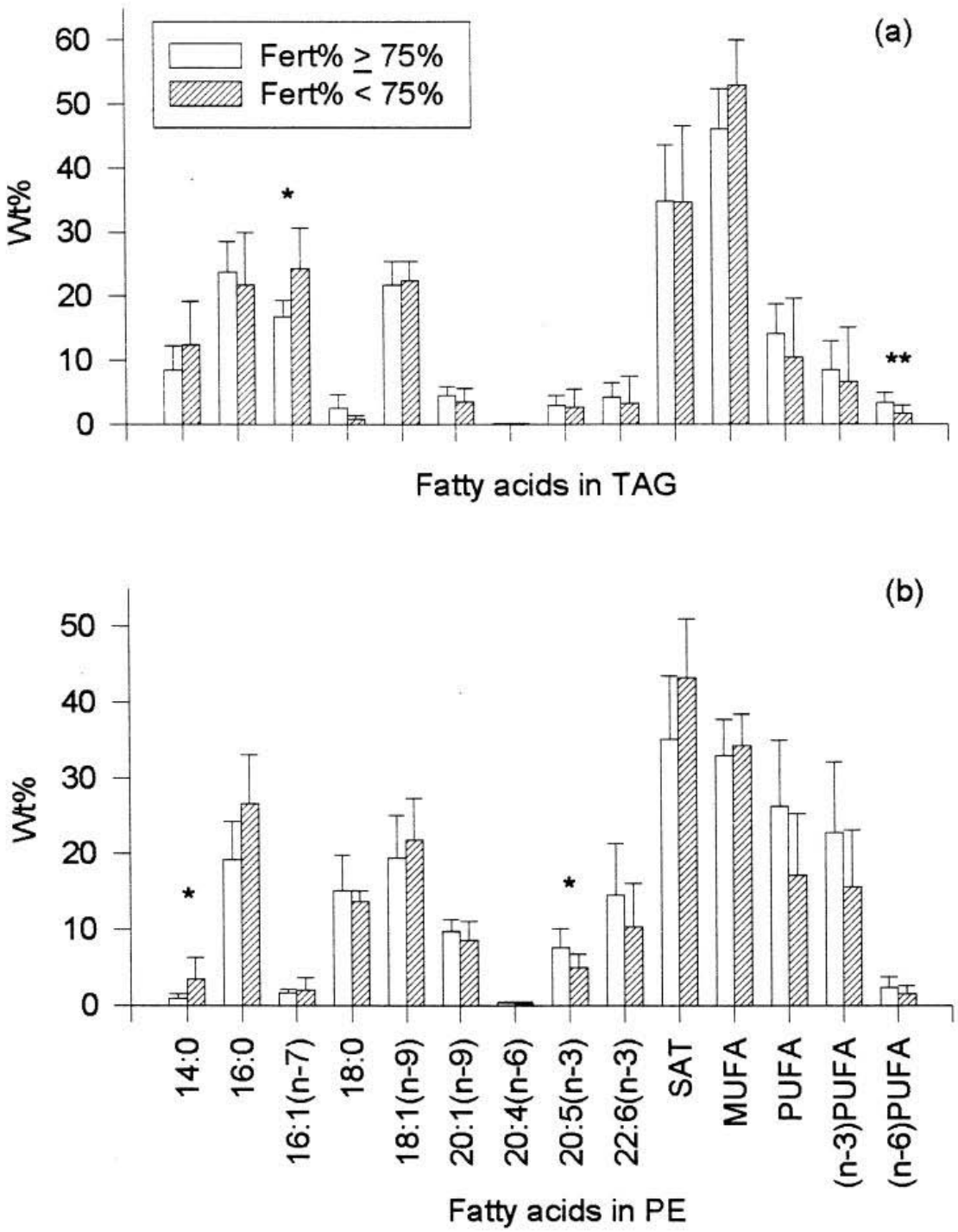

(a)
Fert% ≥ 75%
Fert% < 75%
Wt%
60
50
40
30
20
10
0
*
**
Fatty acids in TAG
(b)
Wt%
50
40
30
20
10
0
*
*
14:0
16:0
16:1(n-7)
18:0
18:1(n-9)
20:1(n-9)
20:4(n-6)
20:5(n-3)
22:6(n-3)
SAT
MUFA
PUFA
(n-3)PUFA
(n-6)PUFA
Fatty acids in PE

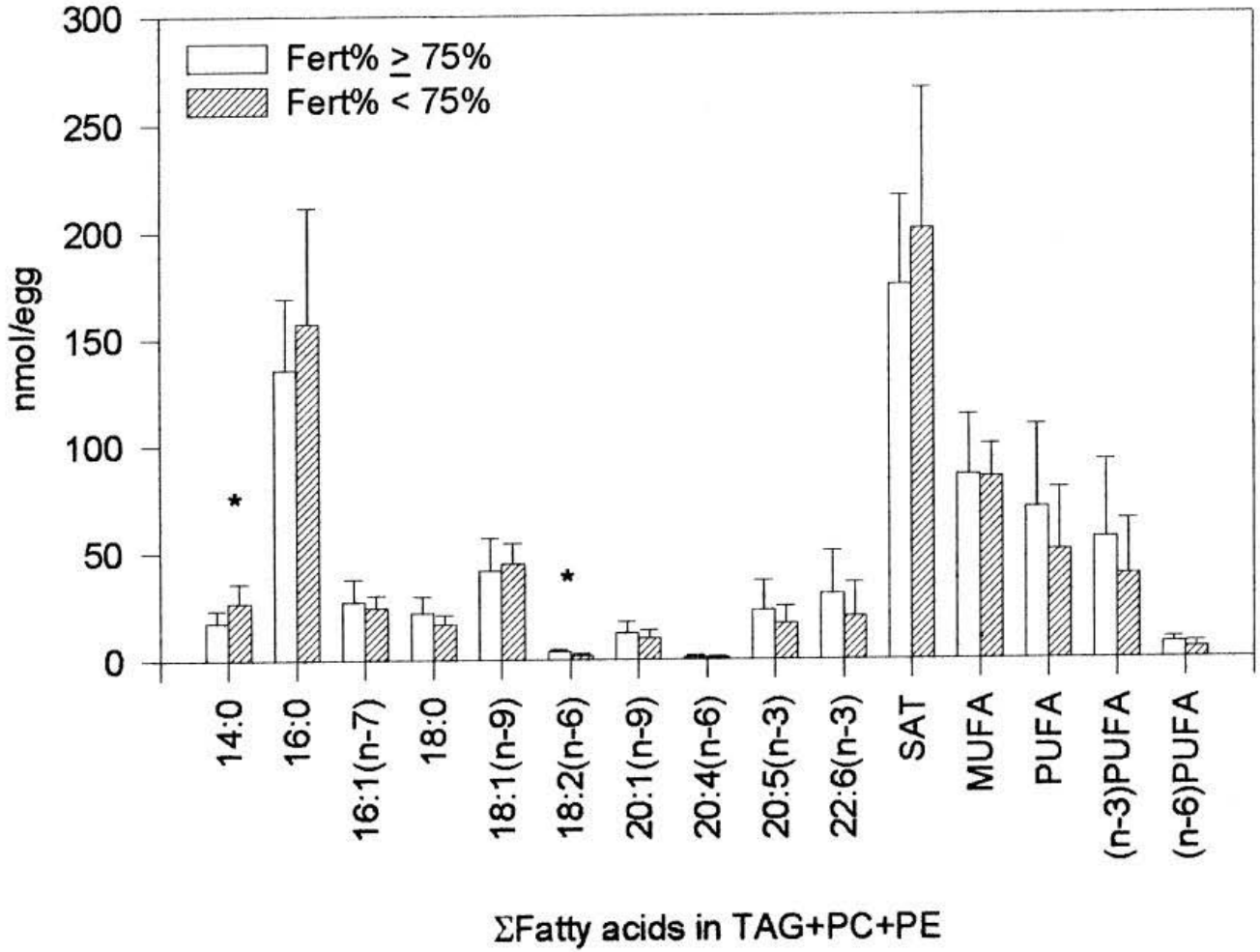
Figure 4. Comparison of the sum of the principal fatty acids in Σ (TAG+PC+PE) in Atlantic halibut eggs with fertilization success above and below 75% (n=11, n=16, respectively).

Each bar represents the average ± S.D. of "n" batches of eggs. For each batch, triplicate samples of 15 eggs were collected randomly.

PC: phosphatidylcholine; PE: phosphatidylethanolamine; TAG: triacylglycerol; SAT: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA polyunsaturated fatty acids; S.D: standard deviation.

* $P<0.05$.

Figure 4.


300
250
200
150
100
50
0
nmol/egg
Fert% ≥ 75%
Fert% < 75%
*
*
14:0
16:0
16:1(n-7)
18:0
18:1(n-9)
18:2(n-6)
20:1(n-9)
20:4(n-6)
20:5(n-3)
22:6(n-3)
SAT
MUFA
PUFA
(n-3)PUFA
(n-6)PUFA
ΣFatty acids in TAG+PC+PE

As shown in Figure 5, the eggs that produced larvae with higher viability contained a significantly lower ($P<0.05$) amount of TAG (20.80 ± 4.04 μg/egg) than eggs that produced less viable larvae (24.25 ± 3.69 μg/egg). No significant differences were found in fatty acid compositions in higher viability eggs and lower viability eggs, presumably due to insufficient larval viability data in the 1994 and 1995 spawning season.

When lipid classes data was expressed as a percentage of egg dry weight, results were similar as those expressed as μg/egg.

3.3. Condition of broodstock and variation in biochemical composition of eggs

3.3.1. Food intake of broodstock and total lipid in eggs

The lipid and dry weight of eggs from one fish ("A") were followed in successive spawning seasons from 1991 to 1995. The samples in 1991 and 1992 were fertilized eggs, while the samples from 1993 to 1995 spawning seasons were unfertilized eggs. Lipid classes in fertilized eggs were not significantly different from those in unfertilized eggs (data not presented). Food intake of the broodstock was expressed as percentage of body weight per day.

Atlantic halibut usually spawn in late winter and early spring (i.e., from December to March), therefore, the biochemical composition of the eggs produced in a specific spawning season may be affected by the food intake of the broodstock during the previous year. The food intake of female "A" decreased continuously from 1990 to 1992. In 1993, there was a

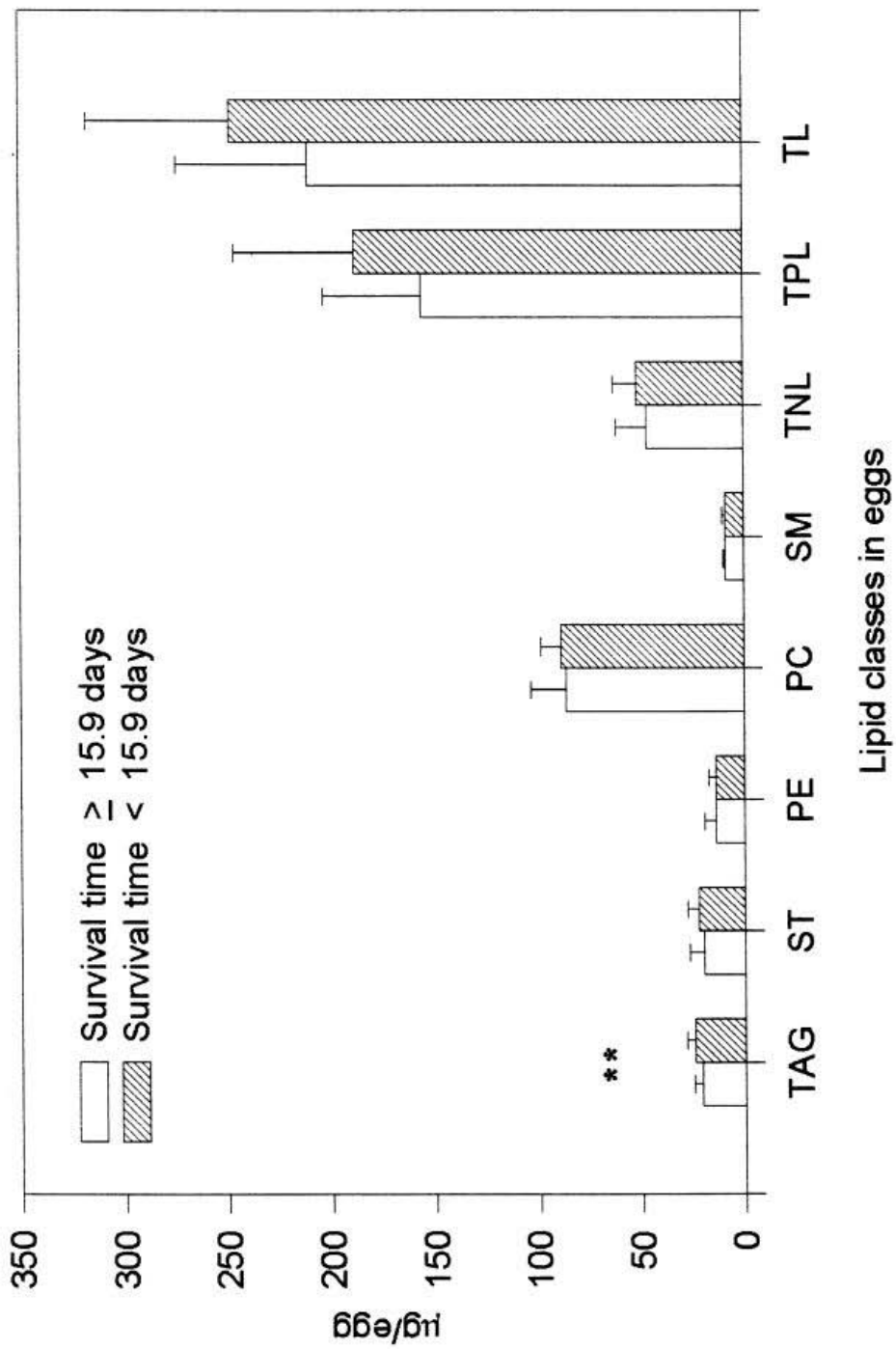
Figure 5. Comparison of lipid classes in Atlantic halibut eggs that produce larvae with higher and lower viability (n=19, n=18 respectively).

Each bar represents the average ± S.D. of "n" batches of eggs. For each batch, triplicate samples of 15 eggs were collected randomly.

TAG: triacylglycerol; ST: sterol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; TNL: total neutral lipid; TPL; total polar lipid; TL; total lipid; S.D.: standard deviation.

** $P<0.01$.

Figure 5.


Survival time ≥ 15.9 days
Survival time < 15.9 days
µg/egg
350
300
250
200
150
100
50
0
**
TAG
ST
PE
PC
SM
TNL
TPL
TL
Lipid classes in eggs

large increase of food intake compared to 1992. Food intake decreased again in 1994 (Figure 6a). The variation of dry weight of eggs produced in 1991-1995 shared a similar trend with the change of food intake in 1990-1994 (Figure 6b). The lipid content in eggs decreased concurrently with egg dry weight from 1991 to 1993 (Figure 6c). The increase in egg dry weight in 1994 following the elevated food intake level in 1993 was not accompanied by an increase in egg lipid. On the contrary, the total lipid content continued to drop in 1994. The increase in egg lipid was delayed until 1995, despite the decrease in food intake in 1994 (Figure 6c). Despite the variation of food intake, the body weight of female "A" increased continuously from 1990 to 1994 (Table 4).

3.3.2. Spawning rhythm and biochemical composition of eggs

Atlantic halibut is a multi-batch spawner, usually producing several batches of eggs in a spawning season, thus the variation of biochemical composition of eggs from different batches over the spawning season was investigated.

As presented in Figure 7, the AFDW of eggs decreased over spawning seasons ($n = 23$, $P<0.05$). There was also a strong negative correlation ($n = 12$, $P<0.01$) between the protein content of the unfertilized eggs and time since the first batch was stripped (Figure 8). No significant correlation was found between lipids or FAA and the time of batch stripping.

Although variation of lipids did not show a consistent trend over the spawning season, some fatty acids did change. 18:0 and AA in TAG (Figure 9a, b) decreased continuously over the season ($n = 14$, $P<0.01$; $n = 6$, $P<0.05$ respectively). Total (n-6) PUFA level (Figure 9d) in

Figure 6. Changes in dry weight and total lipid content in Atlantic halibut eggs in relation to food intake of broodstock fish.

(a) Variation of food intake of broodstock fish in successive years, (b) Variation of average dry weight in eggs over successive spawning seasons, (c) Variation of average total lipid in eggs over successive spawning seasons.

Figure 6.

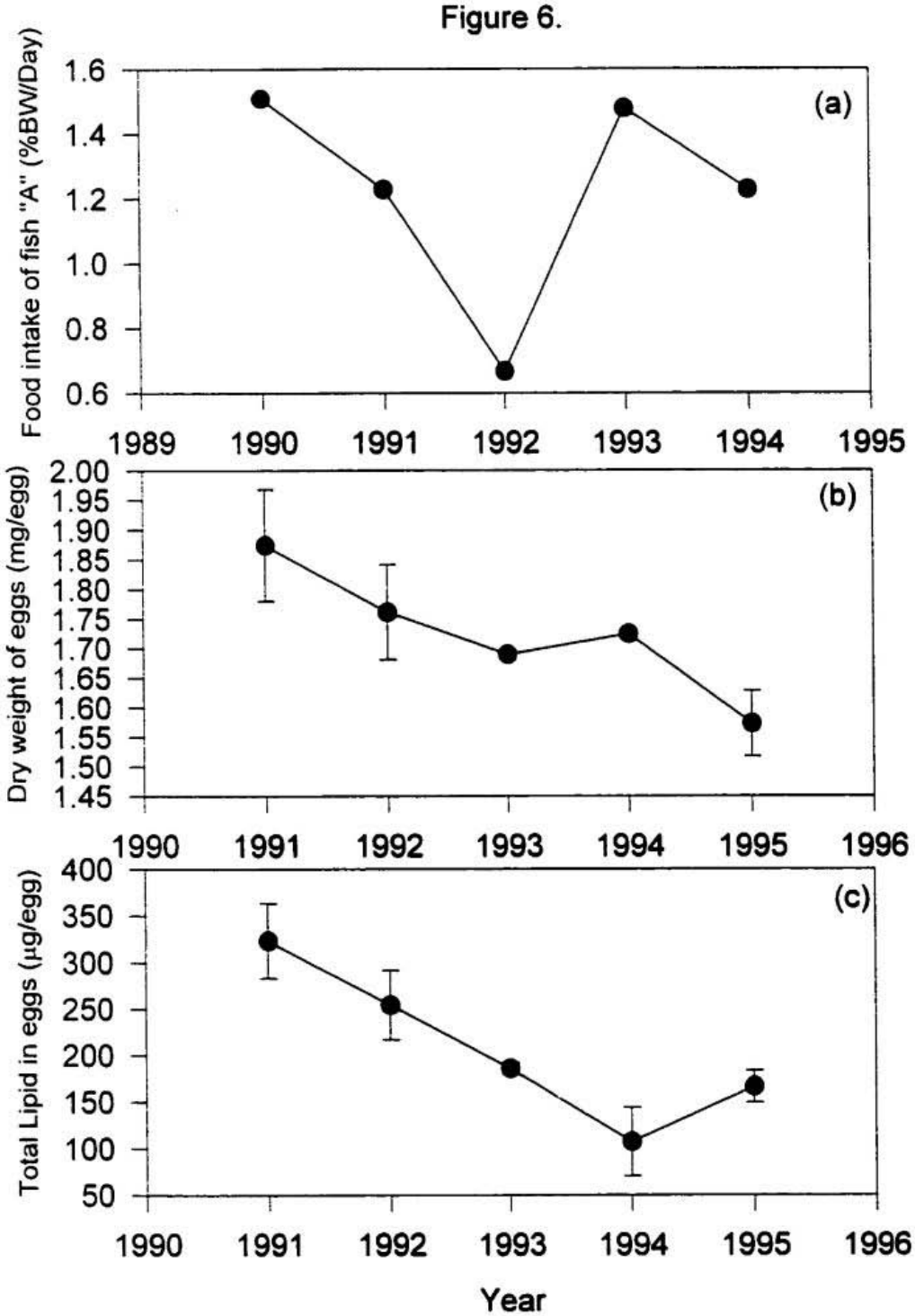

Food intake of fish "A" (%BW/Day)
1.6
1.4
1.2
1.0
0.8
0.6
(a)
1989
1990
1991
1992
1993
1994
1995
Dry weight of eggs (mg/egg)
2.00
1.95
1.90
1.85
1.80
1.75
1.70
1.65
1.60
1.55
1.50
1.45
(b)
1990
1991
1992
1993
1994
1995
1996
Total Lipid in eggs (µg/egg)
400
350
300
250
200
150
100
50
(c)
1990
1991
1992
1993
1994
1995
1996
Year

Table 4. The body weight, food intake and fecoundity of broodstock "A". (BW: body weight; S.D.: standard deviation).

Year	Body weight(Kg)	Food intake Mean(BW%/day)	S.D.	No. of batches	Total No. of eggs
1990	43.90	1.51	0.46	--	--
1991	45.82	1.23	0.33	7	354,750
1992	47.90	0.67	0.20	9	623,700
1993	50.04	1.48	0.39	3	229,350
1994	58.07	1.23	0.35	2	178,860
1995	--	--	--	4	330,000

Figure 7. Changes of AFDW in unfertilized Atlantic halibut eggs over the spawning season.

The straight line represents the linear regression ($y = 1.462\text{-}0.0073x$, $n = 23$, $r^2 = 0.176$, $P<0.05$), the dotted lines are the 95% confidence levels. The open symbols are data from eggs produced by broodstock female "A", solid symbols are data from eggs produced by broodstock female "J". Circles are data from1993, squares are data from 1994, and triangles are data from 1995.

Figure 7.

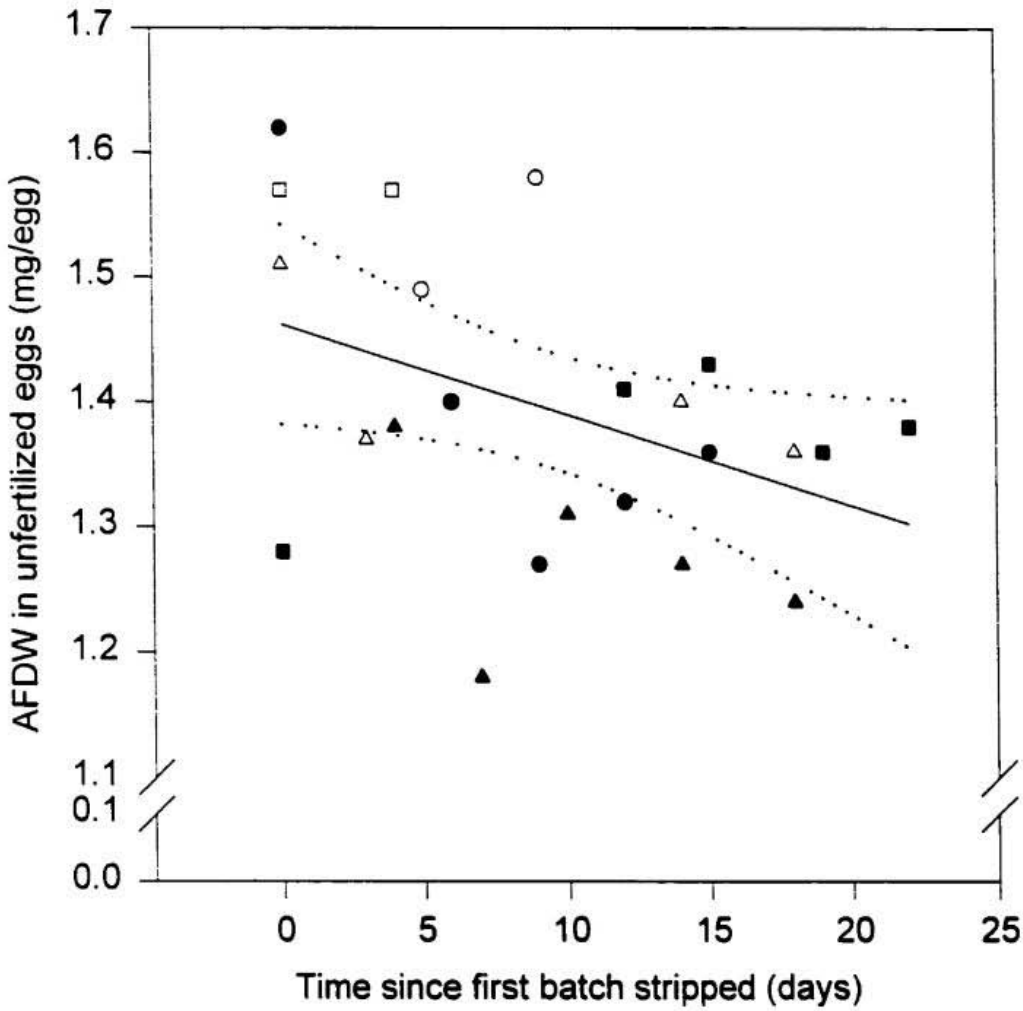

AFDW in unfertilized eggs (mg/egg)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
0.1
0.0
0
5
10
15
20
25
Time since first batch stripped (days)

Figure 8. Changes in protein content in different batches of unfertilized Atlantic halibut eggs over the spawning season.

The straight line represents the linear regression ($y = 986.001\text{-}9.148x$, $n = 12$, $r^2 = 0.554$, $P<0.01$), the dotted lines are the 95% confidence levels. The open symbols are data from eggs produced by broodstock female "A", solid symbols are data from eggs produced by broodstock female "J". Circles are data from 1994, squares are data from 1995.

Figure 8.

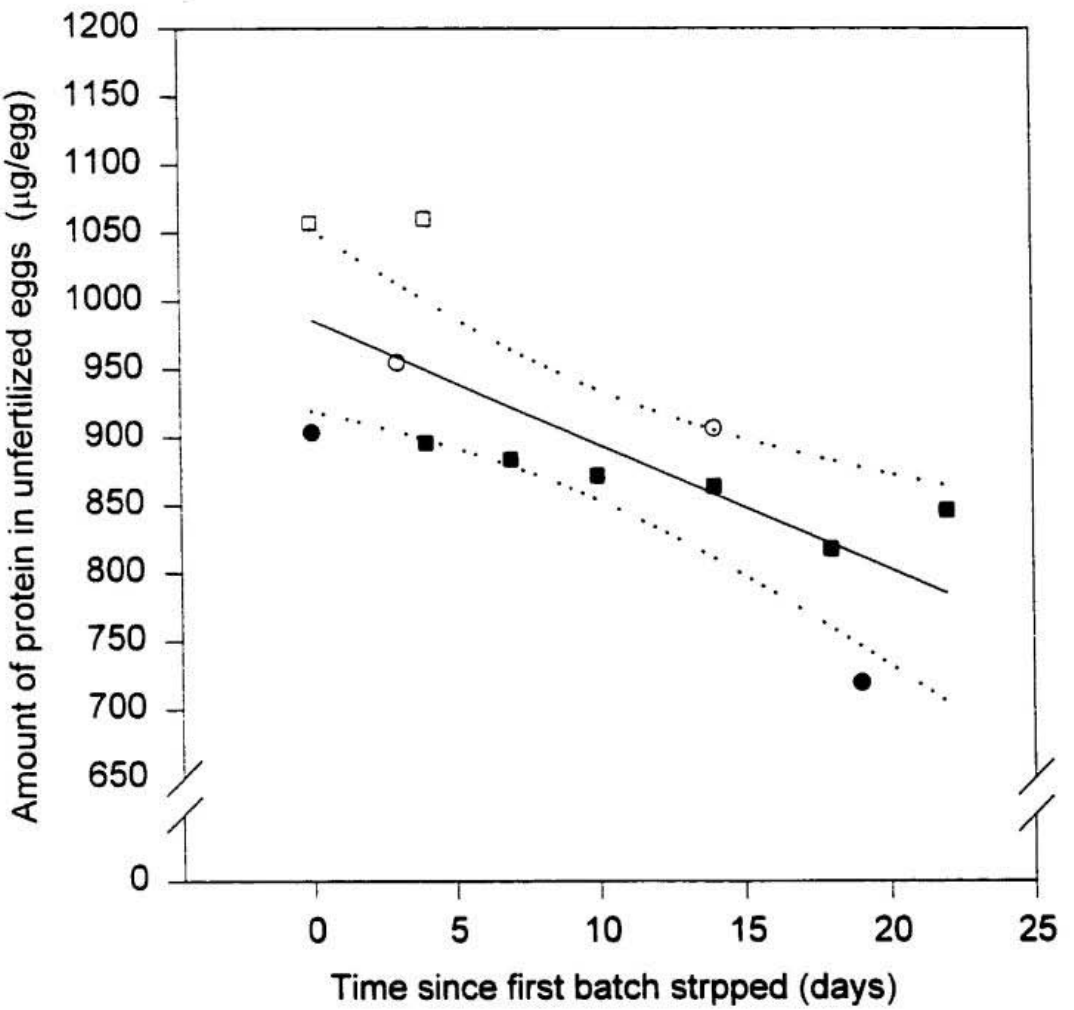
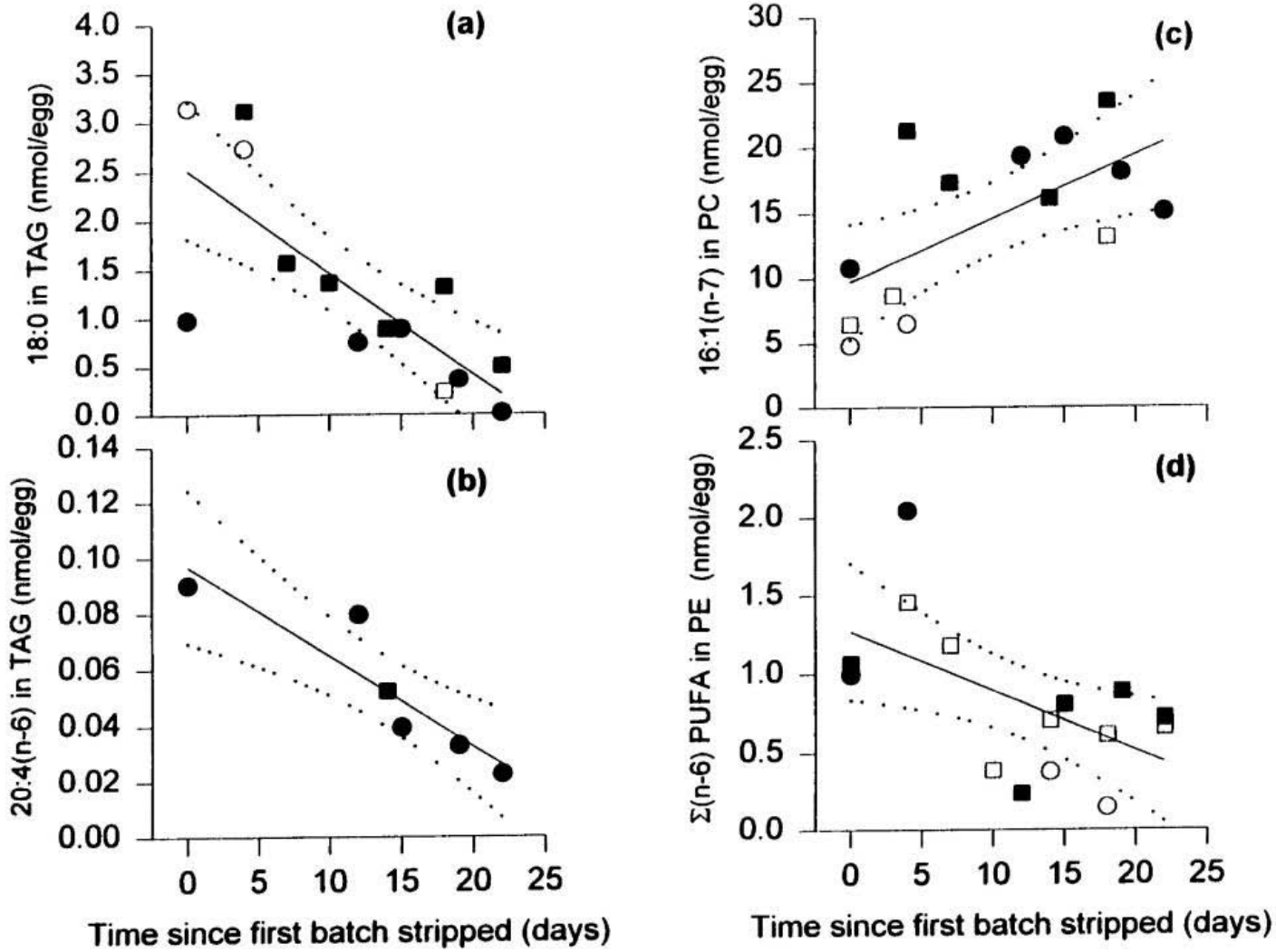

Amount of protein in unfertilized eggs (µg/egg)
1200
1150
1100
1050
1000
950
900
850
800
750
700
650
0
0
5
10
15
20
25
Time since first batch strpped (days)

Figure 9. Changes in fatty acids in unfertilized Atlantic halibut eggs over the spawning season.

(a) 18:0 in TAG (Regression: $y = 2.514 - 0.105x$, $n=14$, $r^2=0.617$, $P<0.05$), (b) 20:4(n-6) in TAG (Regression: $y = 0.0968 - 0.0032x$, $n=6$, $r^2=0.832$, $P<0.05$), (c) 16:1(n-7) in PC (Regression: $y = 9.738 + 0.486x$, $n=14$, $r^2=0.402$, $P<0.05$), (d) Σ(n-6)PUFA in PE (Regression: $y = 1.27 - 0.0376x$, $n=15$, $r^2=0.327$, $P<0.05$). The straight line represents the linear regression, the dotted lines are the 95% confidence levels. The open symbols are data from eggs produced by female broodstock "A", solid symbols are data from eggs produced by female broodstock "J". Circles are data from 1994, squares are data from 1995.

Figure 9.


(a)
18:0 in TAG (nmol/egg)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(b)
20:4(n-6) in TAG (nmol/egg)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
25
Time since first batch stripped (days)
(c)
16:1(n-7) in PC (nmol/egg)
30
25
20
15
10
5
0
(d)
Σ(n-6) PUFA in PE (nmol/egg)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time since first batch stripped (days)

PE also decreased (n=15, P<0.05). 16:1(n-7) in PC (Figure 9c) showed a significant increase over the season (n = 15, P<0.05).

3.3.3. Maturity of the broodstock and lipids in eggs

As indicated in Figure 10, the fertilization success of repeat spawner eggs (68.8 ± 17.2%, n = 37) was significantly higher (P<0.05) than that of first-time spawner eggs (50.9 ± 27.2%, n = 15).

In order to observe the effect of maturity of broodstock females on lipid content of eggs, the lipid classes of unfertilized eggs produced by three first-time spawners and two repeat spawners in different years were compared (Figure 11). Repeat spawner eggs contained significantly lower (P<0.05) amounts of ST (18.0 ± 5.4 μg/egg, n=36) than first-time spawner eggs (22.2 ± 6.8 μg/egg, n=16). Total NL in eggs from repeat spawners (42.8 ± 11.1 μg/egg, n=36) was significantly less (P<0.01) than in eggs from first-time spawners (53.9 ± 13.8 μg/egg, n=16). Repeat spawner eggs contained a significantly higher amount of PE (15.5 ± 5.2 μg/egg, n=17) than first-time spawner eggs (12.1 ± 1.9 μg/egg, n=5). When lipid classes were expressed as a percentage of egg dry weight, similar results were obtained. No significant differences were found in other lipid classes.

3.4. Changes in biochemical content during development of eggs and larvae

Changes in biochemical content in Atlantic halibut eggs and larvae over six

Figure 10. Comparison of fertilization success of Atlantic halibut eggs from first-time spawners and repeat spawners (n = 16, n = 36 respectively).

* $P < 0.05$.

Figure 10.

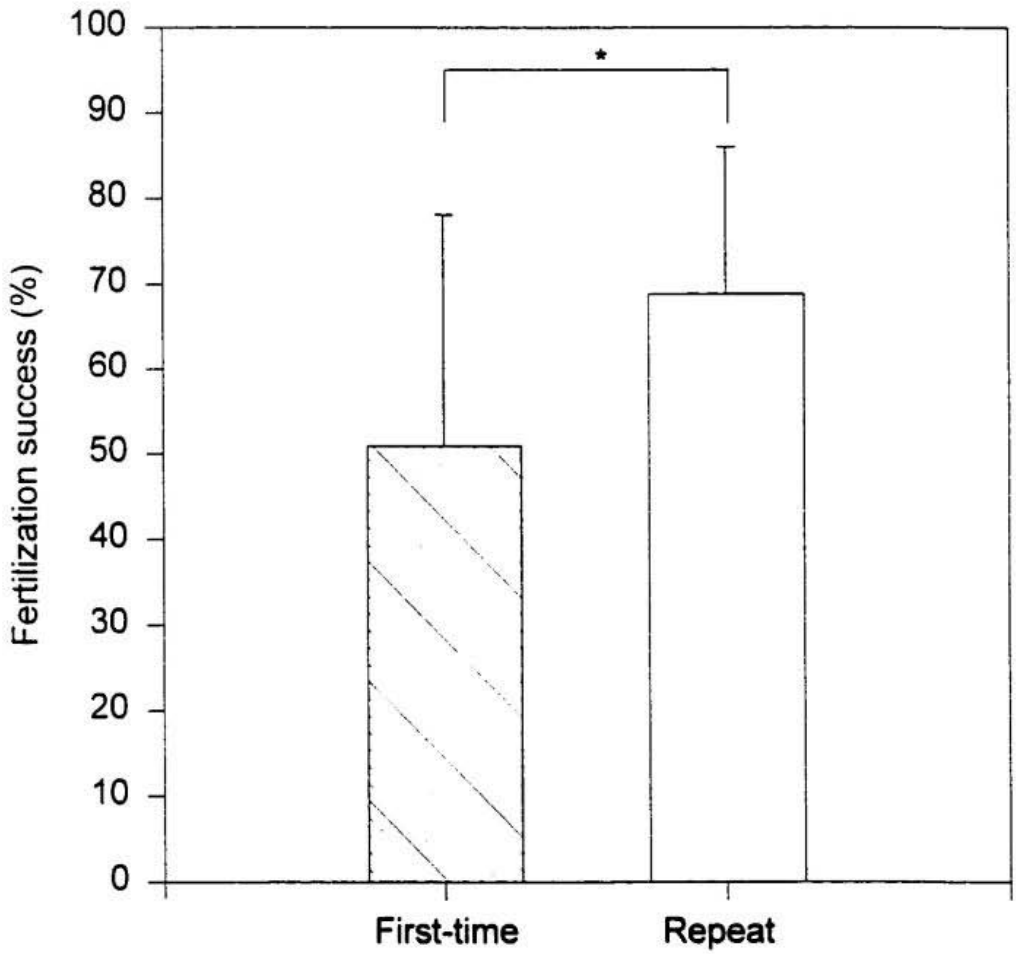

100
90
80
70
60
50
40
30
20
10
0
Fertilization success (%)
*
First-time
Repeat

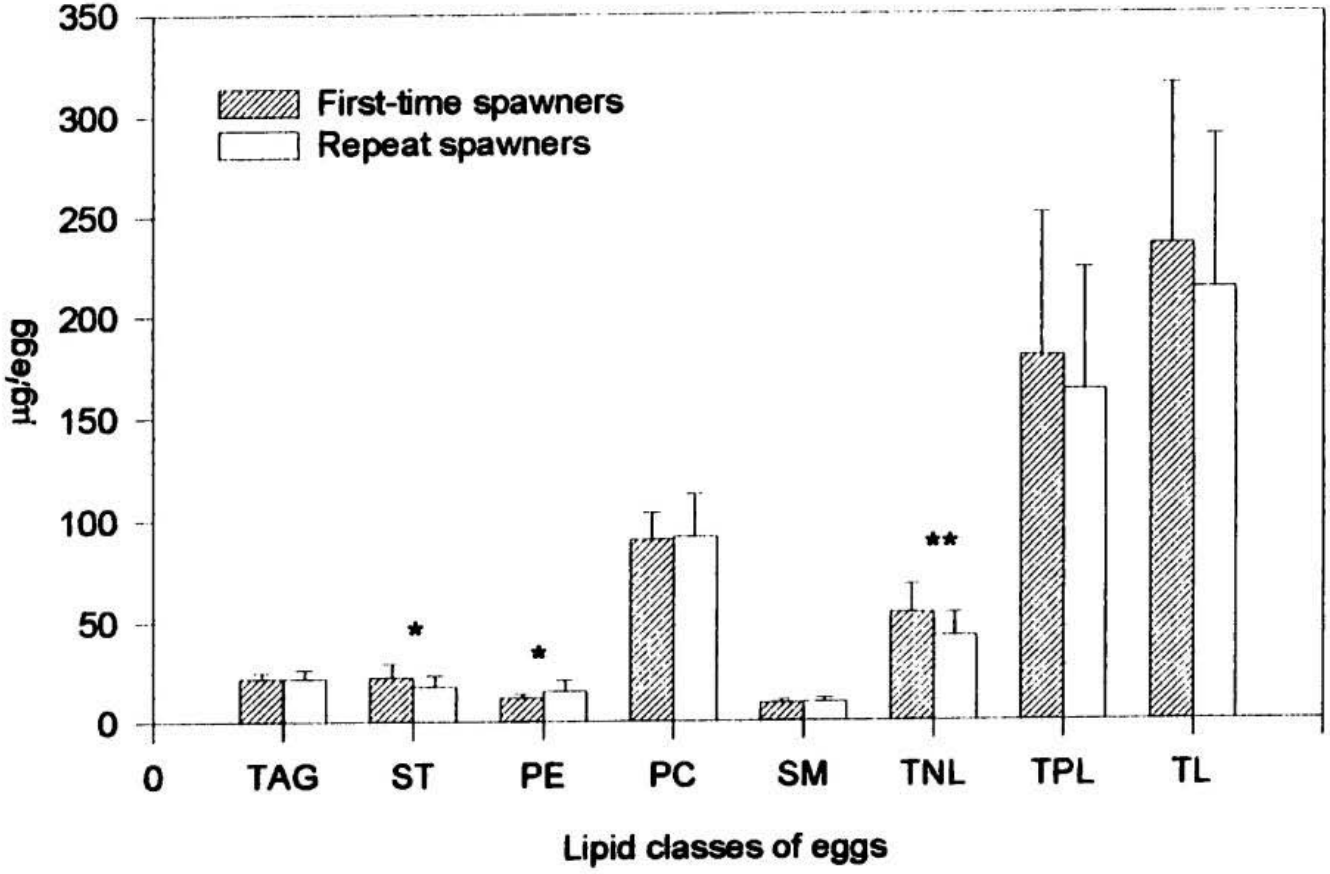
Figure 11. Comparison of lipid classes in Atlantic halibut eggs produced by repeat spawners and first-time spawners (n=36, n=16, respectively).

Each bar represents the average ± S.D. of "n" batches of eggs. For each batch, triplicate samples of 15 eggs were collected randomly.

TAG: triacylglycerol; ST: sterol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; TNL: total neutral lipid; TPL; total polar lipid; TL; total lipid; S.D.: standard deviation.

* $P<0.05$, ** $P<0.01$.

Figure 11.


350
300
250
200
150
100
50
0
μg/egg
First-time spawners
Repeat spawners
*
*
**
0
TAG
ST
PE
PC
SM
TNL
TPL
TL
Lipid classes of eggs

developmental stages were investigated. These stages were: unfertilized (samples were collected immediately after spawning), fertilized (eggs were fertilized within 8 hours after spawning), half-way to hatch (1/2W) (samples were collected at 7 days post fertilization), just hatched larvae (hatching usually took place at 14 days post fertilization), 7 days post hatch (DPH) and 14 DPH.

Lipid and AFDW data of eggs and larvae in different developmental stages were obtained from samples produced in the 1993, 1994, and 1995 spawning seasons. Protein and fatty acid data were obtained from samples in the 1994 and 1995 spawning seasons. Free amino acid data were from the samples in the 1995 spawning season.

3.4.1. Ash free dry weight and the sum of protein, FAA and lipid

Both AFDW and Σ(protein + FAA + lipid) were used to approximate the total biochemical contents in the eggs and larvae. Analysis of variance (ANOVA) indicates that both AFDW ($P<0.05$) and Σ(protein + FAA + lipid) ($P<0.01$) decreased throughout the developmental stages investigated (Figure 12). The steepest decrease occurred from "half-way to hatch" stage to hatching. The discrepancies of AFDW and Σ(protein + FAA + lipid) at stages 1/2W and 14 DPH may be caused by the different sample sizes used.

3.4.2. Lipid classes

The changes in each major lipid class over the developmental stages are shown in

Figure 12. Changes in AFDW and Σ(protein + FAA + lipid) in developing Atlantic halibut eggs and larvae.

Circles and solid line represent the changes of AFDW, squares and dotted line represent the changes of Σ(protein + FAA + lipid). Open symbols represent egg stages, solid symbols represent larval stages. Each data point is the average ± S.D. of "n" bathes of eggs/larvae. For each batch, triplicate samples of 10 or 15 individuals were collected randomly. For the AFDW data, n=16 at the unfertilized stage, n=17 at the fertilized stage, n=12 at the half-way to hatch stage, n=11 at hatch, n=8 at 7DPH, n=4 at 14DPH. For the Σ(protein+FAA+lipid) data, n=8 at the unfertilized stage, n=8 at the fertilized stage, n=3 at the half-way to hatch stage, n=4 at hatch, n=2 at 7DPH, n=2 at 14DPH.

AFDW: ash free dry weight; S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 12.

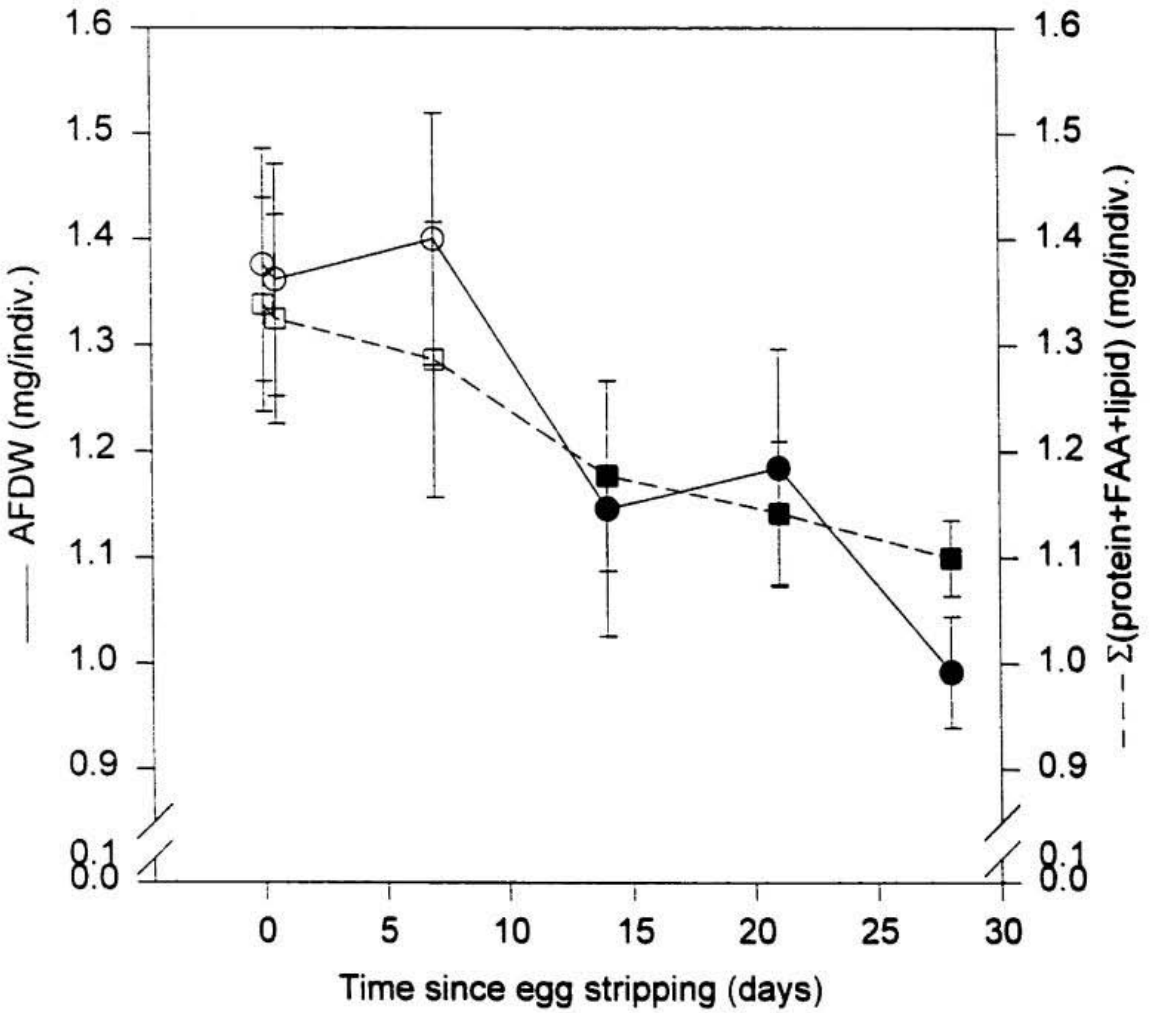

1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.1
0.0
AFDW (mg/indiv.)
Σ(protein+FAA+lipid) (mg/indiv.)
0
5
10
15
20
25
30
Time since egg stripping (days)

Figure 14 and 15. As indicated in Figure 15 (a), PE decreased in the 1/2W stage compared to the fertilized stage ($P<0.05$), making it the only lipid class to decrease in the egg stages (i.e. among unfertilized, fertilized, and 1/2W stages). No differences ($P>0.05$) were found in other lipid classes before hatching occurred. Each lipid class except ST increased ($P<0.05$) from 1/2W to hatching. After hatching, TAG decreased ($P<0.05$, Figure 14a), and all other lipid classes showed a decreasing trend except for PE and SM. As one would expect, the change of total lipid showed the same pattern (Figure 13). Total lipid increased about 25.5 μg in larvae at hatch compared to egg at 1/2W stage. After hatching, total lipid decreased, but it is not significant ($P>0.05$).

3.4.3. Fatty acids

Total saturates in TAG at hatch stage were higher ($P<0.05$) than during the egg stages, i.e. unfertilized, fertilized, and half way to hatch. Total saturates in TAG did not differ ($P>0.05$) in the larval stages investigated (Figure 16). In TAG, two saturates 16:0 and 18:0 changed over the developmental stages. At hatching, TAG 16:0 was higher ($P<0.05$) compared to the level in all other stages. 18:0 was higher ($P<0.05$) at 7 DPH than at fertilization.

3.4.4. Protein

Total protein content of eggs and larvae was measured (Figure 18). Protein content

Figure 13. Changes in total lipid in developing Atlantic halibut eggs and larvae.

Open circles represent egg stages, solid circles represent larval stages. Each data point is the average $\pm$ S.D. of "n" batches of eggs/larvae. For each batch, triplicate samples of 15 individuals were collected randomly. Data points without the same letter are significantly different ($P<0.05$). At the unfertilized stage (day 0) n=17, at the fertilized stage (day 0.3) n=17, at the half-way to hatch stage (day 7) n=12, at hatch (day 14) n=11, at 7DPH (day 21) n=8, at 14DPH (day 28) n=4.

S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 13.

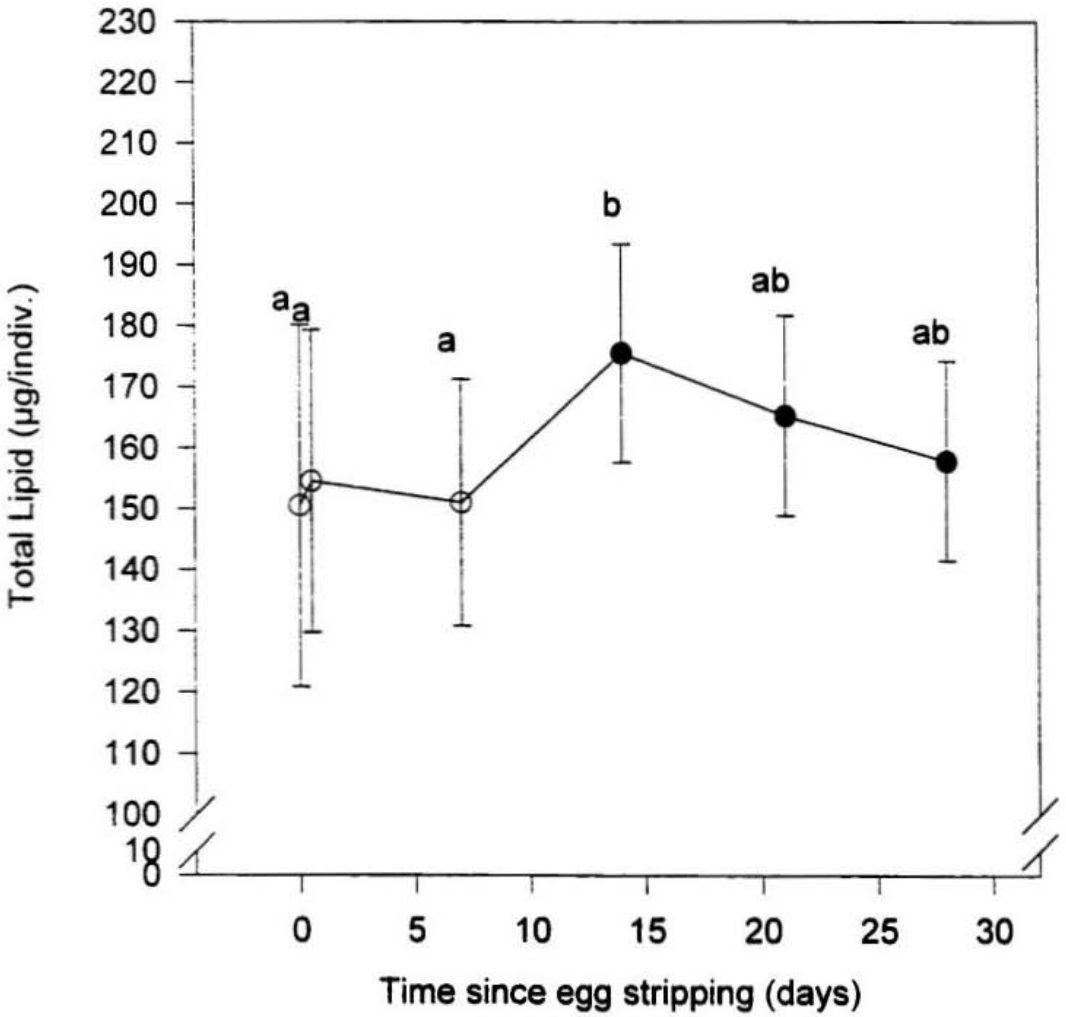

Total Lipid (µg/indiv.)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
10
0
a
a
a
b
ab
ab
0
5
10
15
20
25
30
Time since egg stripping (days)

Figure 14. Changes in neutral lipids in developing Atlantic halibut eggs and larvae.

(a) TAG, (b) ST. Open circles represent egg stages, solid circles represent larval stages. Each data point is the average $\pm$ S.D. of "n" batches eggs/larvae. For each batch, triplicate samples of 15 individuals were collected randomly. Data points without the same letter are significantly different ($P<0.05$). At the unfertilized stage (day 0) n=17, at the fertilized stage (day 0.3) n=17, at the half-way to hatch stage (day 7) n=12, at hatch (day 14) n=11, at 7DPH (day 21) n=8, at 14DPH (day 28) n=4.

TAG: triacylglycerol; ST: sterol; S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 14.

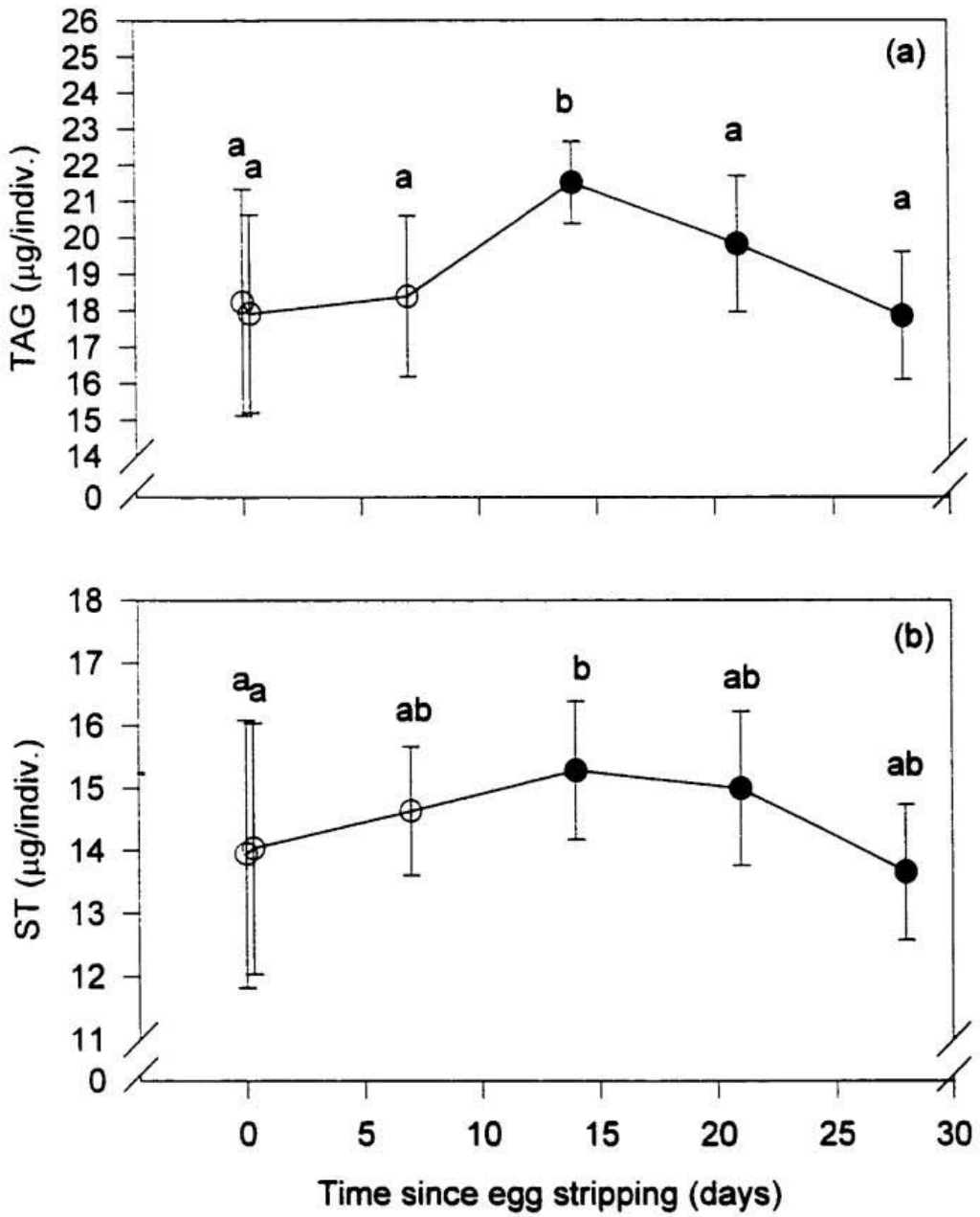

(a)
TAG (µg/indiv.)
26
25
24
23
22
21
20
19
18
17
16
15
14
0
a
a
a
b
a
a
(b)
ST (µg/indiv.)
18
17
16
15
14
13
12
11
0
a
a
ab
b
ab
ab
0
5
10
15
20
25
30
Time since egg stripping (days)

Figure 15. Changes in polar lipids in developing Atlantic halibut eggs and larvae.

(a) PE, (b) PC, (c) SM. Open circles represent egg stages, solid circles represent larval stages. Each data point is the average $\pm$ S.D. of "n" batches of eggs/larvae. For each batch, triplicate samples of 15 individuals were collected randomly. Data points without the same letter are significantly different ($P<0.05$). At the unfertilized stage (day 0) n=17, at the fertilized stage (day 0.3) n=17, at the half-way to hatch stage (day 7) n=12, at hatch (day 14) n=11, at 7DPH (day 21) n=8, at 14DPH (day 28) n=4.

PE: phosphatidylethanolamine; PC phosphatidylcholine; SM sphingomyelin; S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 15.

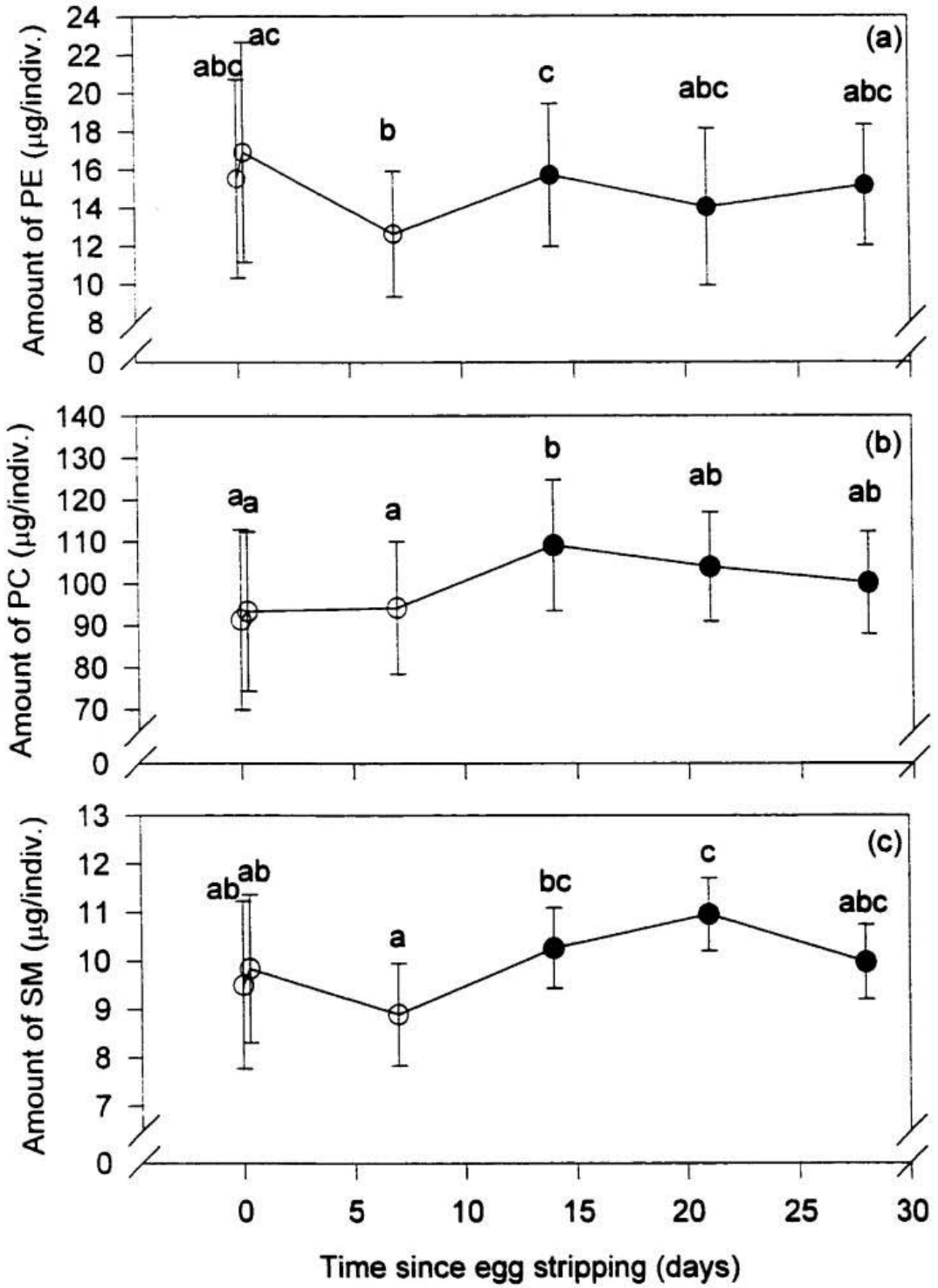
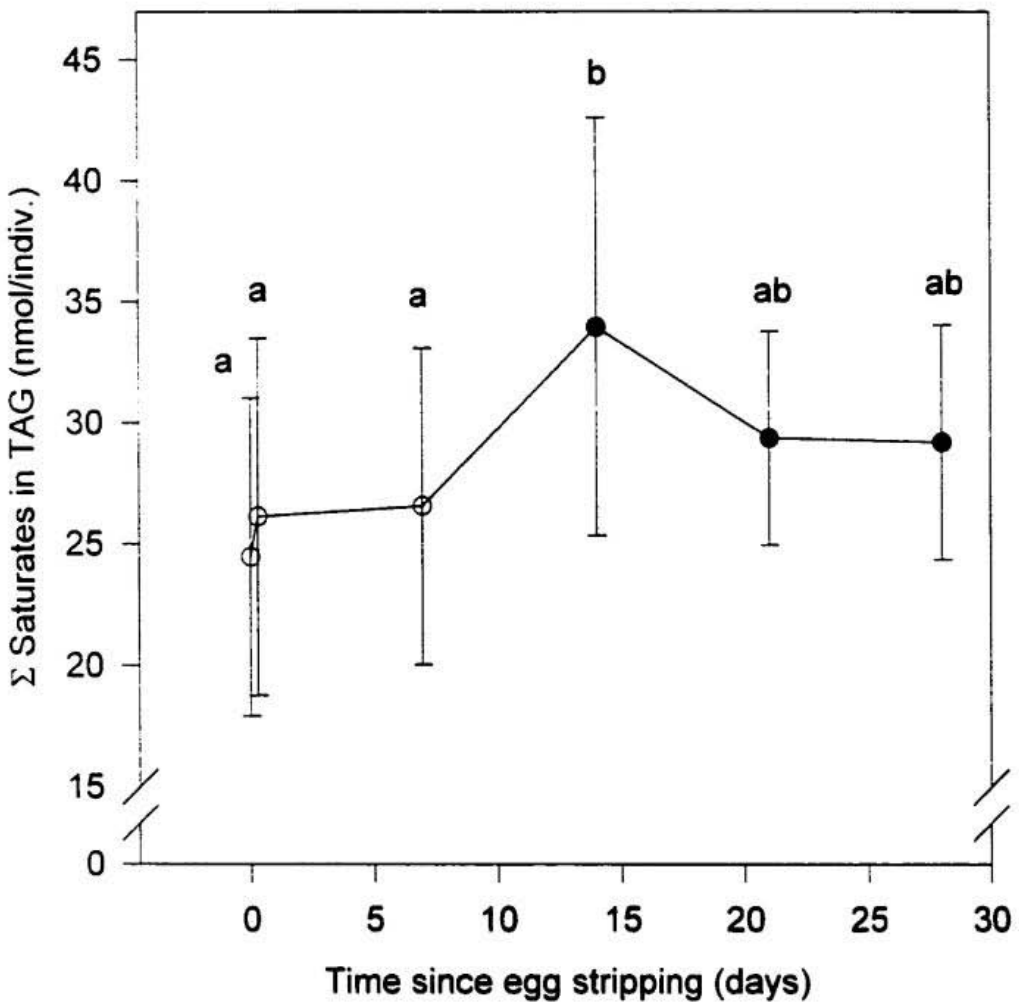

(a)
Amount of PE (µg/indiv.)
24
22
20
18
16
14
12
10
8
0
abc
ac
b
c
abc
abc
(b)
Amount of PC (µg/indiv.)
140
130
120
110
100
90
80
70
0
aa
a
b
ab
ab
(c)
Amount of SM (µg/indiv.)
13
12
11
10
9
8
7
0
ab
ab
a
bc
c
abc
0
5
10
15
20
25
30
Time since egg stripping (days)

Figure 16. Changes in total saturated fatty acids in TAG in developing Atlantic halibut eggs and larvae.

Open circles represent egg stages, solid circles represent larval stages. Each data point is the average $\pm$ S.D. of "n" batches of eggs/larvae. For each batch, triplicate samples of 15 individuals were collected randomly. Data points without the same letter are significantly different ($P<0.05$). At the unfertilized stage (day 0) n=17, at the fertilized stage (day 0.3) n=16, at the half-way to hatch stage (day 7) n=12, at hatch (day 14) n=11, at 7DPH (day 21) n=8, at 14DPH (day 28) n=4.

TAG: triacylglycerol; S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 16.


45
40
35
30
25
20
15
0
Σ Saturates in TAG (nmol/indiv.)
a
a
a
b
ab
ab
0
5
10
15
20
25
30
Time since egg stripping (days)

declined ($P<0.05$) from unfertilized eggs to hatching stage, with a total of 133.2 μg of protein being lost. After hatching there was an increasing trend which was not significant ($P>0.05$).

3.4.5. Free Amino Acids

Total free amino acids (FAA), total essential free amino acids (EAA), and total nonessential free amino acids (NEAA) were continuously depleted throughout the developmental stages (Figure 17 & Table 5). About 50% of the FAA in unfertilized eggs disappeared when the 14 DPH stage was reached. However, the depletion was not significant until after the 1/2W stage. As indicated in Table 5 and Figure 17, the depletion of FAA did not occur equally among the EAA and NEAA; NEAA was more utilized than EAA. Of the depleted FAA, 57.9% was NEAA, this percentage was higher than the original molar percentage (51.5%) of NEAA in FAA of unfertilized eggs (Table 3). All amino acids in the EAA category decreased (ANOVA, $P<0.01$). The most depleted EAA (in a descending order of depletion) were leucine, lysine, arginine, and isoleucine (Table 5). Most of the amino acids in the NEAA group decreased (ANOVA, $P<0.01$), except taurine, α-amino-n-butyric acid, cysteine and tryptophan (ANOVA, $P>0.05$). The most depleted NEAA (in descending order of depletion) were serine, alanine, glutamic acid, and glutamine (Table 5).

3.4.6. Ammonia and urea

Compared to the value reported by Finn *et al.* (1991), NH_3 was low in unfertilized

Figure 17. Changes in free amino acids in developing Atlantic halibut eggs and larvae. Open symbols represent egg stages, solid symbols represent larval stages. Circles are total free amino acids, squares are essential amino acids, and triangles are nonessential amino acids. Each data point is the average ± S.D. of "n" batches of eggs/larvae. For each batch, 15 or 30 individuals were collected randomly. At the unfertilized stage (day 0) n=10, at the fertilized stage (day 0.3) n=10, at the half-way to hatch stage (day 7) n=5, at hatch (day 14) n=6, at 7DPH (day 21) n=4, at 14DPH (day 28) n=4.

FAA: free amino acids; NEAA: nonessential amino acids; EAA: essential amino acids; S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 17.

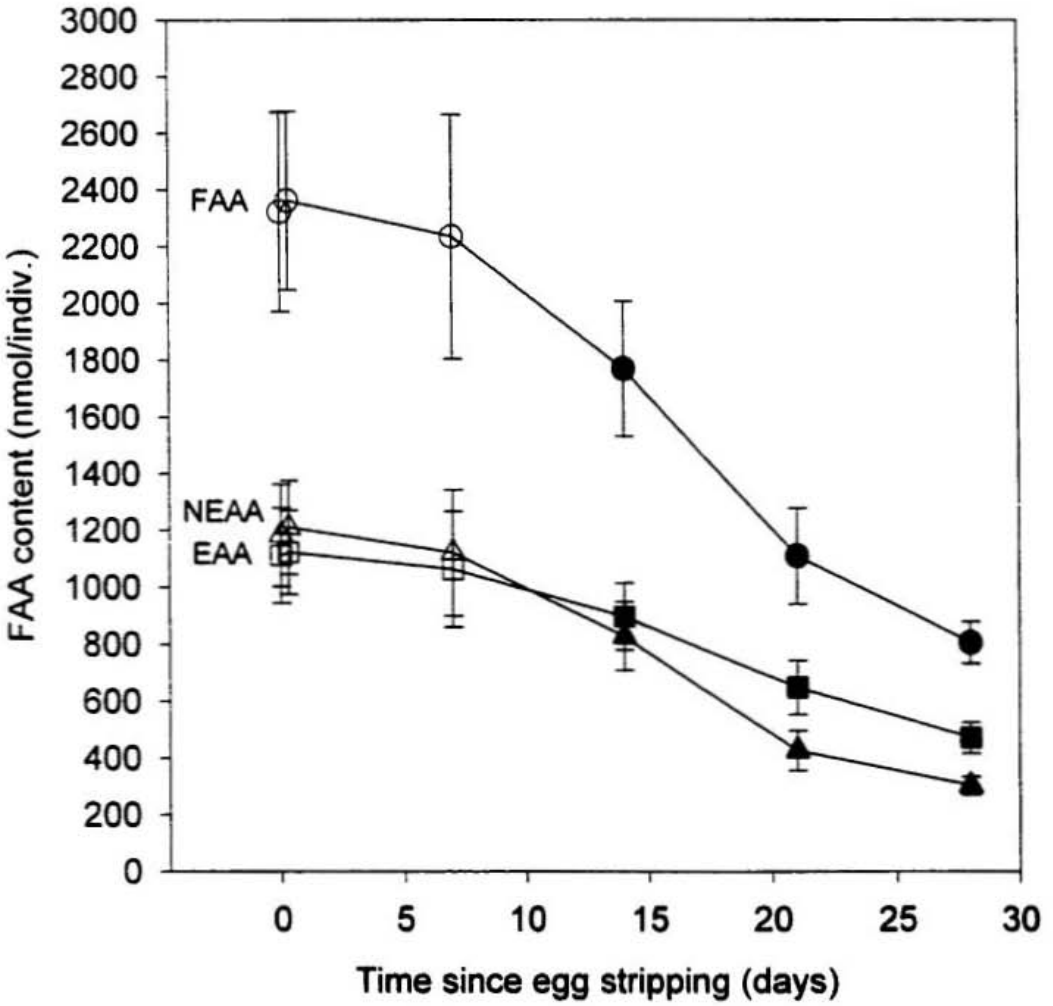

FAA content (nmol/indiv.)
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
FAA
NEAA
EAA
0
5
10
15
20
25
30
Time since egg stripping (days)

Table 5. The changes of free amino acids over developmental stages. Samples are from 10 batches of eggs produced by two brood stocks in 1995 spawning season. For each batch, a sample of 30 or 15 indiv. were collected randomly. (EAA: essential amino acids; FAA: free amino acids; NEAA: nonessential amino acids; 1/2W: half-way to hatch; 7DPH: 7 days post hatch; 14DPH: 14 days post hatch; S.D.: standard deviation; indiv.: individual. * Proportion of the total FAA decrease.)

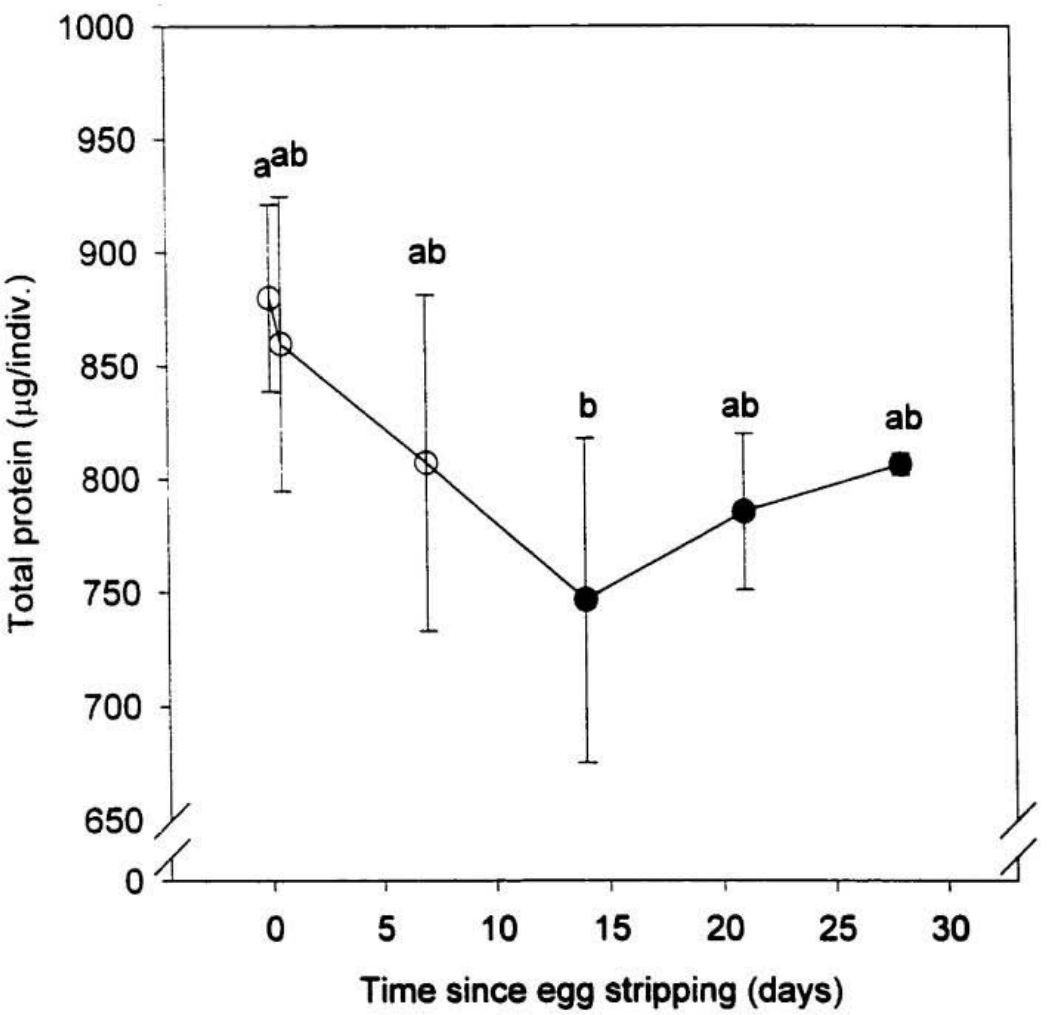
	Unfertilized		Fertilized		1/2W		Hatch		7 DPH		14 DPH			
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Δ FAA	%ΔFAA*
EAA(nmol/indiv.)														
Threonine	95.1	14.5	96.8	13.9	105.3	19.5	77.3	11.3	50.1	12.2	35.2	6.9	-60.0	3.9
Valine	162.0	23.9	164.9	22.5	156.3	23.8	135.3	18.6	118.3	12.3	102.1	8.0	-59.9	3.9
Methionine	60.2	7.8	61.4	7.9	44.4	27.6	50.8	8.7	36.6	5.2	24.9	4.4	-35.3	2.3
Isoleucine	120.6	18.5	120.1	17.5	109.9	19.6	94.3	13.4	72.8	12.4	57.7	6.7	-62.9	4.1
Leucine	237.2	35.6	235.1	28.4	235.6	41.0	191.2	24.7	132.8	20.5	94.2	11.0	-143.0	9.4
Tyrosine	51.6	9.3	52.5	8.5	52.6	10.8	50.1	8.2	37.1	7.0	28.6	2.5	-23.0	1.5
Phenylalanine	52.1	8.0	53.3	7.2	51.4	8.9	45.2	5.8	34.6	4.9	24.9	3.3	-27.3	1.8
Lysine	197.2	29.8	199.8	26.3	186.9	38.0	155.5	20.1	109.6	15.0	68.8	10.6	-128.3	8.4
Histidine	40.9	6.6	42.7	6.3	40.6	4.6	31.9	5.4	18.7	3.5	12.3	3.4	-28.6	1.9
Arginine	94.5	15.0	96.2	14.4	80.1	29.4	65.7	8.7	37.9	8.3	21.6	3.5	-72.9	4.8
ΣEAA	1111.4	166.4	1122.7	147.3	1063.0	202.6	897.3	117.4	648.4	95.6	470.2	54.6	-641.2	42.1
NEAA (nmol/indiv.)														
Cysteic acid & Phosphoserine	7.1	1.2	7.3	1.3	5.5	1.5	2.5	0.3	2.2	0.7	1.5	0.1	-5.6	0.4
Taurine	67.2	10.8	68.3	8.9	70.0	13.3	67.1	10.4	64.4	8.1	75.2	11.6	8.1	-0.5
Aspartic acid	81.4	15.3	81.8	14.1	71.1	13.8	45.7	10.0	20.4	5.7	19.5	2.3	-61.9	4.1
Serine	269.1	42.2	269.3	33.4	269.2	43.9	171.9	31.0	60.6	19.2	32.0	12.3	-237.1	15.6
Asparagine	97.9	16.8	100.9	11.2	98.4	15.7	67.2	10.7	16.2	9.5	1.0	1.7	-96.9	6.4
Glutamic acid	144.3	21.3	144.4	21.1	89.8	66.8	95.2	15.1	47.8	7.0	23.3	2.7	-121.0	7.9
Glutamine	135.5	19.9	145.4	20.7	135.7	19.1	98.8	11.6	63.9	7.7	37.7	6.9	-97.8	6.4
Proline	65.8	12.0	74.4	12.8	76.4	16.9	48.7	10.6	27.5	14.1	28.1	4.2	-37.7	2.5
Glycine	79.2	12.2	80.9	11.7	77.7	14.8	61.4	9.0	31.0	5.6	22.8	6.3	-56.4	3.7
Alanine	221.6	34.5	223.5	34.9	212.4	39.3	155.4	24.3	80.5	15.5	47.6	9.0	-174.0	11.4
α-amino -butyric acid	0.2	0.4	0.5	0.6	1.2	1.5	1.2	1.1	0.4	0.4	1.2	0.9	1.0	-0.1
Cysteine	3.0	1.2	3.0	1.0	4.2	1.5	3.5	1.5	2.6	1.2	2.9	0.7	-0.2	0.0
Tryptophan	10.3	2.5	10.4	1.8	8.6	2.4	9.3	2.2	8.6	2.3	9.0	1.6	-1.3	0.1
ΣNEAA	1182.7	179.6	1210.0	165.1	1120.3	221.5	827.9	119.7	426.2	69.6	301.9	30.9	-880.8	57.9
ΣFAA	2294.1	345.8	2332.7	311.7	2183.3	423.8	1725.2	230.8	1074.6	162.2	772.2	75.2	-1522.0	

Figure 18. Changes in total protein in developing Atlantic halibut eggs and larvae.

Open symbols represent egg stages, solid circles represent larval stages. Each data point is the average $\pm$ S.D. of "n" batches. For each batch, a sample of 15 or 30 individuals were collected randomly. Data points without the same letter are significantly different ($P<0.05$). At the unfertilized stage (day 0) n=12, at the fertilized stage (day 0.3) n=11, at the half-way to hatch stage (day 7) n=10, at hatch (day 14) n=4, at 7DPH (day 21) n=3, at 14DPH (day 28) n=2.

S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 18.


1000
950
900
850
800
750
700
650
0
Total protein (µg/indiv.)
aab
ab
b
ab
ab
0
5
10
15
20
25
30
Time since egg stripping (days)

eggs and at fertilization (about 45 nmol), but was continuously increased during the egg stages (Figure 19). The content of NH_3 at hatch was about 6 fold higher than in unfertilized eggs. After hatching, NH_3 did not change ($P>0.05$). Only *in vivo* NH_3 was detected, NH_3 excreted to the outside was not measured. Urea was not detectable in any of the six developmental stages investigated.

3.4.7. Energy

Table 6 indicates the change of energy during development. The total energy of eggs and larvae were calculated by the sum of energy derived from lipid, FAA and protein. Combustion values of 21.00 kJ/g, 20.14 kJ/g, and 35.56 kJ/g were used for FAA, protein and lipid (Ronnestad *et al.*, 1995). The total energy decreased during development (ANOVA, $P<0.01$), largely due to the decrease of FAA. From the unfertilized stage to the 14 DPH stage, the decrease of energy associated with FAA accounted for about 68% of the decrease of the total energy.

Figure 19. Changes in ammonia in developing Atlantic halibut eggs and larvae.

Open circles represent egg stages, solid circles represent larval stages. Each data point is the average $\pm$ S.D. of "n" batches. For each batch, a sample of 15 or 30 individuals were collected randomly. Data points without the same letter are significantly different ($P<0.05$). At the unfertilized stage (day 0) n=10, at the fertilized stage (day 0.3) n=10, at the half-way to hatch stage (day 7) n=5, at hatch (day 14) n=6, at 7DPH (day 21) n=4, at 14DPH (day 28) n=4.

S.D.: standard deviation; DPH: days post hatch; indiv.: individual.

Figure 19.

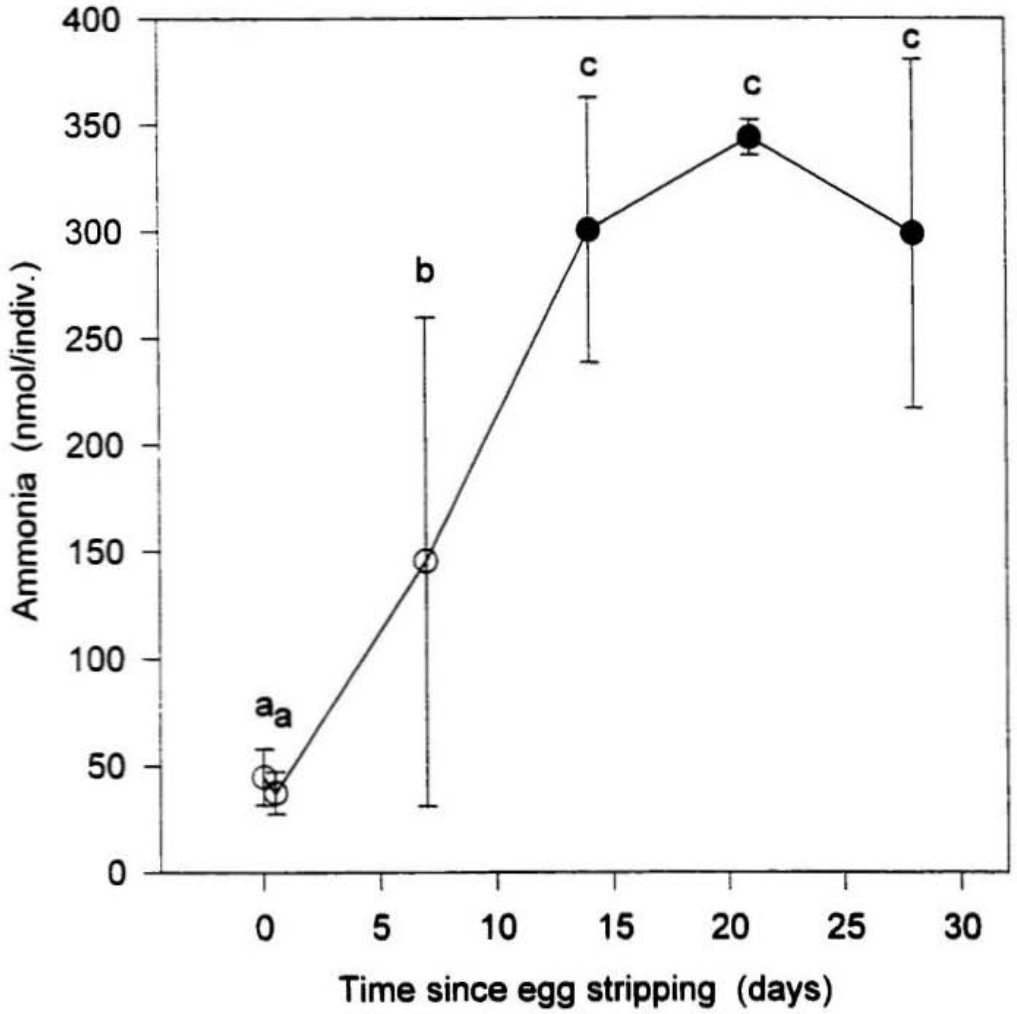

400
350
300
250
200
150
100
50
0
Ammonia (nmol/indiv.)
a a
b
c
c
c
0
5
10
15
20
25
30
Time since egg stripping (days)

Table 6. Changes of energy over the developmental stages of Atlantic halibut embryos and larvae. unit: J/individual (1/2W: half-way to hatch; FAA: free amino acids; 7DPH: 7days post hatch; 14DPH: 14 days post hatch).

Source	Developmental Stages					
of energy	Unfertilized	Fertilized	1/2W	Hatch	7DPH	14DPH
Lipid	5.37	5.55	5.41	6.05	5.75	5.62
FAA	6.28	6.37	6.32	5.49	3.86	2.93
Protein	17.72	17.31	16.61	15.04	15.82	16.25
Total	29.54	29.34	28.51	26.45	25.61	24.63

Chapter 4. Discussion

4.1. Lipid class and fatty acid composition in eggs in relation to egg fertilization success and larval viability

A 75% fertilization success was used as the egg quality index in this study. This method is quick and easy to perform and the egg quality can be determined at a very early embryonic stage. Fertilization success appeared to be positively correlated with the larval survival time, however, the correlation was not significant ($P>0.05$) in this study. This indicates limitations to the value of fertilization success as an index, and suggests other factors within the egg might be sought as determinants of egg and larval viability. The biochemical composition of a healthy egg reflects both the quantitative and qualitative demands for nutrients by developing embryos and larvae, thus this method may be used as an index to determine egg and larval viability.

Lipid, especially NL, is regarded as an important energy substrate in fish eggs, and it is continuously depleted throughout the endogenous feeding stage in different marine species. There are reports that a high fat content in the eggs is associated with high viability of the larvae (Kjorsvik *et al.*, 1990). However, there appeared to be a lower level of total lipid ($P>0.05$) in better eggs (Fertilization $\geq$ 75%) and in eggs that produce more viable larvae (survival time $\geq$ 15.9 days) (Figure 2 and Figure 5). In better quality eggs there were lower amount of ST ($P<0.05$) and TNL ($P<0.01$). In eggs that produce more viable larvae, there were lower amount of TAG ($P<0.05$). Parrish *et al.* (1994b) also documented, in a small

sample of eggs from captive halibut, that a high fertilization success was associated with a low total lipid content. Devauchelle *et al.* (1988) found that there was a higher lipid content in overripening trout eggs. Devauchelle *et al.* (1982) indicated that a higher lipid level was generally associated with lower viability of the eggs of turbot, sole and seabass. As indicated by Kamler (1992), during the period of intensive growth of fish ovaries, there is a rapid increase in the percentage of proteins in ovarian matter and a decrease in the percentage of lipids. Decreased fertilizability of eggs containing an augmented percentage of lipids was attributed to incomplete maturation of the eggs. Kjorsvik *et al.* (1990) and Kamler (1992) both mentioned the high concentration of cholesterol in eggs produced by young and old females, as well as in overripening eggs. Bruce *et al.* (1993) also reported a significantly higher percentage of cholesterol in non-viable Atlantic halibut eggs. Both incomplete maturation and overripening may cause excessive lipid accumulation in the egg. The low viability of eggs and larvae may not result directly from the high lipid content, but from the insufficiency or deterioration of other nutrients in the eggs.

The average (n-3) and (n-6)PUFA levels in this study (Figure 1) were much lower than the levels reported by Falk-Petersen *et al.* (1989), Rainuzzo *et al.* (1992) and Bruce *et al.* (1993). This low level is possibly the cause of the poor larval survival observed in this study. Since fatty acids elongase and desaturase activities are limited in marine fish, (n-3)PUFA and (n-6)PUFA levels are essentially dependent on food ingestion. In this context, the impact of dietary fatty acids is larger in marine teleost species compared to freshwater fish (Silversand, 1996). (n-3)PUFA (especially 22:6(n-3) and 20:5(n-3)) and (n-6)PUFA (especially 20:4(n-6)) are defined as EFA for marine fish. Although some authors (Wiegand, 1996) have reported

that there is a strong selection pressure to maintain the proportions of (n-3)PUFA in yolk polar lipids within a relatively narrow range, broodstock fed with (n-3)PUFA deficient diets may produce eggs with decreased (n-3)PUFA levels (Leger *et al.*, 1981; Mourente & Odriozolar, 1990; Harel *et al.*, 1994). There are also some reports of significant differences between (n-3)PUFA levels in domestic and wild fish eggs (Abi-ayad *et al.*, 1995; Harrell & Woods III, 1995; Silversand *et al.*, 1996), with significantly higher amounts of (n-3)PUFA, and higher (n-3)/(n-6) ratios, in wild fish eggs. This difference may be associated with the difference between wild and artificial food intake of broodstock. However, in the present study, due to limited time and manpower, no fatty acid data on the broodstock diet were collected.

It is well documented that in marine species (Watanabe, 1991, 1993; Harel *et al.*, 1994), higher (n-3)PUFA (especially 22:6(n-3) and 20:5(n-3)) levels are usually associated with higher egg and larval quality. However, Bruce *et al.* (1993) reported no difference in fatty acid composition of viable and non-viable halibut eggs, although the levels were higher than the present study. In this study we found that in better quality eggs there tended to be higher levels of PUFA, especially higher levels of (n-6)PUFA in both TAG, PE, as well as in the Σ(PC+PE+TAG) which approximated the fatty acid profile of the total lipids (Figure 4). In better eggs, 20:5(n-3) was significantly higher in PE ($P<0.05$) (Figure3b). In poorer eggs, there were higher amounts of 16:1(n-7) in TAG ($p<0.05$), and higher amounts of 14:0 in PE and total lipid ($P<0.05$).

Our results indicate that (n-6)PUFA sufficiency may have been important to egg quality. In TAG of better quality eggs, Σ(n-6)PUFA was significantly higher ($P<0.01$)

(Figure 3a). The level of 18:2(n-6) in total lipid of better eggs was also significantly higher than in poorer eggs (Figure 4, $P<0.05$). The (n-6)PUFA are precursors for the synthesis of eicosanoids or prostaglandins which are involved in cell signal transduction, water transport, and osmoregulation. However, the minimum requirement of (n-6)PUFA in fish is not as well defined as that of (n-3)PUFA. As reported by Sargent (1995), excessive amounts of (n-6)PUFA will have a negative effect on fish larvae. Since some (n-3)PUFA, especially 20:5(n-3) (which itself is an eicosanoid precursor), can modulate the synthesis of eicosanoid, the ratio of (n-3)/(n-6) may be more important than the absolute amount of each PUFA. Sargent (1995) suggested a (n-3)/(n-6) ratio in the range of 5:1-10:1 is ideal. In this study, the average (n-3)/(n-6) ratio in Σ(PC+PE+TAG) was about 8.2, but the standard deviation was as high as 4.3 due to the effects of compound errors in this calculation. No correlation was found between this ratio and egg fertilization success or larval viability.

We did not find significant differences in egg fatty acid profiles between more viable and less viable larvae. This indicates that yolk-sac larvae survival may not be a very precise egg quality index, since larvae often develop high rates of malformations although survival may be high (Kjorsvik & Holmefjord, 1995).

4.2. Condition of the female broodstock and egg biochemical composition

4.2.1. Food intake of female broodstock and egg total lipid

Atlantic halibut spawn several hundred thousand eggs in one single spawning season

(Table 4). The calculated total weight of these eggs accounts for about 20% of the body weight of the female broodstock. Therefore the broodstock allocates a large amount of the energy ingested to gonad maturation and oogenesis. It is known that food availability is one of the main factors affecting reproductive processes in fish, and that food restriction generally reduces total fecundity (Cerda *et al.*, 1994). The reduction in food ingestion in 1991 and 1992 may cause a decline in the number of eggs produced in 1993 and 1994, but did not seem to slow down the increase of broodstock body weight (Table 4). In order to maintain the increase in body weight under conditions of low food intake, there had to be less energy used in reproduction. Bromage (1995) states that, fish age appears to be less important in determining fecundity than food intake. Therefore the decrease of fecundity reported here is not likely to be caused by aging of the broodstock. Not only did the broodstock food intake in the previous year appear to affect the total number of eggs produced in the following years, it also seemed to affect the average DW and amount of total lipid in eggs. The change of average egg DW in successive years shared a similar trend with the fluctuation of food intake (Figure 6a & b). The average total lipid in eggs declined from 1991 to1993, corresponding to the continuous decrease of food intake from 1990 to 1992. However, the drastic increase in food ingestion in 1993 was not followed by an increase in egg lipid in 1994. The increase in egg lipid was delayed to 1995, and was unaffected by the food intake decrease in 1994. The discrepancy between the changes of egg DW and lipid over the spawning season suggests that the allocation of lipid into oocytes may involve a mechanism that is different from that of other biochemical contents. The lipid in eggs might be affected by the storage of lipid in the body of maternal fish as well as the food intake of the fish, while the amount of egg

protein is probably more affected by the food intake.

4.2.2. Changes of egg biochemical composition over the spawning season

As shown in Figures 7 and 8, egg AFDW ($P<0.05$), and total protein ($P<0.01$), decreased significantly over the spawning season. Vitellogenin is generally considered to be the precursor of major biochemical substances such as protein and lipid in the oocytes. The uptake of radiolabelled vitellogenin in rainbow trout and killifish oocytes is correlated with the oocyte size, being highest in the largest oocytes, even when the rate was expressed relative to surface area (Silversand, 1996). According to Kjorsvik and Holmefjord (1995), egg size decreases during the spawning season. In this study, egg diameter decreased over the spawning season, although the decrease is not significant ($P>0.05$). Plasma estradiol-17β declined in female Atlantic halibut as the season progresses (Methven *et al.*, 1992; Hyllner *et al.*, 1994). Kjesbu *et al.* (1996) suggests that this decline of estradiol-17β is the result of a decrease in the number of ovarian follicles, i.e. steroid producing cells, as spawning proceeds. As maternal Atlantic halibut do not ingest food during the whole spawning season, the body deposition will be depleted at late stages in the spawning season. This may result in a lower amount of protein and lipid being allocated into the oocytes. However, total lipid in the egg did not change significantly over the spawning season.

The fatty acids in eggs changed over the spawning season (Figure 9). The level of 18:0 and 20:4(n-6) in TAG, and Σ(n-6)PUFA in PE decreased significantly ($P<0.05$). In a smaller study of Atlantic halibut eggs, Parrish *et al.* (1994b) also found there were significant

decreases in 20:5(n-3) and Σ(n-3)PUFA in TL and 14:0 in TPL with the progress of the spawning season. McEvoy *et al.* (1993) reported a similar significant decrease of 20:5(n-5) in turbot eggs over the season. Like halibut, turbot fast while spawning. The decrease of these fatty acids reflects their depletion in the ovary and other body lipid deposition sites in late season as a result of selective incorporation into oocytes (Schwalme *et al.*, 1993; Silversand & Haux, 1995). In this study, the significant decrease ($P<0.05$) of Σ(n-6)PUFA in PE (Figure 9d) and the significantly higher level ($P<0.01$) of (n-6)PUFA in TAG (Figure 3a) in better quality eggs suggests that (n-6)PUFA may be important in embryonic and larval development. No significant decrease of Σ(n-3)PUFA were found, probably because of the large variability in these components. The increase of 16:1(n-7) in PC ($P<0.05$) (Figure 9c) may be a compensation for those depleted fatty acids.

4.2.3. Maturity of spawners

It is generally accepted that middle aged fish produce eggs of the best quality and poorer egg quality is found in first time spawners (Kjorsvik *et al.*, 1990). In the present study the fertilization success of eggs produced by repeat spawners was significantly higher ($P<0.05$) than that of eggs produced by first time spawners (Figure 10). The significant lower amount of ST ($P<0.05$) and TNL ($P<0.01$) found in the eggs produced by repeat spawners (Figure 11) is in agreement with the aforementioned lower ST and TNL in better eggs. The eggs of repeat spawners contained significantly higher amounts of PE ($P<0.05$). The importance of PE in embryonic and larval stages will be discussed later. The poorer quality

of eggs produced by first time spawners may relate to under-developed reproduction systems in first time spawners.

4.3. Biochemical changes in developing embryos and larvae

4.3.1. Protein, FAA and ammonia

During prefeeding developmental stages, i.e., endogenous feeding stages, all nutrients needed for development, growth and homeostasis of the embryos and larvae come from the yolk, and very little amount of exogenous nutrients are ingested. Therefore a continuous decrease in organic nutrients is expected. This theory is consistent with the data presented in Figure 12. Both AFDW and Σ(Protein + FAA + Lipid) decreased significantly as development proceeded (ANOVA $P<0.01$ and $P<0.05$ respectively). The steepest decrease found between embryos at half way to hatch and larvae that had just hatched may be related to the loss of chorion and perivitilline colloids during the hatching process. According to Finn *et al.* (1991), in Atlantic halibut eggs, the dry weight of chorion is about 12% of the egg dry weight, and 84% of the chorion dry weight is composed of protein. Therefore the loss of AFDW at hatch is mostly the loss of protein.

Protein is the largest caloric component in eggs (Table 1), accounting for ca. 60% of total dry weight. There is a significant difference ($P< 0.05$) in protein content between just hatched larvae and unfertilized eggs (Figure 18). Protein content decreased about 133 μg. This value is very close to the chorion protein value of 155 μg reported by Finn *et al.* (1991),

especially considering that the average diameter of the egg samples used in this study were smaller than eggs used in their study. The total protein content decreased during embryogenesis. This decrease, though not significant ($P>0.05$), may reflect the partial digestion of chorion protein before hatching occurred. Whether the breakdown products of choriolysis are utilized by the embryo is controversial. Finn *et al.* (1991) reported that in Atlantic halibut, the dry weight of chorion decreased significantly before hatching, and they suggested that the breakdown products were retained in the perivitelline space. Although the biochemical composition of perivitelline fluid is not known in halibut eggs, the data from fertilized Atlantic salmon eggs suggests that perivitelline contains substantial amount of protein (22.7% of total protein), lipid (7.7% of total lipid), and carbohydrate (46% of total lipid) (Hamor & Garside, 1977). On the other hand, there is an indication of the choriolysis product being used by winter flounder embryos (Cetta & Capuzzo, 1982).

The average FAA content in unfertilized eggs was 2294 nmol/indiv (Figure 17), this value is similar to that reported by Finn *et al.* in 1991 (2308 nmol/indiv). Total FAA decreased about 88 nmol/egg at half way to hatch stage (the first week after fertilization) compared to the unfertilized stage. The differences were not statistically significant ($P>0.05$) in unfertilized, fertilized, and half way to hatch stages. However, the average level of NH_3 /NH_4^+, which is believed to be the only N-end product in the embryonic stage (Ronnestad, 1992), increased significantly ($P<0.05$) from ~45 nmol/indiv. in unfertilized eggs to ~145 nmol/indiv. in fertilized eggs at the half way to hatch stage (Figure 19). According to Ronnestad (1992), 1 mole of average amino acid molecules in the FAA pool contains 1.34 mole of N, therefore catabolism of 88 nmol of amino acid should produce a total of 117

nmol of NH_3/NH_4^+. Since the secretion of the NH_3/NH_4^+ to the ambient water by the embryos at this early stage (1- 5.5 day) is less than 20 nmol (Finn *et al.*, 1991), the increase of 100 nmol of NH_3 in the embryo (Figure 19) is in good agreement with the calculated value. The cause of the large standard deviation in the NH_3/NH_4^+ value at the half-way to hatch stage is not known.

Ronnestad (1992) and Finn *et al.* (1995c) suggested that, in embryos of turbot, lemon sole, and cod, FAA are metabolized after the half way to hatch stage, i.e., after the completion of gastrulation stage, when the vitelline syncytium layer is fully developed. This tissue is believed to be responsible for the utilization of yolk nutrients. Ronnestad (1992) suggested that in Atlantic halibut, FAA were not used until after hatch. The present study indicated that FAA and/or protein were possibly catabolized in the early embryonic stages, before completion of the gastrulation stage.

The FAA pool decreased significantly ($P<0.05$) in just-hatched larvae compared to the embryos at half way to hatch (Figure 17). The decrease of 466 nmol in FAA was accompanied by a 155 nmol increase in NH_3/NH_4^+ (Figure 19), which is only one fourth of the calculated total NH_3/NH_4^+ production, assuming that the FAA that have disappeared are completely deaminized. The NH_3/NH_4^+ secreted during this period of time would be ~ 100 nmol/indiv. as calculated by Finn *et al.* (1991). Taking the sum of these two values from the total calculated value still leaves a residue as high as ~ 370 nmol of NH_3/NH_4^+. However, since the NH_3/NH_4^+ level in the later embryonic stages in this study was much higher than the level reported by Finn *et al.* (1991), the NH_3/NH_4^+ excreted to the media must be higher than the reported ~ 100 nmol/indiv. The discrepancy may arise from the different methods

used in NH_3/NH_4^+ measurement and differences in experimental conditions such as temperature and size of the container.

The depleted FAA can be used both as energy substrates, and precursors for the synthesis of lipid, glucose, protein and some other quantitatively unimportant N-rich substances (polyamines, nucleic acids etc.). There is no net increase of protein before hatching (Figure 18), and total protein content decreased significantly ($P<0.05$) at hatch. Therefore the depletion of FAA pool may not be a result of protein synthesis, although there is probably an active exchange of amino acids between FAA and PAA pools. According to Finn *et al.* (1995a), in cod embryos, both glucose and glycogen increased as hatching approached then decreased shortly before hatching. Whyte *et al.* (1993) reported total saccarides increased in Pacific halibut embryos as well. FAA is believed to be the precursor for gluconeogenesis. However, carbohydrate is a minor component in marine fish eggs, accounting for about 1% of dry weight in Pacific halibut eggs (Whyte *et al.*, 1993). Therefore, it is unlikely that gluconeogenesis will utilize substantial amounts of FAA. The possibility of FAA as substrates for lipid synthesis will be discussed later.

There was a net increase of ~ 60 μg protein in larvae from just hatched to 14DPH (Figure 18). At the same time, FAA decreased significantly from 1770 nmol/indiv. at hatch to 806 nmol/indiv. at 14DPH (Figure 17), resulting in a difference of 960 nmol. Taking 130 Da as the approximate average molecular weight of the free amino acids (Finn *et al.*, 1995c), 100% polymerization of 960 nmol of FAA would produce 107.5 μg of protein. Therefore, less than 60% of the FAA pool that disappeared was polymerized in newly synthesized protein. This finding is similar to that reported by Finn *et al.* (1995c). Pittman *et al.* (1990)

also concluded from the RNA/DNA ratio that each cell's capacity for protein synthesis increases during the first two week after hatching. The synthesis of protein during the first two weeks after hatching can be further substantiated by the total NH_3/NH_4^+ production of the larvae during this period of time, since FAA channeled to protein synthesis will not go through deamination. When FAA is used as the carbon source for energy metabolism, lipogenesis or glucogenesis, NH_3/NH_4^+ production will be increased. Unfortunately, NH_3/NH_4^+ secreted into the system was not measured in this study. Both systems used to rear eggs and larvae make the measurement of NH_3/NH_4^+ level very difficult. The egg incubator is not a stagnant system, but one with a constant flow of seawater which makes the concentration of NH_3/NH_4^+ too dilute to be measured. The larvae are reared in petri-dishes. Although water in petri-dishes is changed and bodies of dead larvae are removed regularly, the water is easily contaminated with NH_3/NH_4^+ released from decomposed bodies in between changes.

As shown in Figure 17 and Table 5, the utilization of the FAA pool is not distributed evenly between EAA and NEAA. The NEAA level is slightly higher than EAA level during the embryonic stage, but since NEAA are utilized more than EAA, a reversal of the ratio is found during the larval stage. In body protein and yolk protein, EAA were more abundant than NEAA, and the ratio of the two did not change throughout the larval stage (Finn *et al.*, 1995c). It is possible that EAA in the FAA pool are retained for protein synthesis. The higher depletion rate of NEAA is due to the selective utilization of serine, alanine and glutamic acid (Table 5). Among EAA, valine is selectively retained in the FAA pool. Ronnestad (1992) also reported a similar preferential uptake of these amino acids.

The initial level of NH_3/NH_4^+ in unfertilized and fertilized eggs (45 and 35 nmol/egg, Figure 18) is substantially lower than the level reported by Finn *et al.* in 1991 (~149 and 157 nmol/embryo). This large discrepancy may be the result of different methods used in collecting eggs and in NH_3/NH_4^+ analysis. The NH_3/NH_4^+ level increased continuously throughout the embryonic stage from 45 nmol/indiv. in unfertilized eggs to ~300 nmol/indiv. in newly hatched larvae. In this study (Figure 18) as well as that of Finn *et al.* (1991), NH_3/NH_4^+ did not decrease substantially after hatch, unlike the sharp decrease of NH_3/NH_4^+ occurs in newly hatched larvae of other species such as cod (Finn *et al.*, 1995a), turbot, lemon sole (Ronnestad, 1992), and gilthead sea bream (Ronnestad *et al.*, 1994). This may be related to the fact that Atlantic halibut larvae hatch at a relatively undeveloped stage compared to other species studied, therefore, the secretion of NH_3/NH_4^+ is slower. The mechanism of NH_3/NH_4^+ secretion in embryonic and early larval stages is not clear. As the gill is not developed at this stage, the skin may be the site for NH_3/NH_4^+ secretion.

The role of FAA and the end product, NH_3/NH_4^+ as osmolytes in invertebrate and marine fish eggs and larvae is well documented (Riis-Vestergaard, 1987; Fyhn, 1990). Marine invertebrate execrete FAA into the seawater for the purpose of osmoregulation. In contrast, marine fish larvae regulate the concentration of FAA by using it in energy production and biosynthesis. Ronnestad (1992) documented that there was no significant amount of FAA excreted by Atlantic halibut larvae. Finn *et al.* (1995a, 1995c) found that in cod and Atlantic halibut larvae the resorbtion of FAA was linearly correlated with the yolk volume, so that the FAA concentration remained stable and the osmolality equivalence was maintained.

4.3.2. Lipids and fatty acids

Total lipid did not change significantly ($P>0.05$) in the early embryonic stages, i.e., before the half-way to hatch stage (Figure 13). PE is the only lipid class that decreased significantly ($P<0.05$) at the half-way to hatch stage (Figure 15a). Falk-Petersen *et al.* (1989) also found a sharp decrease of PE in 4-10 day old halibut embryos compared to the 0-3 day old embryos. This selective metabolism of PE may be related to the release of (n-3) PUFA in order to synthesize or regulate biochemically active substances such as eicosanoids at the early embryonic stage when rapid cell division and differentiation occurs.

Total lipid increased ($P<0.05$) ca.16% in newly hatched larvae compared to the embryo at the half way to hatch stage (Figure 13). All the major lipid classes except for ST increased significantly at hatching. The observed increase of lipid at hatch is in contrast to the continuous decrease of total lipid in embryos and larvae of herring (Tocher *et al.*, 1985b), cod (Freaser *et al.*, 1988; Finn *et al.*, 1995b), Pacific halibut (Whyte *et al.*, 1993), and goldfish (Wiegand, 1996). However, Cetta and Capuzzo (1982) found that in winter flounder, lipid increased ca. 57.7% at the end of the embryonic stage compared to the initial level at fertilization. After deamination, the carbon skeleton of FAA can be used as carbon units in lipid synthesis in adult fish (Henderson & Tocher, 1987). In rainbow trout liver, more radioactivity from ^{14}C-alanine than from ^{14}C-glucose was incorporated into fatty acids (Henderson & Sargent, 1981). In the FAA pool of unfertilized Atlantic halibut eggs, ketogenic amino acids accounted for more than half of the total FAA (Table 3). These amino

acids can produce acetyl-CoA after deamination. Acetyl-CoA can be used as a precursor of fatty acids and lipid synthesis in trout embryos (Terner *et al.*, 1968) or it can enter the tricarboxylic acid cycle (TCA) to produce ATP by combining with oxaloacetate to form citrate. The calories associated with the increase of lipid accounted for ~71% of the total calories associated with the decrease of FAA from unfertilized eggs to the hatch stage (Table 6).

Under conditions of anoxia or hypoxia, amino acids can also be directed to fatty acid chain elongation. van Raaij *et al.* (1994 a&b) suggested amino acids as acetyl donors for lipid synthesis, and found fatty acid elongation as well as incorporation of 1-^{14}C-acetate into free fatty acids, TAG, and polar lipids in anoxic goldfish. Metabolism of 1-^{14}C-acetate and 1-^{14}C-leucine in anoxic goldfish both produce $^{14}CO_2$ in an amount higher than one would expect from ethanol production (van Raaij *et al.*, 1994b). It is suggested that the excessive $^{14}CO_2$ may come from the TCA cycle. Under normoxia the TCA cycle is tightly coupled to the operation of the electron transport chain, where the reducing equivalents produced by the TCA cycle (mainly NADH) are transferred to O_2, and the energy released leads to the production of ATP. In anoxic fish, excessive NADH may accumulate due to lack of O_2. The reverse reaction of fatty acid β-oxidation in mitochondria leads to fatty acid elongation, and consumes NADH at the same time, so that TCA cycle operation under anoxia becomes possible (van Waarde, 1988). When there is excessive succinate, another form of reducing equivalents produced by the TCA cycle, $FADH_2$, can be directed to the formation of NADH through the reversal of the respiratory chain (van Waarde, 1988).

In the case of fish embryos, under normal culture conditions anoxia or hypoxia is

unlikely to happen. Although the gill may not become functional until near the end of the larval period, the skin remains a major site of gas exchange throughout the embryonic and larval periods. In many species microvilli in early embryonic stages and the well-developed vascular networks that form just under the skin during early organogenesis may assist respiratory gas exchange. It is believed that the chorion and perivitelline fluid are not the major barrier to diffusive gas exchange during embryonic life (Rombough, 1988). During the process of hatching, the hatching enzyme, a chorion specific proteinase, is excreted to the perivitelline space by the embryo, and the chorion is degraded extensively from within. The mature embryo exhibits vigorous muscular activity afterward to tear open the chorion. A hypoxic condition may result from this intense movement associated with the hatching process. Hypoxia is involved in the natural hatching process of Atlantic salmon larvae, and can be used as an artificial method to induce hatching in the hatchery (Oppen-Berntsen *et al.*, 1990). Helvik and Whalther (1992) suggested that hypoxia did not affect hatching in halibut. However, the rate of O_2 uptake increased with the development of halibut embryo (Finn *et al.*, 1991). Pelagic embryos such as halibut embryos are susceptible to hypoxia. Since they are usually found in well-oxygenated waters, they tend to have a relatively poorly developed capillary plexus near the body surface compared to demersal embryos (Rombough, 1988). Therefore when hatching occurs, it is possible that parts of the embryo may experience hypoxia, and stimulate the process of fatty acid chain elongation. An increase of lipid content has been observed in anoxic perch embryos (van Waarde, 1988).

There were no significant changes in fatty acid profiles associated with the increase of PC and PE at hatch. These two lipid classes are rich in (n-3)PUFA compared to TAG.

Since the ability of (n-3)PUFA synthesis is limited in marine fish, the maintenance of fatty acid profiles in PC and PE requires the transfer of (n-3)PUFA from other lipid classes. There is a net increase of total saturated fatty acids in TAG (Figure 16) at hatch largely due to the increase of 16:0 and 18:0.

Total lipid decreased during the larval stage (Figure 13). TAG is the only lipid class that decreased significantly ($P<0.05$). At 14 DPH, there was ~17% decrease in TAG compared to just hatched larvae (Figure 14a). PC showed a decreasing trend, but this was not significant ($P>0.05$). PE remained at a constant level after hatching. No significant change in fatty acids was found during the larval stages studied. This agrees with Finn *et al.* (1995b), who suggested that there was a non-selective utilization of lipid classes between hatch and 200 degree-days post hatch. Towards first-feeding, they found a selective catabolism of PC and a net synthesis of PE. Synthesis or retention of PE in larvae prior to first feeding was also found in Atlantic herring, cod, Atlantic halibut, plaice, turbot, and Senegal sole (Tocher *et al.*, 1985b; Fraser *et al.*, 1988; Vazquez *et al.*, 1994). PE is the only phospholipid containing plasmalogens in fish roe (Tocher *et al.*, 1984). Plasmalogen-linked phospholipids seem to play a role both as membrane components and as cellular mediators (Snyder, 1985). Some ether lipids appear to have the capacity to serve as protective storage reserviors for PUFA. The protective nature of ether-linked group is due to their ability to retard the rate of hydrolysis of acyl moities at the sn-2 position in the same molecule by phospholipase A_2 (Snyder, 1985). In Atlantic halibut larvae, 38% of the DHA released from PC catabolism was retained by PE (Ronnestad *et al.*, 1995). Therefore, the plasmalogens in PE may serve as protectors of the PUFA reservior. The retention and synthesis of PE in different species

suggests its importance in marine fish larvae. Sargent (1995) has suggested that the high levels of PE and its constituent fatty acid, DHA, in the brain and eye assemblage of fish indicates a primary role for these compounds during embryonic and larval development which might involve both structural and behavioral functions.

4.4. Conclusions and directions for future research

From this study, it was found that lipid and fatty acid composition in eggs from captive Atlantic halibut was associated with egg quality. Higher TL content, especially higher NL content, was associated with poorer egg quality. Whether there is a cause and effect relationship between high neutral lipid content and poor egg quality requires further investigation. In better quality eggs, there tended to be a higher amount of (n-3)PUFA as well as (n-6)PUFA. There was a positive correlation (although not statistically significant) between egg protein and FAA and larval viability. A larger data set is needed to determine if this correlation is real.

The biochemical content of eggs can be affected by the food intake of broodstock female, maturity of the maternal fish, and the time the egg batch was spawned during the season. The more experienced spawners produce better quality eggs than first-time spawners, and egg from the former contain less lipid than the latter. Reduction of food intake by the spawner resulted in decreased average egg dry weight and average egg lipid. But these changes did not seem to affect the egg quality. No change of egg quality could be found over the spawning season, although total protein and several fatty acids changed significantly with

the progress of the spawning season.

FAA are the biochemical components that are most utilized during the embryonic and early larval stages. While significant amounts of FAA may be catabolized to produce energy, a larger part of the depleted FAA may be directed to lipogenesis (70% of depleted FAA during the embryonic stage and just-hatched larvae), or protein synthesis (60% in the larval stage before 14 DPH). Lipids accumulated during the embryonic stage, and were utilized after hatch. The increase of lipids around hatching may be a biochemical adaptation to hypoxia induced by embryo muscular movement when hatching occurred.

The role of yolk lipids and fatty acids as energy substrates has been well studied in fish embryos and larvae. However, the connection between yolk lipid composition and the role of lipid and fatty acids as biomembrane components is not fully understood. Lipid molecular species and fatty acid composition analyses of plasma membranes of embryos and larvae will help greatly in understanding the connection between lipid and fatty acid composition and egg quality, especially for species which live in a low temperature and high salinity environment like Atlantic halibut. In this type of environment, the structure of the biomembrane may be equally important or even more important than the energy content in the yolk.

To understand the direction of FAA metabolism in developing embryos and larvae more completely, an experiment designed specifically to measure NH_3/NH_4^+ excretion to the ambient water should be undertaken. Use of radio-labeled amino acids would also be useful in future studies.

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