LIPIDS, FATTY ACIDS AND FREE AMINO ACIDS AS INDICATORS OF EGG AND LARVAL VIABILITY IN ATLANTIC COD (Gadus morhua)

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Lipids, fatty acids and free amino acids as indicators of egg and larval viability in Atlantic cod (*Gadus morhua*)

by

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Abstract

In aquaculture, the ability to predict future performance potential of eggs and larvae can help optimize hatchery production and lower overall production costs. In this study, egg batches spawned by Atlantic cod (*Gadus morhua*) broodstocks were collected, incubated to hatch, and the larvae reared to yolk-sac absorption. Objectives were to determine whether lipid classes, fatty acids, and amino acids could be used as indicators of egg and larval quality; to compare the utility of biochemistry and blastomere morphology as indicators of future performance potential; and to study changes in egg and larval biochemistry during ontogenetic development. Variability in several lipids and free amino acids was significantly correlated with fertilization and hatching success, particularly the phospholipids and the monosaturated fatty acid 24:1. Lipids showed conservation throughout embryo development, while free amino acids were catabolized as a primary energy source. Several blastomere morphological measures in newly fertilized eggs (eg. cell symmetry, uniformity, margins and adhesions) showed positive correlations with hatching success. These results show that both biochemistry and blastomere morphology can be useful tools for determining the performance potential of egg batches in culture, and that free amino acids serve as a primary energy source for endogenous-feeding eggs and larvae.

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List of abbreviations

AMPL – acetone mobile phospholipid

ARDF – Aquaculture Research and Development Facility

AA – arachidonic acid

CF – condition factor

dph – days post-hatch

dd – degree days

DG - diacylglycerol

DHA – docosahexenoic acid

EPA – eicosapentaenoic acid

FA – fatty acids

FAA – free amino acids

GC-FID – Gas chromatograph with flame ionization detector

GE – glyceryl ethers

HC - hydrocarbons

KET – ketones

MUFA – monounsaturated fatty acids

PE – phosphatidylethanolamine

PC – phosphatidylcholine

PL – phospholipids

PUFA – polyunsaturated fatty acids

SFA – saturated fatty acids

TL – total lipids

TAG – triacylglycerols

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1 Introduction and background

1.1 Introduction

The Atlantic cod, *Gadus morhua*, of the family Gadidae, is found on both sides of the North Atlantic, and ranges as far south as North Carolina to Greenland in the north. A fish with a heavy body and large head, the mature cod averages ~ 70 cm in length, with offshore stocks growing even larger (Stares et al. 2007). It is a groundfish, living at depths of 60 – 120 m, with some stocks showing seasonal migratory patterns. Patterns of migration can differ among stocks. For example, Labrador cod moves north and south along the coastline, but remains in cold waters, while cod in the Gulf of St. Lawrence move into the Gulf in spring and south-east in the fall (Leim and Scott 1966).

While spawning patterns differ among stocks, those off the coast of Newfoundland generally spawn around May. The mature female produces ~ 0.5 to 3 million eggs in one spawning season, with egg production changing according to population densities and fish size (Stares et al. 2007). The growth rates of cod differ depending on stock and season. When mature, the cods' diet consists mainly of other fish (for example, herring and capelin), while younger cod depend more on a diet of amphipods, euphausiids, polycheates and molluscs, and some plants (Leim and Scott 1966).

For centuries, cod have been fished for both private and commercial use, and have been used for food, liver oil and even in glue production. Their abundance on the coast of Newfoundland and Labrador resulted in a thriving fishery. However, overfishing and an

increase in market demand have put great strain on Northwest Atlantic cod stocks. These cod populations have experienced a severe drop in population numbers since the 1960s, with the total population estimated currently at 1-2% of historical levels. As a result, the Newfoundland cod fishery experienced a moratorium in 1993 and is now limited to a restricted commercial and recreational fishery (Hutchings 2003).

With the decline of the wild stocks, there has been a rising interest in cod aquaculture in order to meet international market demands. Norway currently has a cod industry well underway, while Canada, Scotland and the USA are following suit. Continued intensive research into developing protocols for the rearing of these fish at all stages of their life history can help cod aquaculture be a successful and sustainable industry,.

Temperature, lighting regimes, salinity levels and nutrition have been manipulated in order to develop effective protocols for rearing marine fish in captivity (Bjornsson and Olafsdottir 2006, Clark et al. 1995, Davie et al. 2007, Purchase and Brown 2001, Puvanandren et al. 2006). However, further research is necessary so that the growth and quality of stocks can be further improved. Of particular interest is broodstock management and its effect on egg and larval quality indices. The growth and survival of fish in early life stages affects the recruitment and final product (Zhao et al. 2001). By developing methods to increase growth and survival rates at this stage, hatchery production can be maximized.

An understanding of the factors affecting egg and larval quality can serve as a means to separate poor eggs from highly successful eggs, thus allowing producers to select viable

eggs that will result in maximum rates of fertilization, hatch, and larval survival. The viability of fish eggs – that being the success of fertilization and hatch – has been found to be dependent on a number of factors. These include genetic, environmental, and biochemical factors. Several parameters measured in newly spawned eggs of marine finfish species have been proposed and/or used as indicators of future egg viability or hatching success. For cod, these include both morphological and biochemical indices (Kjørsvik et al. 1990, Thorson et al. 2003, Vallin and Nissling 1998). This thesis looks in particular at the potential of biochemical parameters (lipid, fatty acid (FA) and free amino acid (FAA)) in comparison with blastomere morphology parameters as indicators of egg viability and hatching success and their utilization during egg development.

1.2 Objectives

By analyzing lipid, fatty acid and free amino acid levels in newly spawned eggs, and tracing the catabolism of these through development, the above variables may be correlated with the viability of eggs and larvae in culture. Thus they can be potentially useful indicators of future performance potential such as egg survival and hatching success. Similar studies have been done on sea bass (Nocillado et al. 2000), common wolffish (Halfyard and Parrish 2002), and Atlantic halibut (Zhu et al. 2003, Evans et al. 1995).

In this research several batches of eggs spawned from cultured cod broodstock at the Aquaculture Research and Development Facility (ARDF) were followed from spawning to yolk-sac absorption. Relationships among lipid classes, fatty acids, free amino acids,

fertilization success, and hatching success were examined in a manner similar to that of Penney et al. (2006), but here the full suite of FA as proportions (%), ratios and concentrations (per egg or larva) were used. As well, low fertilization success samples were obtained in order to extend the viability range beyond that of Penney et al. (2006). In addition to lipids and FA, levels of FAA were also analysed in relation to egg and larval viability.

Comparisons are made between the use of morphology and biochemistry as indicators of egg and larval viability. Studying lipid and amino acid utilization during ontogenetic development increases the knowledge of which nutrients are essential for the developing embryo and endogenous larvae. Identification of new biochemical indices of egg and larval quality contributes to developing broodstock management and husbandry protocols that allow maximum quality and survival of early life cod in culture. It also provides information that will allow producers to use the best means for selecting high quality eggs to ensure better survival during the early life history of their stocks.

1.3 Background

Currently, the most common method used to evaluate egg quality is to examine blastomere morphology. Fertilized eggs are examined under a stereoscope to evaluate such things as cell symmetry and uniformity (see Materials and Methods for further explanation). In general, batches of eggs showing an unusually high percentage of abnormal development are considered of poor quality (Kjorsvik et al. 1990, Bromage et al. 1994).

Studies on several marine fish have looked at correlating blastomere morphology with hatching success and larval survival. Symmetry has been correlated with hatching success in haddock (Rideout et al. 2004) and halibut (Shields et al. 1997). However, there are still some problems with using blastomere morphology as an indicator of success. For example, Vallin and Nissling (1998) found that these abnormalities often correct later in embryo development. Therefore, another measure needs to be found to improve predications about the quality and potential viability of eggs and larvae.

The following is a brief overview of some of these, followed by a review of the importance of lipid biochemistry in egg and larval development and why it may be useful as an indicator of potential success.

1.3.1 Broodstock effect

Differences in life histories are seen between different fish populations, signifying a need for stock selection in culture situations. Maternal condition has been closely linked with the quality and condition of eggs. Ouellet et al. (2001) found that cod egg production correlated with number of batches from females in high pre-spawning condition, that is length and condition factor ($CF = w/l^3$). However, there was no relationship of maternal condition and size with survival of eggs and larvae, nor with hatching success. There is a positive relationship between egg size and larval viability parameters – age at first feeding, swimbladder development, and growth rate after 15 days post hatch (dph) – with size, condition and age of female Icelandic cod (Marteinsdottir and Steinarsson, 1997). A positive correlation between egg size and larval size at hatch in Atlantic cod was noted by

Knutsen and Tilseth (1985). It is possible that maternal condition is only a factor in some, but not all, cod populations.

In research on Baltic and Skagerrak cod, Pickova et al. (1997) found that the factors which most influenced the phospholipids (PL) in eggs was more related to stock differences than to diet. It is likely a combination of these that affects egg quality, but studies on improving diets is important for improving quality of eggs in any given stock.

Broodstock nutrition has a major effect on egg quality. Broodstock diet affects growth of the fish as well as of the gonads, thus affecting fecundity (Watanabe 1985). Wroblewski et al. (1998) captured wild cod and fed them in captivity over several years. These captive cod experienced better growth and a fecundity 2-4 times that of wild cod on a natural diet.

Nutrients in eggs come from glycophospholipoprotein vitellogenin, which is synthesized in the liver of the female and incorporated into the oocytes to eventually be processed into yolk proteins (Ohkubo and Matsubara 2001). The yolk can be defined as all the material which is deposited into an oocyte (via vitellogenesis) that subsequently serves as nutrients for the developing embryo and endogenous larvae (Wiegand 1996).

Lipids are a major nutrient in fish diets, and thus can have influence on both the broodstock and the subsequent individual egg batches. Lipids and fatty acids (FA) in the eggs can reflect that of the broodstock diet (Rainuzzo et al. 1997). Thus the condition of the female should be expected to influence that of the egg. In a comparison of wild and farmed cod stocks, eggs from farmed cod showed lower levels of phosphatidylinositol, arachidonic acid (AA) and pigment than those of wild cod. These three factors were also

positively linked with success (fertilization success and survival to hatch). It was therefore recommended that the diet of farmed cod include higher proportions of AA (Salze et al. 2005).

Another factor which can affect egg and larval quality is the genetics of the stocks used. Genetics in turbot and halibut have been found to account for about 30% of growth variation (Imsland and Jonsdottir 2002), and can account for many of the differences between fish populations.

1.3.2 Biochemical factors: Lipids, fatty acids and amino acids

The majority of past research has focused on the formulation of diets for larval and adult stages and, while broodstock nutrition has been well documented, less is known about the roles of lipids during the very early stages of life history – that is, the lipid stores of eggs and endogenously feeding larvae. There is a need for specific indicators that can help predict the success (or quality) of spawns in culture. With a controlled environment and selected stock being a fixed variable it is likely that the best indicator is biochemical – specifically, lipids, FA and amino acids.

From egg to first-feeding larval stage, cod depend on an endogenous supply of nutrients. These come from proteins, carbohydrates, and a supply of FA and lipids within the egg and yolk. Protein is a large nutrient component in marine fish eggs, up to 59% of egg composition in Atlantic halibut (Zhu et al. 2003). It seems logical that there is a necessary requirement of these nutrients for certain developmental changes to occur. As one might

expect, both egg and yolk size varies according to the requirements of different species of fish, as well as the total lipids (TL) which are stored.

Lipid analyses may show a relationship between the levels of these lipids and FA, and the success of the eggs and larvae. The PL and triacylglycerols (TAG), along with many of the ω3 and ω6 fatty acids, are found in great abundance in most fish species, and at all life stages. Of particular importance are the polar PL phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and neutral lipid TAG, which serve as the main sources of stored energy in eggs (Pickova et al. 1997). PL are also important in the formation of cells and tissues (Parrish 1999). These polar lipids can make up 61 - 71% of total lipids in some Gadidae and flounder species (Wiegand 1996). Also of great importance are the ω3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosohexaenoic acid (DHA), which are shown to be required at some level by most fish species (Pickova et al. 1997).

Lipids and FA act as sources of energy for the growing embryo and newly hatched larvae, as well as providing building blocks for physical development. While all lipid classes may be used as fuel (Wiegand 1996), the presence of particular lipids and FA in the egg and yolk-sac are essential for survival. Studies have shown that by profiling these in early life stages of marine fish, one may be able to predict the nutrient requirements for larval diets (Nocillado et al. 2000). Also, by understanding the influence of their levels and ratios it may be possible to predict the success of individual batches of eggs. This can include such things as fertilization and hatching potential. In wolffish, *Anarhichas lupus*, significant relationships have been found between egg lipid and fatty acid variability,

fertilization success, hatching success, and larval mortality (Halfyard and Parrish 2002, Tvelten et al. 2004).

Fraser (1988) found that PC in cod was the only phospholipid that declined in absolute terms during cod embryogenesis, while the neutral lipids were catabolized during the first week following hatch. According to the Rainuzzo et al. (1997), polar lipids (particularly PC) are preferentially catabolized following hatch, whereas PE is synthesized or conserved. Simlar results were found for rockfish, *Dentex dentex* (Mourente et al. 1999). It is likely that this synthesis comes from the fatty acids produced by PC catabolism.

While sufficient levels of these lipids and FA are needed to support a developing embryo and the newly hatched larvae, research has shown that it depends not only the base level of these, but also the relations between them. Ratios of DHA:EPA affect hatching, growth and neural development of both the common and spotted wolffish (Halfyard and Parrish 2002, Tvelten et al. 2004), yellowtail flounder (Copeman et al. 2002), and cod (Pickova et al. 1997). These studies have all found that DHA:EPA has a positive correlation with growth, survival and neural function. There are a variety of reasons that may explain this, including the fact that there is competition between DHA and EPA for some enzymes in order to esterify FA into phospholipids, and also that there are high levels of DHA found in neural membranes (Copeman et al. 2002). Thus a low DHA:EPA may compromise neural development. Rainuzzo et al. (1997) state that high amounts of EPA in relation to DHA can create an imbalance in the structure of PL. Pickova et al. (1997) found that the AA content of the phospholipid fraction also influences egg viability and symmetry in cod, and is involved in prostaglandin formation. AA and EPA are both used as a substrate

for eicosanoids (Copeman et al. 2002), which are prostaglandins and other oxygenated FA responsible for a variety of physiological functions.

Nocillado et al. (2000) found by sorting sea bass eggs into two groups (zero fertilization *vs* fertilization and hatch) there were differences in TL, saturated fatty acids (SFA), EPA, and DHA. Their results show that egg components may serve as quality measures in spawns of sea bass. Pickova et al. (1997) suggest that the DHA:EPA ratio serves as an important factor influencing egg viability in cod. Halfyard and Parrish (2002) found that in both eggs and larvae, the total and specific fatty acid and lipid class content (DHA, EPA, DHA:EPA, TAG and PL) were positively correlated with survival and growth in the common wolffish.

A recent paper published by Penney et al. (2006) looked at the morphology as well as lipid biochemistry of Atlantic cod eggs. This paper compared eggs from three different groupings (according to broodstock). Factors measured included egg size and dry weights, as well as blastomere morphology, TL and lipid classes, and FA and ratios. Because of the low variability in blastomere morphology characteristics, Penney et al. (2006) were unable to correlate these factors with hatching success, and also found that neither TL, lipid classes, FA, nor their ratios, could be correlated with hatching success. It is worth noting that the range of fertilization in this study was somewhat narrow at 93.7 - 98.7%. It was suggested at the end of the paper that biochemical indicators for cod egg viability should include lipid profiling in combination with measurements of free amino acids (FAA).

FAA are also important factors to consider when studying egg quality. While lipids have been the main focus of energy metabolism in developing eggs and larvae of marine fishes, studies on FAA have shown an important role in energetics as well. While some of the FAA are incorporated into protein of the developing embryo (Rønnestad et al. 1992b, Rønnestad et al. 1993, Sivaloganathan et al. 1998), the decline in the FAA pool cannot be wholly attributed to protein synthesis. Instead, the breaking down of FAA is used as an energy source either along with other nutrients, or as a separate resource (Fyhn and Serigstad 1987, Rønnestad et al. 1992a, Rønnestad et al. 1992b, Rønnestad et al. 1993, Rønnestad et al. 1994, Finn and Fyhn 1995, Fyhn and Govani 1995, Sivaloganathan et al. 1998). As well, some FAA are depleted at a significant rate during development, while others seem to be conserved. In the common wolfish, taurine – a non-essential amino acid – was found in higher levels in the eggs of higher quality (Halfyard and Parrish 2002). This may indicate an important role for particular FAA in the successful development of the fish.

It is also possible that FAA contribute to the synthesis of lipids and FA. Zhu et al. (2003) found that FAA serve as the carbon skeleton on which lipids are built in eggs and endogenous larvae of Atlantic halibut. It was found that throughout development, FAA decreased while phospholipids increased, meaning that lipid synthesis from FAA might be related to the restructuring of membranes.

As both a source of energy and as building blocks for proteins and cell membranes, FAA play an important biochemical role in the development success of marine fish eggs and

larvae. The contribution of FAA to early life success in species such as cod is important to consider, and may provide a useful indicator of viability for commercial hatcheries.

2. Lipids and fatty acids in eggs and yolk-sac larvae of Atlantic cod

2.1 Introduction

From both an ecological and commercial viewpoint, egg quality and larval viability go hand in hand. This experiment looks at the relationship among lipid classes, fatty acids, fertilization success, and hatching success in cod egg batches from communal spawning groups. Seeing if there are thresholds, minimum levels and ratios that allow maximum viability can then help in the development of broodstock diets that will lead to high quality eggs, as well as more efficient first-feeding diets that provide requisite nutrients to the larvae.

The second purpose of this experiment was to examine the use and conservation of the lipids and FA in cod throughout development of the embryo and endogenous larvae. This can thereby increase the understanding of which nutrients are important for energy as well as for incorporation into membranes and tissues, and thus contribute to the development of broodstock diets which give the best advantage to offspring as well as to formulating diets for larval cod in the first days of feeding (Whyte et al. 1993).

2.2 Materials and methods

2.2.1 Sampling methods

A total of twelve separate egg batches were collected throughout the experiment. Ten batches of eggs were collected from the cod broodstock held in a communal spawning tank in the Aquaculture Research and Development Facility at the Ocean Sciences

Centre, Logy Bay, Newfoundland. Another two batches (LowFert B and LowFert C) were acquired from paired matings. A total of 150 - 200 ml of eggs (~ 52,500 – 70,000 eggs) were used for each batch and were immersed in a Perosan disinfectant bath for one minute before being transferred to tanks. The eggs were reared in 300 L flow-through incubators at 6 ± 1°C, with a density of approximately 50 eggs/L, with each separate batch kept in its own tank. The tanks were monitored regularly for mortalities and for hatched larvae, and dead eggs were flushed out daily.

At collection, a subsample of one hundred eggs from each batch was examined under a stereoscope to estimate fertilization success (percentage of eggs showing cell cleavage), and blastomere morphology (adapted from Shields et al. 1997). This was performed at the two - sixteen cell stage. Morphological characteristics included the following:

1) Cell symmetry: the percentage of eggs showing normal symmetry in blastomere cell division.

2) Uniformity: the percentage of eggs showing blastomere cells which were uniform in size and shape.

3) Adhesions: the percentage of eggs showing normal adhesion of blastomere cells.

4) Margins: the percentage of eggs showing clear margins between separate blastomere cells.

5) Clarity: the percentage of eggs showing clarity in blastomere cells (no cloudiness)

6) Cell number: the percentage of eggs showing expected number of cells with proper division (e.g. two, four, eight, sixteen).

Eggs were from all batches were sampled immediately after spawning, at fertilization (Day 0), halfway to hatch (~ 40 degree days), pre-hatch (~ 78 dd), post-hatch (80-90 dd), half-way to yolk-sac absorption (114 dd) and at yolk-sac absorption (145 dd). The unfertilized eggs were collected separately for analysis along with the fertilized eggs from the same batch at Day 0. The separate batches were classified as high fertilization success (> 70% of eggs fertilized: HighFert), or low fertilization success (< 70%: LowFert). Each egg batch was sampled in triplicate. Triplicate samples (fifty eggs/sample, one hundred larvae/sample) were taken from each batch for each sampling stage. Egg samples were rinsed with filtered seawater, gently blotted dry, and then placed in 10 mL lipid-clean vials with ~ 2 mL of $CHCl_3$. Larvae were collected on glass-fibre filters, rinsed with filtered sea water, and placed in 10 mL vials. The vials were then filled with N_2 (g), sealed with Teflon tape, and stored at -20°C until lipid and fatty acid analysis took place. Triplicates of twenty eggs and fifty larvae were also sampled for measuring wet and dry weights.

2.2.2 Lipid extraction and analysis

Lipids were extracted from the samples using a modified Folch (1957) method with 2:1 chloroform-methanol. In brief, one ml methanol was added to the vial, and the sample

was ground into a pulp and sonicated in an ice bath for four minutes. The tube was then placed in a centrifuge and spun at >1000 rpm for approximately two minutes. The organic (bottom) layer was removed using the double pipetting technique, and placed in a lipid-clean 7 ml vial. This process was repeated three to four times. The extract was then concentrated using nitrogen and transferred into a lipid-clean 2 ml vial.

The lipid extract was separated into classes using a silica gel coated Chromarods-SIII in four solvent systems, and lipid analysis was carried out using an Iatroscan Mark V TLC/FID analyzer. The separation of the hydrocarbon to ketone groups (HC to KET) was achieved by first focusing the rods in acetone, and then developing twice in hexane:diethyl ether:formic acid (98.95:1:0.05) for 25 minutes, followed by 5 minutes drying and then developing another twenty minutes. Rods were then scanned to the lowest point behind the KET peak. To separate the glyceryl ether (GE) to diacylglycerol (DG) groups, the rods were developed for 40 minutes in hexane:diethyl ether:formic acid (79:20:1) and scanned to the lowest point behind the DG peak. The last development system, for separating the acetone mobile polar lipid (AMPL) and the phospholipid groups, consisted of two 15 minute developments in acetone, followed by two ten minute developments in chloroform:methanol:choloroform-extracted-water (5:4:1). The entire length of the rods was then scanned. Peaks were compared with a 9-component lipid standard to identify lipid classes.

2.2.3 Fatty acid derivatization and analysis

Fatty acid methyl ester derivatives were made using hexane and BF_3/CH_3OH. In brief, approximately half of the lipid extract was transferred into a 7 ml lipid-clean vial and evaporated with nitrogen to dryness. Approximately 0.5 ml hexane and 1.5 ml BF_3/CH_3OH were added to the vial, which was then vortexed and sonicated for four minutes. The headspace of the vials was then flushed with nitrogen, capped and placed at 85°C for 1.5 hours. Next, 0.5 ml of chloroform extracted water and 2 ml of hexane were added and the resulting upper organic phase transferred to a 2 ml vial. The sample was blown dry with nitrogen and refilled with 2 ml hexane. Vials were filled with nitrogen and capped, sealed with Teflon tape, and vortexed and sonicated once more.

The fatty acid component of the sample was analyzed using a Hewlett Packard 6890 Series II gas chromatograph with a flame ionization detector. Resulting peaks were compared with a Supelco 37-component standard in order to identify FA.

2.2.4 Statistical analysis

Samples were sorted according to fertilized *vs* unfertilized eggs and fertilization and hatching success to see if there are statistical relationships between lipid and fatty acid profiles and the egg viability. Those FA examined were those which comprised greater than 1% of the total FA. All statistical tests were carried out using SigmaStat Version 13. Pearson correlations were used for fertilization and hatching success, and levels of particular lipid classes and FA. Comparisons between fertilized eggs and unfertilized eggs were made using a paired t-test, and between groups of high fertilization success (>70%)

and low fertilization success (<70%) comparisons were made using a t-test. For samples which did not pass normality or equal variance tests, and could not be made to pass by transformation, the median was tested using a Mann-Whitney Rank Sum test.

To examine changes through development, one-way ANOVAs were performed to look for changes in levels of lipids and FA over time. A Dunn's test was used to perform pair-wise comparisons where significant differences were found. For those samples which did not pass normality or equal variance, and could not be made to pass by transformation, a Kruskal-Wallis one-way ANOVA on ranks was performed.

2.3 Results

Fertilization success ranged from a low of 46% (LowFert C) to 99% (HighFert G and H). Hatching occurred between 82.6 dd (HighFert A) and 100 dd (LowFert B). Of the egg batches used in this study, ten batches survived to hatch. Of the two which did not survive, HighFert B was of 98.0% fertilization success and LowFert C was of poor success at 46% (Table 2.1).

Cell number was significantly correlated with fertilization success ($r = 0.615$, $p = 0.044$), so that an increase in cells with abnormal cell division correlated with an decrease in fertilization success. High abnormalities in blastomere symmetry ($r = 0.848$, $p < 0.001$), cell adhesion ($r = 0.809$, $p = 0.001$), cell uniformity ($r = 0.693$, $p = 0.018$) and cell margins ($r = 0.658$, $p = 0.028$) correlated with a lower hatching success (Table 2.1). Correlations between individual lipid classes and FA can be found in Appendix I. While most correlations were relatively weak ($r < 0.8$), 24:1 showed a positive correlation with

fertilization success ($r = 0.671$, $p = 0.024$), hatching success ($r = 0.682$, $p = 0.021$), and cell symmetry ($r = 0.688$, $p = 0.019$) and adhesions ($r = 0.699$, $p = 0.017$).

No significant differences were found between the dry weight of fertilized and unfertilized eggs, nor between eggs in groups of high or low fertilization success (Table 2.2). From 0 dd to 85 dd, there was no significant difference in egg dry weight, however at 40 dd the eggs in the LowFert groups were significantly smaller than those in the HighFert group.

Total lipid (TL) made up about 10% of the dry weight of eggs, with no differences seen between fertilized eggs and unfertilized eggs nor between eggs of high and low fertilization success (Table 2.3). Phospholipid was the predominant lipid class, with all other classes making up less than 1% of the total dry weight. The only significant differences found were in the means of TAG and PL between groups of high and low fertilization success. Higher levels of TAG were found in egg batches with low (< 70%) fertilization success (Mann-Whitney Rank Sum test, $p = 0.003$), and higher levels of PL in batches with high (>70%) fertilization success (Tukey's t-test, $p = 0.026$).

The saturate 16:0 was the predominant fatty acid found in all eggs, followed by 18:1ω9 and 22:6ω3 (DHA). Levels of EPA (20:5ω3) were the next abundant. No significant differences were found between any of the fatty acids for fertilized *vs* unfertilized eggs. On the other hand, high fertilization success batches had much higher levels of 24:1 than those with low fertilization success (Mann-Whitney Rank Sum test, $p = 0.037$). While saturates and MUFA make up most of the fatty acid profile (~ 70-80% total FA), there

were no significant differences between these nor PUFA, P/S ratios or total ω3s (Tables 2.3 and 2.4).

No significant differences were found in larval weights over time, or between groups (Table 2.5). Among the lipid classes, the only one which showed significant changes over time on a per individual basis was PL. There were no changes during embryo development, but the total phospholipid levels were significantly lower at yolk-sac absorption (Figure 2.1). PL was significantly different between high success and low success eggs at Day 0 and at 40 dd.

The fatty acid saturates 14:0 and 18:0 also changed significantly. The saturate 14:0 showed significantly lower quantities at the end of the sampling period, while 18:0 had significantly higher quantities at the end (Figure 2.2). For these FA, there was a significant difference between fertilization groups at 40 dd. Low success eggs showed significantly lower quantities only at this stage, while high success eggs did not change significantly.

The PUFA 20:5ω3, 22:5ω3 and 22:6ω3 all showed significantly lower quantities from post-hatch to yolk-sac absorption (Figure 2.3). From day 0 to post hatch, levels were conserved, and the significant difference only occurred by 114 dd. Total PUFA (Figure 2.4) show significantly lower quantities post-hatch, and essential FA (DHA + EPA + AA) also followed this pattern (Figure 2.8). The sum of ω3 and ω6's are also similar (Figure 2.6).

The ratio of PUFA to SAT, or P:S, shows significantly lower values post-hatch (Figure 2.5). Ratios of ω3 to ω6 is significanly lower at 145 dd (Figure 2.7), while AA:EPA changed from 0.06 at 0 dd to 0.25 at 145 dd, as EPA levels drop. DHA:EPA experienced only a difference of 1.89 at 0 dd to 2.27 at 145 dd (Figure 2.9).

Table 2.1*: *Blastomere morphology at day 0, fertilization success and hatching success of individual Atlantic cod egg batches. No significant differences exist in morphology of eggs of high success and low success.

Batch	Fertilization Success (%)	Symmetry** (%)	Uniformity** (%)	Adhesions** (%)	Margins** (%)	Clarity (%)	Cell number* (%)	Degree days to hatch	Hatch Success (%)
HighFert A	97.0	97.0	92.0	99.0	89.0	95.0	100.0	82.6	88.0
HighFert B	98.0	70.0	88.0	91.0	72.0	94.0	96.0	N/A	0
HighFert C	97.0	94.0	96.0	99.0	88.0	100.0	99.0	88.4	91.3
HighFert D	97.0	80.0	92.0	96.0	93.0	96.0	96.0	91.2	4.0
HighFert E	95.0	90.0	98.0	98.0	100.0	100.0	100.0	88.3	77.3
HighFert F	96.0	88.0	92.0	96.0	76.0	98.0	96.0	89.4	89.3
HighFert G	99.0	97.0	93.0	100.0	97.0	97.0	95.0	87.2	95.5
HighFert H	99.0	97.0	93.0	100.0	92.0	100.0	94.0	85.0	77.3
LowFert A	66.0	88.0	92.0	93.0	98.0	99.0	94.0	90.3	40.0
LowFert B	52.0	80.0	84.0	94.0	74.0	100.0	90.0	100.0	23.0
LowFert C	46.0	80.0	88.0	94.0	62.0	98.0	92.0	N/A	0
LowFert D	69.0	90.0	95.0	99.0	96.0	97.0	99.0	91.8	66.0

* Correlates with Fertilization Success

** Correlates with Hatching Success

Table 2.2: Mean dry weight (μg) per individual Atlantic cod egg. No significant differences exist between groups..

	Fertilized eggs	Unfertilized eggs	High Fertlization	Low Fertilization
HighFert A	9.28	9.18	9.28	--
HighFert B	11.3	12.2	11.3	--
HighFert C	9.78	7.70	9.78	--
HighFert D	11.4	9.40	11.4	--
HighFert E	9.68	9.75	9.68	--
HighFert F	9.65	10.2	9.65	--
HighFert G	12.9	12.7	12.9	--
HighFert H	12.5	16.0	12.5	--
LowFert A	8.52	12.3	--	8.52
LowFert B	11.7	10.8	--	11.7
LowFert C	9.43	10.8	--	9.43
LowFert D	10.0	8.45	--	10.0
			--	
Total Means	**10.5 ± 0.40**	**10.8 ± 0.65**	**10.8 ± 0.49**	**9.91 ± 0.66**

Table 2.3: Lipids and fatty acids in fertilized and unfertilized Atlantic cod eggs. Values are μg ind $^{-1}$, mean ± standard error. Fatty acids shown are those which appeared in samples at > 1% total FA content in either group. No significant differences existed between groups.

	Fertilized eggs (n=12)	Unfertilized Eggs (n=12)
Lipids (μg/individual)		
Hydrocarbons	0.13 ± 0.04	0.14 ± 0.05
Steryl Esters/Wax Esters	0.14 ± 0.05	0
Ethyl Ketones	0.06 ± 0.04	0.06 ± 0.03
Methyl Ketones	0.32 ± 0.11	0.57 ± 0.24
Triacylglycerols	0.42 ± 0.13	0.05 ± 0.30
Sterols	0.79 ± 0.14	0.66 ± 0.14
Acetone Mobile Polar Lipids	0.42 ± 0.08	0.29 ± 0.07
Phospholipids	6.69 ± 0.58	5.85 ± 0.61
Total Lipids	9.66 ± 0.90	9.79 ± 1.29
Fatty Acids (μg/individual)		
14:0	0.11 ± 0.01	0.10 ± 0.02
16:0	1.85 ± 0.17	1.98 ± 0.29
16:1ω9?	0.13 ± 0.01	0.13 ± 0.02
16:1ω7	0.18 ± 0.02	0.17 ± 0.02
18:0	0.24 ± 0.02	0.40 ± 0.07
18:1ω9	0.93 ± 0.08	0.91 ± 0.12
18:1ω7	0.31 ± 0.03	0.32 ± 0.04
20:1ω9	0.22 ± 0.02	0.20 ± 0.03
20:5ω3	0.41 ± 0.08	0.47 ± 0.12
22:5ω3	0.05 ± 0.01	0.05 ± 0.01
22:6ω3	0.89 ± 0.18	0.95 ± 0.26
24:1	0.17 ± 0.03	0.13 ± 0.02
∑ SAT	2.25 ± 0.21	2.34 ± 0.34
∑ MUFA	2.08 ± 0.19	1.88 ± 0.26
∑ PUFA	1.66 ± 0.29	1.72 ± 0.42
P:S	0.75 ± 0.10	0.79 ± 0.12
ω3	1.46 ± 0.27	1.51 ± 0.39
ω6	0.13 ± 0.02	0.15 ± 0.03

Table 2.4: Lipids and fatty acids in Atlantic cod egg batches of high (> 70%) and low (< 70%) fertilization success. Values are μg ind $^{-1}$, mean ± standard error. Fatty Acids shown are those which appeared in samples at > 1% total FA content in both groups.

	High fertilization (n=8)	Low Fertilization (n=4)
Lipids (μg individual^{-1})		
Hydrocarbons	0.12 ± 0.05	0.14 ± 0.07
Steryl Esters/Wax Esters	0.20 ± 0.07	0.01 ± 0.01
Ethyl Ketones	0.08 ± 0.06	0
Methyl Ketones	0.45 ± 0.15	0.04 ± 0.04
Triacylglycerols	0.17 ± 0.06^{a}	0.99 ± 0.34^{b}
Sterols	0.65 ± 0.19	1.10 ± 0.12
Acetone Mobile Polar Lipids	0.49 ± 0.10	0.26 ± 0.15
Phospholipids	7.53 ± 0.76^{a}	4.76 ± 0.41^{b}
Total Lipids	11.04 ± 1.18	7.95 ± 1.07
Fatty Acids (μg/individual)		
14:0	0.11 ± 0.01	0.11 ± 0.01
16:0	1.85 ± 0.24	1.84 ± 0.24
16:1ω9?	0.13 ± 0.02	0.12 ± 0.02
16:1ω7	0.18 ± 0.02	0.17 ± 0.02
18:0	0.25 ± 0.03	0.22 ± 0.03
18:1ω9	0.96 ± 0.12	0.87 ± 0.11
18:1ω7	0.32 ± 0.04	0.30 ± 0.04
20:1ω9	0.22 ± 0.03	0.22 ± 0.03
20:5ω3	0.49 ± 0.12	0.27 ± 0.05
22:5ω3	0.06 ± 0.01	0.06 ± 0.01
22:6ω3	1.12 ± 0.27	0.47 ± 0.10
24:1	0.21 ± 0.04^{a}	0.09 ± 0.02^{b}
∑ SAT	2.27 ± 0.28	2.23 ± 0.29
∑ MUFA	2.16 ± 0.26	1.97 ± 0.23
∑ PUFA	2.02 ± 0.41	0.96 ± 0.18
P/S	0.89 ± 0.15	0.48 ± 0.08
ω3	1.80 ± 0.38	0.82 ± 0.17
ω6	0.15 ± 0.03	0.10 ± 0.01

*Treatments in the same row followed by different superscript letters are significantly different (P<0.05)

Table 2.5: Dry weights of Atlantic cod eggs and larvae through development: Fertilization (Day 0) to Yolk-sac absorption (~ 10 dph). Significant differences between high success and low success indicated by superscript letters. All weights are μg individual^{-1}.

	Fertilized Eggs (n=12)	Half-way to hatch (n=12)	Pre-hatch (n=10)	Post-hatch* (n=10)	Half- way yolk-sac absorption* (n=10)	Yolk absorption* (n=10)
HighFert A	9.28	11.2	12.2	7.74	7.73	7.15
HighFert B	11.3	11.0				
HighFert C	9.78	11.9	8.60	7.97	7.94	6.60
HighFert D	11.4	13.0	15.7	7.63	7.35	5.88
HighFert E	9.68	12.7	12.6	7.87	6.37	4.83
HighFert F	9.65	13.3	12.6	8.26	5.70	4.83
HighFert G	12.9	14.2	13.5	7.59	10.1	8.87
HighFert H	12.5	12.9	9.75	9.80	9.47	
LowFert A	8.52	11.1	13.0	8.17	8.00	5.43
LowFert B	11.7	10.6	10.3	7.29	5.34	4.60
LowFert C	9.43	9.83				
LowFert D	10.0	9.50	8.70	8.60	6.43	6.65
HighFert Mean	10.8 ± 1.41	12.5 ± 1.09[a]	12.1 ± 2.35	8.12 ± 0.77	7.81 ± 1.57	6.36 ± 1.54
LowFert Mean	9.91 ± 1.34	10.3 ± 0.73[b]	10.7 ± 2.17	8.02 ± 0.68	6.59 ± 1.34	5.56 ± 1.03
Total Mean	10.5 ± 1.39	11.8 ± 1.46	11.7 ± 2.29	8.09 ± 0.71	7.44 ± 1.54	6.09 ± 1.38

* Indicates larval weights.

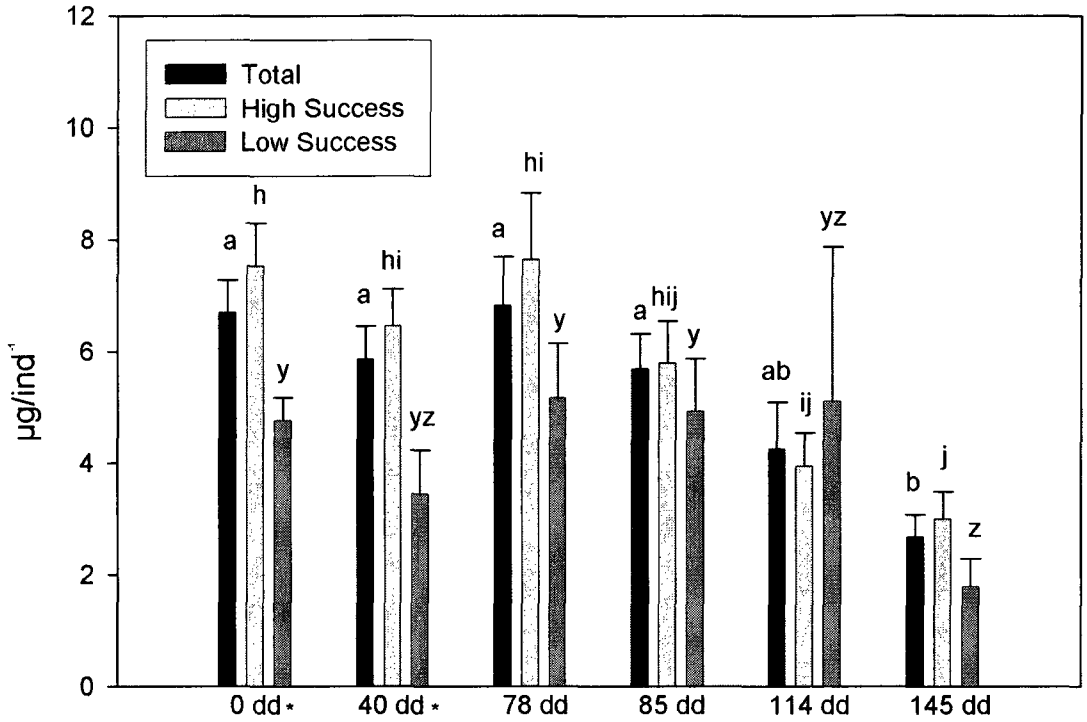


Figure 2.1: Total phospholipids (mean + standard error, as µg individual^{-1}) from egg fertilization to larval yolk-sac absorption for Atlantic cod. Line indicates time of hatch. Bars with different letters are significantly different within groups. (One-way ANOVA, $p<0.05$).

** indicates sampling period where high success and low success groups are significantly different.*

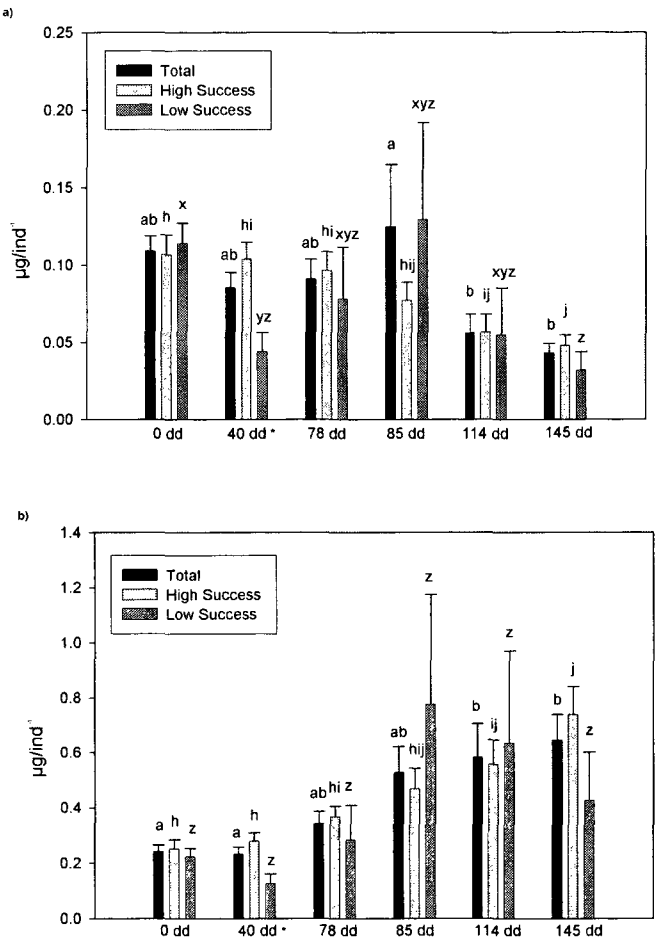


Figure 2.2: Saturates from egg fertilization to larval yolk-sac absorption for Atlantic cod (mean + standard error, as µg individual^{-1}). a) 14:0 b) 18:0. Line indicates time of hatch. Bars with different letters are significantly different within groups. (One-way ANOVA, $p<0.05$).

** indicates sampling period where high success and low success groups are significantly different.*

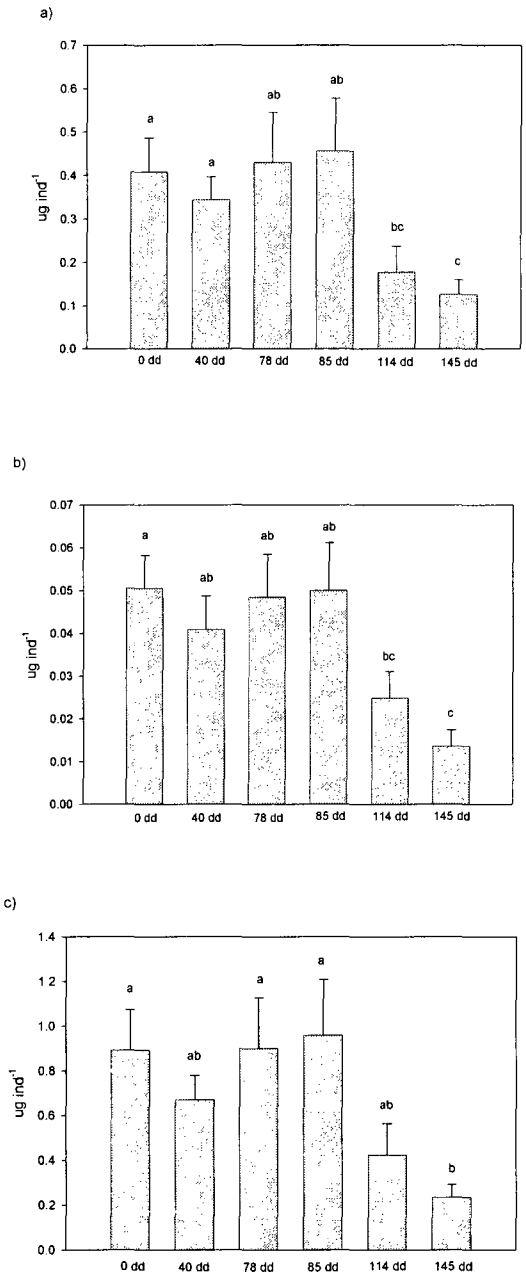


Figure 2.3: Omega-3 polyunsaturates from egg fertilization to larval yolk-sac absorption for Atlantic cod (mean + standard error, as µg individual^{-1}). a) 20:5ω3 b) 22:5ω3 c) 22:6ω3. Line indicates time of hatch. Bars with different letters are significantly different (One-way ANOVA, $p<0.05$).

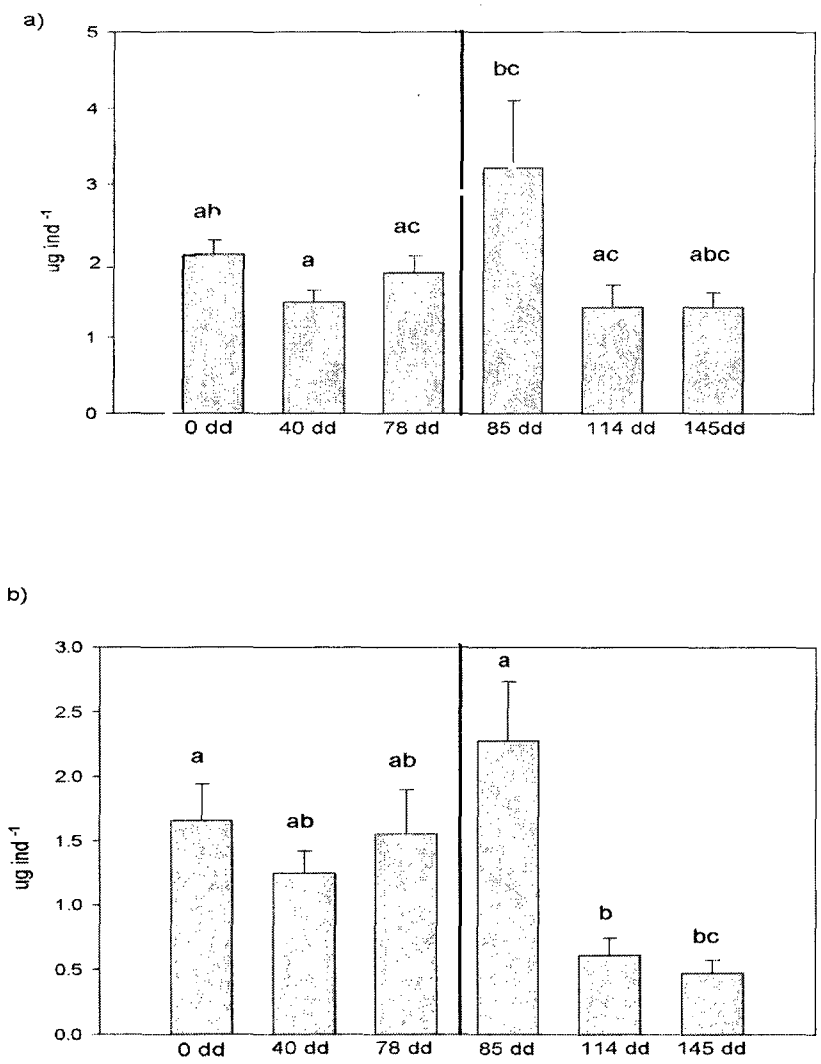


Figure 2.4: Sum patterns from egg fertilization to larval yolk-sac absorption for Atlantic cod (mean + standard error, as µg individual^{-1}). A) ∑ MUFA b) ∑ PUFA. Line indicates time of hatch. Values are for all eggs/larvae from all groups in sample period. Bars with different letters are significantly different (One-way ANOVA, p<0.05).

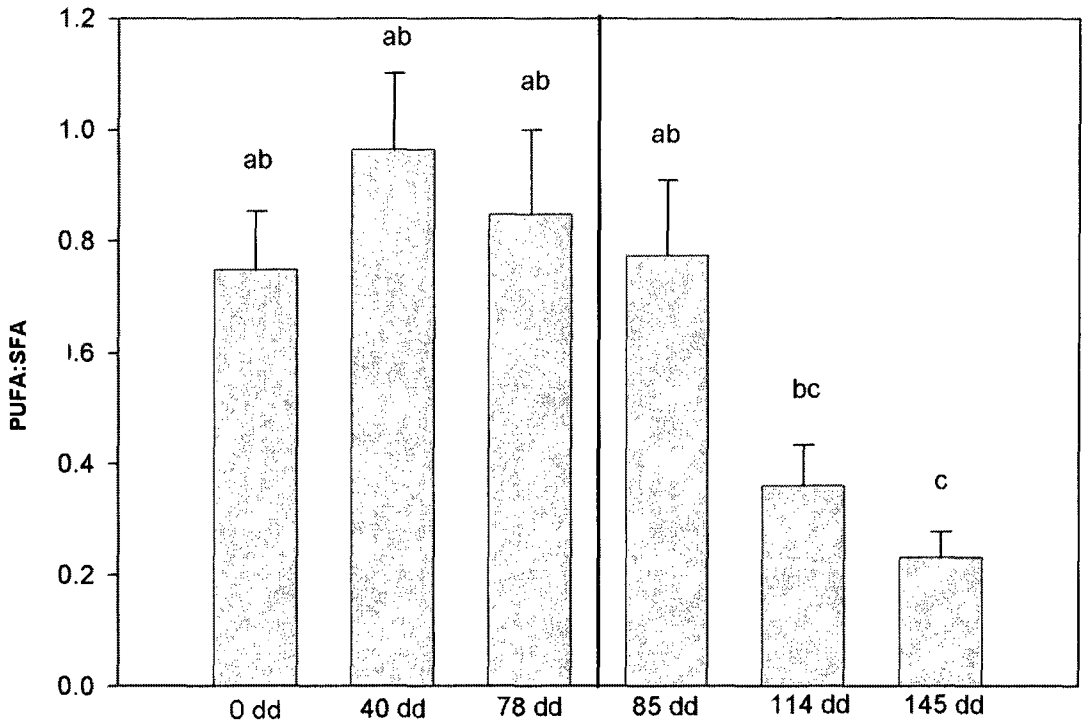


Figure 2.5: Ratio of PUFA to SFA (P:S) over time (mean + standard error). Values are for al lAtlantic cod eggs/larvae from all groups in sample period. Line indicates time of hatch. Bars with different letters are significantly different (One-way ANOVA, p<0.05).

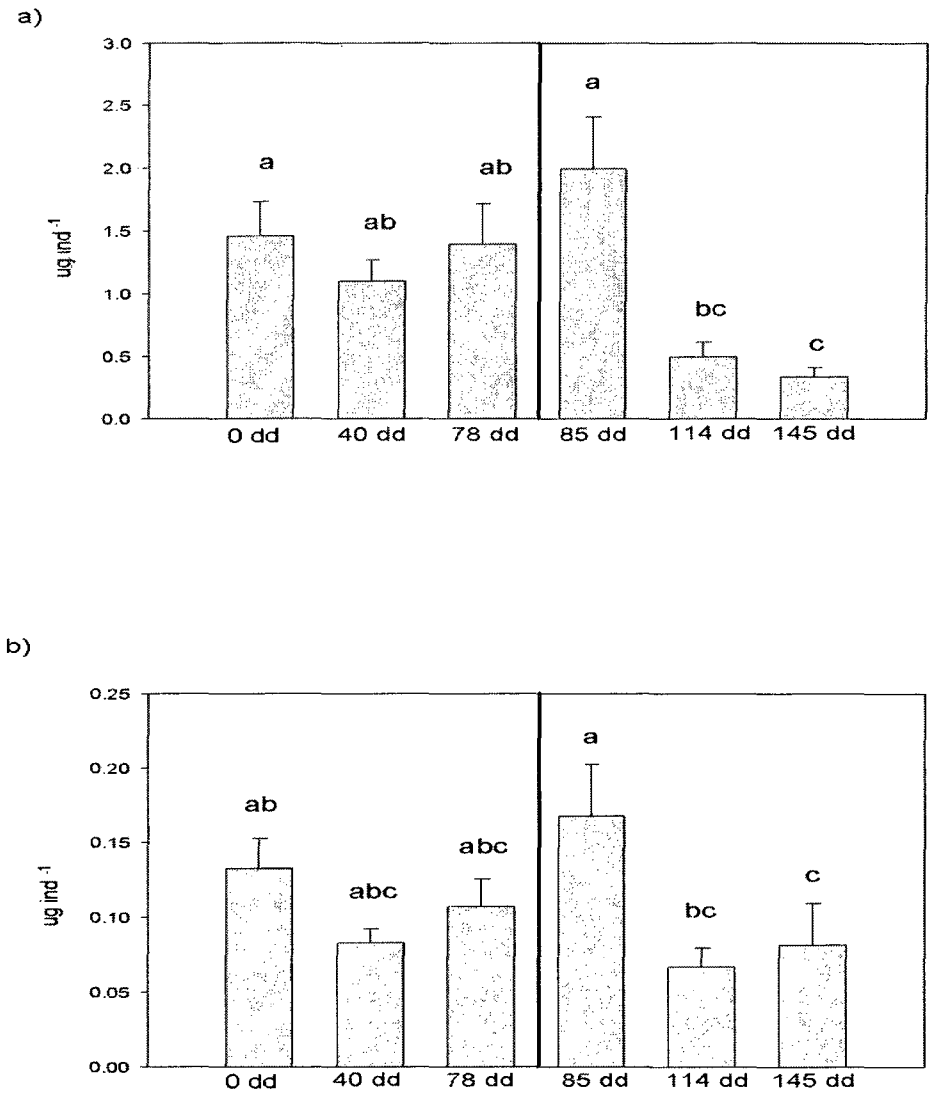


Figure 2.6: Sum of total a) omega-3 and b) omega-6, over time (mean + standard error). Values are for all Atlantic cod eggs/larvae from all groups in sample period. Line indicates time of hatch. Bars with different letters are significantly different (One-way ANOVA, $p<0.05$).

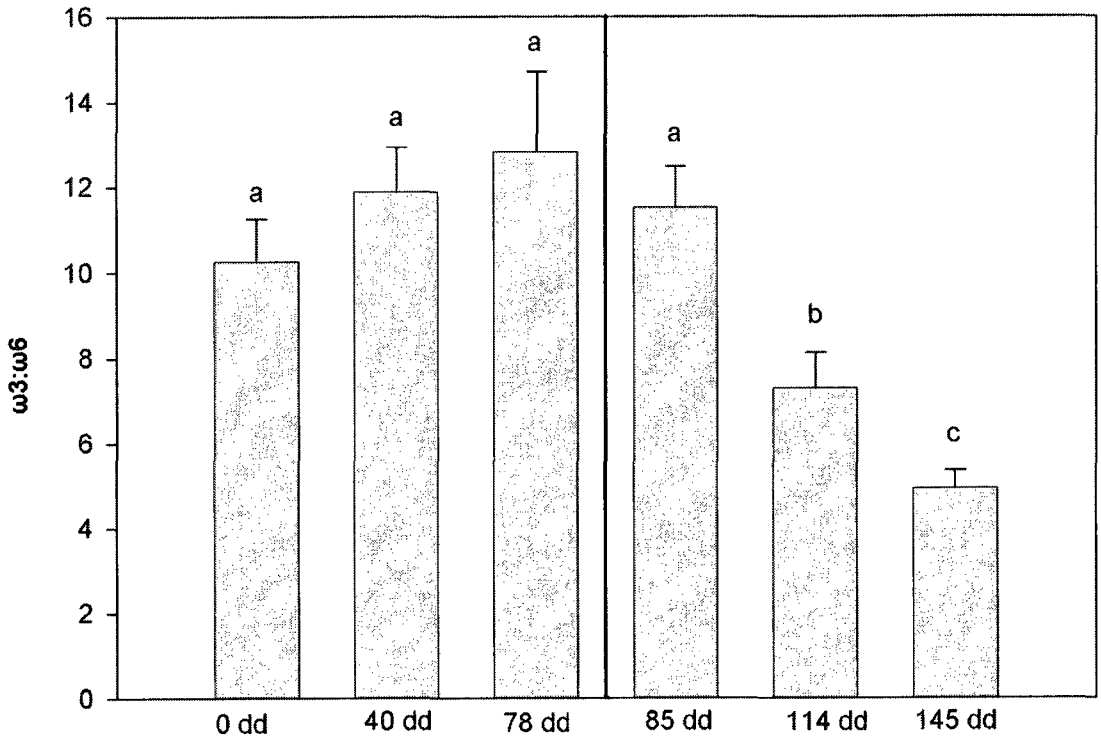


Figure 2.7: Ratio of total ω3 to ω6 over time (mean + standard error). Values are for all Atlantic cod eggs/larvae from all groups in sample period. Line indicates time of hatch. Bars with different letters are significantly different (One-way ANOVA, $p<0.05$).

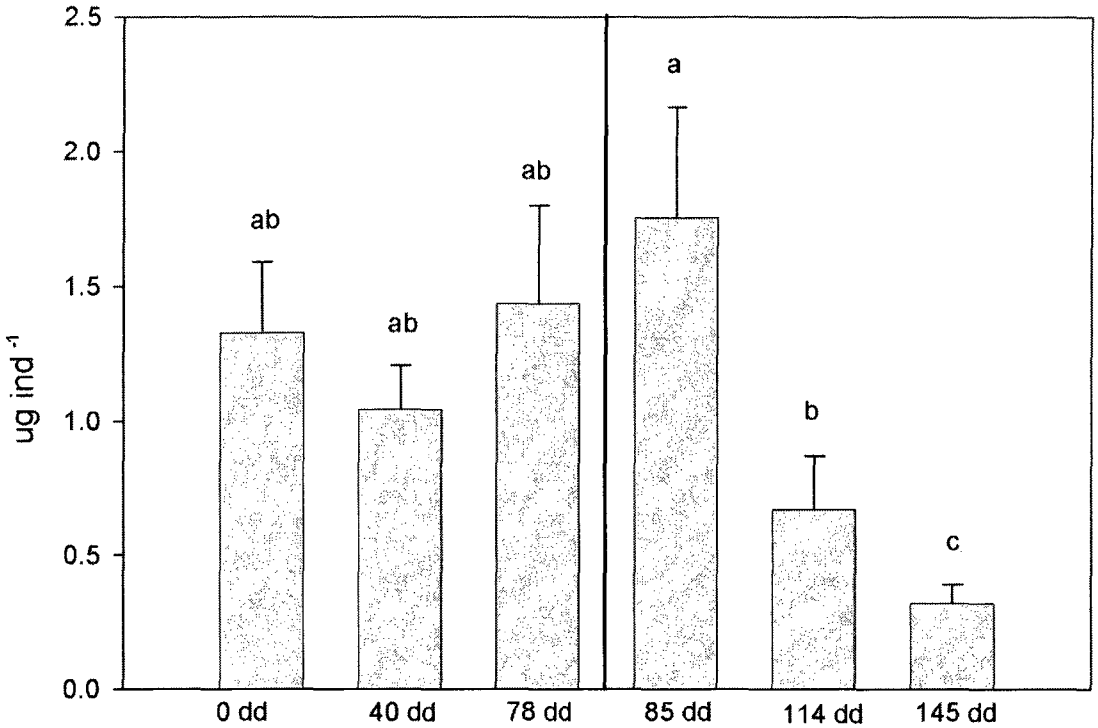


Figure 2.8: Sum of essential FA: DHA + EPA + AA (mean + standard error, ug individual $^{-1}$) across time. Values are for all Atlantic cod eggs/larvae from all groups in sample period. Line indicates time of hatch. Bars with different letters are significantly different (One-way ANOVA, $p<0.05$).

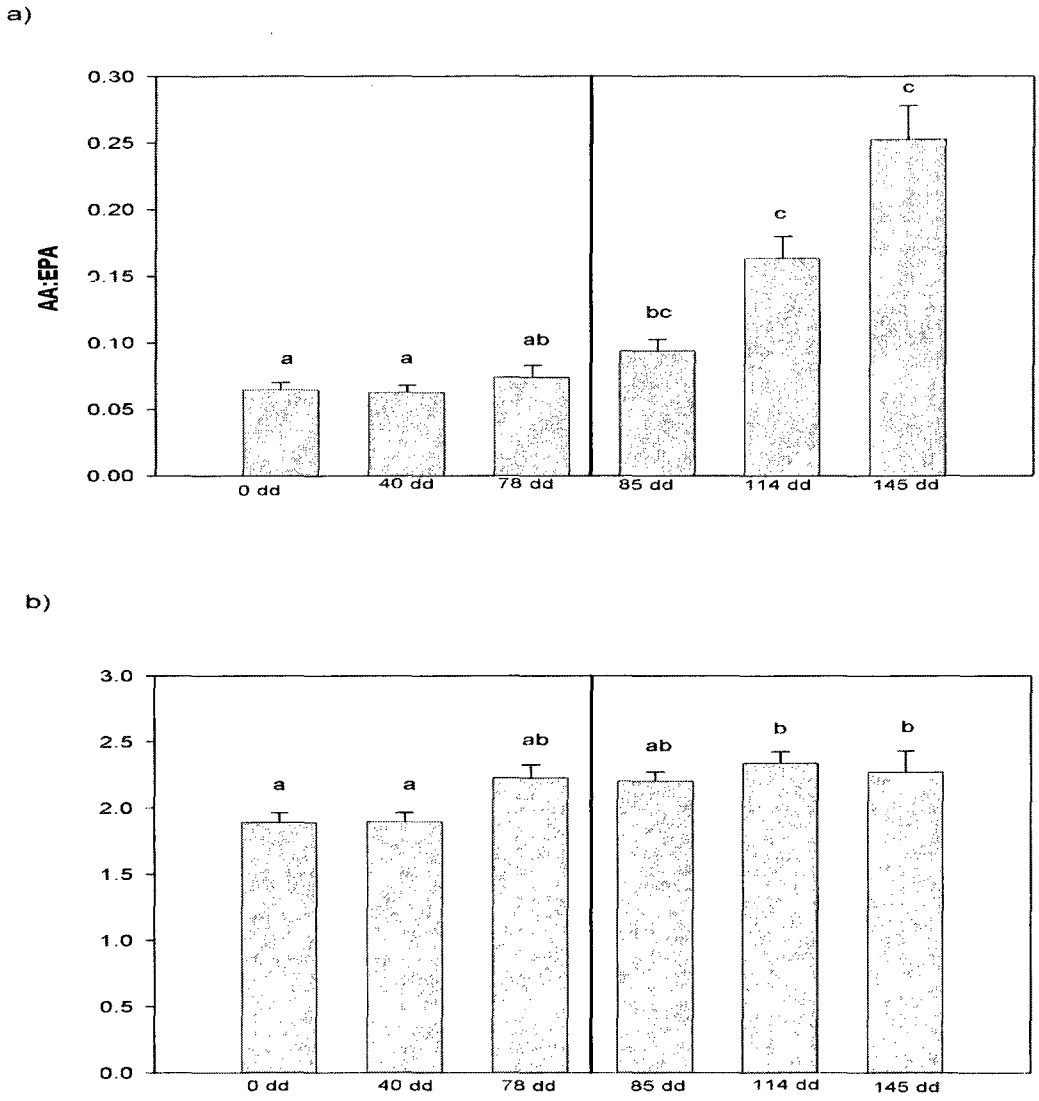


Figure 2.9: Ratios of a) AA:EPA and b) DHA:EPA across time (mean + standard error). Values are for all Atlantic cod eggs/larvae from all groups in sample period. Line indicates time of hatch. Bars with different letters are significantly different (One-way ANOVA, $p<0.05$).

2.4 Discussion:

2.4.1 Lipids and fatty acids in day 0 eggs

Kjorsvik et al. (1990) defines egg quality as the potential of an egg to produce viable larvae. Naturally, in order for this to occur the egg must first successfully fertilize, and then must hatch successfully. Thus, these two aspects are essential for good quality eggs. Some early indicators of egg quality include egg shape, buoyancy, yolk osmolarity, chromosomal aberrations, nutritional condition of broodstock, and occurrence of overripening in the egg. However, early morphological abnormalities can also serve as reliable and quick indicators of egg quality (Kjorsvik 1990, Bromage et al. 1994).

Correlations among blastomere morphology, fertilization success, and hatching success show that blastomere morphology is a useful indicator of egg viability in Atlantic cod. Symmetry and adhesion had particularly strong positive correlations with hatching success and thus are strong candidates for indicators. While this was not found in the research done by Penney et al. (2006) it may be due to the high quality of batches in that study, whereas ranges of viability in the present study are much broader. This may indicate, however, that morphology is no different to using fertilization success itself as the sole parameter. Correlations between individual lipid classes and FA can be found in Appendix I. While most corrlations were relatively weak ($r < 0.8$), 24:1 showed a positive correlation with fertilization success, hatching success, and cell symmetry and adhesions, which means that the fatty acid 24:1 is more important than thought, and should be examined further (see below).

Similar to Penney et al. (2006), positive correlations were also shown between morphologies, showing that morphological abnormalities usually co-occurred. In a study on haddock embryos, Rideout et al. (2004) observed similar results, but it was also found that occurrence of asymmetry in blastomere cells could be used to predict hatching success. A study on Atlantic halibut by Shields et al. (1997) showed that hatch rate correlated positively with symmetry, cell size, adhesions and cell margins. In both of these studies, fertilization rates were generally high – > 95% in Rideout et al. (2004) – but did not correlate with hatching success.

Vallin and Nissling (1998) report that malformations during early blastula (2-32 cells) cannot be considered as a consistent indicator of poor viability because evidence has shown that these abnormalities may be repaired later in development. In that study, some egg batches with irregular morphology actually resulted in higher hatch rates than those without. The recommendation from this study was that a more consistent measurement would be malformations at gastrulation, when egg mortality more often occurs. This being said, a large number of studies on various marine fish species do suggest that there is some validity in using cleavage patterns to estimate early embryo viability (Shields et al. 1997, Rideout et al. 2004, Penney et al. 2006).

Two of the egg batches did not survive to hatch. HighFert B and LowFert C (98% and 46% fertilization success respectively) did well for the first week of incubation, with losses comparable to that of other batches. However, at 40.5 dd for HighFert B and at 76.4 dd for LowFert C, a sudden and unaccountable loss of eggs occurred. Not enough eggs remained for further sampling, and thus survival to hatch was 0. Other batches being held in the same system did not experience these sudden losses. Since HighFert B was

considered the high success group with a 98% fertilization success, and LowFert C was low at 46%, and there were no significant differences between these groups, something aside from lipids and FA contributed to this high loss. Since water temperatures remained within the 5.5-7.5 range for all incubation times, seawater was UV filtered, and oxygen levels were always at maximum, water quality does not appear to be a factor. Possible factors may include biochemical, such as FAA, proteins, carbohydrates, vitamins or minerals; unknown factors related to genetic variability among parental broodfish; problems with blastula development later than the 2-16 cell stage; or some unidentified husbandry problem such as bacterial or viral infection. Morphological development could be a problem, as Vallin and Nissling (1998) stated that abnormalities could occur at the gastrula stage.

The other factor affecting egg and larval viability which was considered in this thesis is lipid biochemistry. While lipid and fatty acid profiles may be reported according to % TL or FA, or as concentrations (% dw or µg/g), these data do not indicate how much lipid is available to an individual (egg or larvae). Therefore, the values are mainly expressed in this thesis as mass per individual. Values expressed as per unit of dry weight can be found in the Appendices.

Overall values of PL in Day 0 eggs, at 70.5% TL, were comparable with those levels found in the study by Penney et al. (2006), and higher than in Finn et al. (1995). However, Finn et al. had higher TL at 14.8 ± 0.50 µg ind^{-1}. TAG in this study was lower than that of Penney et al. (2006), at only 3.67% TL, or 0.42 ± 0.13 µg ind^{-1}. This may be due to differences in the amount of lipid storages in the TAG of the eggs.

While levels of 16:0, 18:0, and 18:1ω9 were comparable with that of Finn et al. (1995), and higher than in Penny et al. (2006), the total DHA was much lower (at 0.89 ± 0.18 μg ind^{-1} / 11.75 ± 1.91% FA) as were the levels of EPA (at 0.41 ± 0.08 μg ind^{-1}/ 5.90 ± 0.65% FA) than both. Although these levels were low, the eggs successfully hatched and likely exceeded a minimum requirement for essential FA. In the PUFA, the high level of ω3 (averaging 88% of PUFA) reflects the important structural role of PL containing these FA in developing embryos (Wiegand 1996).

A comparison of lipid and fatty acid profiles between eggs of high fertilization success and those of low fertilization success showed that there were significant differences, on a per individual basis, in levels of TAG, PL and the MUFA 24:1. While higher levels of PL and 24:1 were found in those batches with high fertilization success, the levels of TAG were actually higher in the low success groups (Table 2.4). The high level of TAG may possibly be attributed to an overripening of the eggs, which in turn contributed to the low fertilization success in the batches (Kjorsvik et al. 1990). Overripening can result in morphological and compositional changes in the egg, and therefore a loss in quality (Evans et al. 1995). PL are essential for energy as well as for membrane formation, and thus the high levels in all batches may increase fertilization potential. The higher presence of 24:1 in high success groups can be attributed to the concurrent high amount of PL. This fatty acid is found in high levels in the sphingomyelin of fish tissue (Hellgren 2001, Sérot et al. 1998). Sphingomyelin is important for the formation of myelin sheaths around nerve fibers. Nervonic acid, 24:1, is found in high amounts in the phosphoglycerides of herring brains, however its exact function has not been thoroughly explored (Mourente and Tocher 1992).

There were no significant differences between groups in the sums of SFA, MUFA or PUFA, nor in the sums of total ω3 and ω6 FA. The lack of differences between other lipid and fatty acid levels in the eggs of high and low fertilization success batches may be due to several factors. First of all, all the eggs sampled were those which were successfully fertilized. It can be assumed therefore that in order for fertilization to be successful these eggs had to contain a requisite amount of certain nutrients to support a developing embryo. Thus those eggs which were sampled, even if coming from low success batches, were the eggs which would be expected to be of the highest quality in that particular group. Also, the broodstock from which these eggs were acquired were all fed the same diet. Therefore it can be assumed that the same nutrients, at the same levels, are available to the fish. It is expected that the eggs produced by these fish would have similar levels of FA and lipids, assuming that nutrition is the only factor affecting egg production. However, it is also possible that lipids and FA alone are not the primary factors. responsible for differences in egg quality. In other words, lipid and FA indicators alone are not adequate to predict future viability among batches of fertilized eggs in Atlantic cod.

In order to see if there are differences in eggs which successfully fertilized and those which did not, samples were taken of unfertilized eggs when fertilized eggs were collected at Day 0. The comparison could then be made between fertilized and unfertilized eggs from the same batches, to see if lipid and fatty acid levels differed. These comparisons were made between the 2 - 16 cell stage of cleavage, when original nutrient levels would not be expected to be greatly affected by embryo development.

Results showed, however, that there were no significant differences between the levels of lipids and FA of these eggs. When ratios of FA were also looked at in comparing high and low fertilization eggs, and fertilized and unfertilized eggs within batches, which included looking at DHA:EPA, AA:EPA, ω3:ω6 FA, P:S, and also the sums of the essential FA (EPA, AA and DHA), no significant difference was found for any of these (See Appendix). The results were similar in a study on eggs of Atlantic halibut by Evans et al. (1995).

Lipid and fatty acid content of eggs may not influence the ability to be fertilized. Instead it may be attributed to other factors, which may include outside influences such as the quality of sperm (Dushkina 1975, Rurangwa et al. 2004). It is possible that these eggs did have potential to be successfully fertilized but for some other reason did not. However, there may also be factors involving the egg itself, including other nutrients such as protein or carbohydrate content. As well, though found in much smaller amounts, biochemicals such as vitamin and mineral levels can play a role in embryo and larval success (Brooks et al. 1997). It may be that lipids and FA cannot be used as the sole indicator, as other variables may contribute to the total energy in eggs and yolk-sac larvae. One of these factors may include free amino acids, which will be looked at further in this thesis.

Because ten of the egg batches were collected from communal spawning tanks, it is possible that eggs collected came from more than one female. This can mean that eggs in one 'batch' could have been a combination of eggs from more than one female. In this case, sample from a single batch may actually be pooled eggs from more than one group of eggs. This makes it difficult to attribute values such as fertilization success with full

confidence as applying to a single spawn from a single female. However, communal spawning tanks are common practice, and economical in relation to space and labour in a commercial setting. As well, variation in morphologies and biochemistry of eggs can occur over time with one single female. In situations where fish from the same stock are kept in the same tank, in identical health and condition, and from the same age group, then these variations are expected to be normal. Hatching success averaged 56%, which is less than averaged from the study by Penney et al. (2006) at 74.7 - 83.1% and the range was broader at 0 - 95.5%. This was also comparable to Vallin and Nissling (1998) who had an average hatch rate of 64% in Baltic cod, and Marteinsdottir and Steinarsson (1998) whose hatching success ranged from 0 -100%. However, two of the LowFert batches were acquired from paired matings, where eggs could be attributed to a known female. Any variations in this data is known to come from within a single batch of eggs.

2.4.2 Changes in lipid profiles from fertilization to yolk-sac absorption

Examining the lipid profile of eggs and larvae throughout development gives insight into which classes and particular FA are catabolized or conserved, and thus indicates which are important as sources of energy or building blocks for membranes and tissues. By the use of the lipid in endogenous larvae it is possible to know which FA need to be available in first-feeding diets. Knowing which FA are essential for developing embryos can contribute to the formulation of broodstock diets which give eggs the best biochemical advantage.

In lipid classes in this study, the only one which showed significant changes on a per individual basis was phospholipids. The total phospholipid levels was found in lower

quantities after hatch (Fig 2.1). This was also found in the barfin flounder in Ohkubo and Matsubara (2001). However, in the white seabream, PL only showed a decline after embryogenesis, and remained constant post-hatch (Cejas 2004). On a per dry weight basis, there was no significant change in levels of PL (See Appendix I).

The saturates 14:0 and 18:0 also changed significantly in this study. The shorter chain 14:0 decreased post hatch, while 18:0 increased post-hatch (Fig 2.2). This shows while 14:0 was catabolized, 18:0 was not. A similar increase in 18:0 was also evident in the Atlantic halibut (Zhu et al. 2003), where 16:0 also increased, and in the common dentex (Mourente et al. 1999) where 18:0 increased as μg/mg total lipid, though decreased as ng/individual as total lipids decreased 1.6 μg per individual per day. However, in the Pacific halibut 18:0 showed a significant decrease during development (Whyte et al. 1993). Wiegand (1996) states that saturates, particularly 18:0, are retained by most marine fish larvae. The monosaturates showed no differences across stages. This conflicts with the common idea that MUFA are preferentially catabolized by developing embryos (Wiegand 1996). Since catabolization patterns may differ between species, it may be that this is not the case at all for cod. This stability during embryogenesis may mean that cod are using another source for energy – perhaps carbohydrates, proteins or free amino acids.

The polyunsaturates 20:5ω3, 22:5ω3 and 22:6ω3 all showed significantly lower quantities at 114 dd. Total PUFA decreased post-hatch, and the essential FA (DHA + EPA +AA) decreased significantly in the same fashion. The sum of ω3 and ω6's followed the same pattern. Because the PUFA decrease, and no significant difference was found for total SFA, the sum ratio of PUFA to SFA, or P:S, decreased significantly post-hatch. However, AA:EPA and DHA:EPA levels were higher post-hatch. While the

polyunsaturates show a decrease, these increasing ratios indicate that DHA are preferentially conserved compared to EPA, and may therefore be more important to the embryo and pre-feeding larvae.

In a normal culture situation, food is introduced to larvae as early as 2 dph. The larvae in this study were starved to 10 dph (145 dd) for utilization of endogenous yolk sac nutrients in the absence of exogenous feeding. The fact that the ω3 FA, essential for development, are being used at this point indicates that it is important that larvae are fed before this depletion can occur. Avoiding loss of essential fatty acids can contribute to more viable larvae.

Oxidative depletion of FA is possible, due to no increase in higher carbon chains and the lack of ability of larvae to elongate or desaturate. The decrease in ω3 and ω6, as well as the C_{20}'s (particularly EPA), may partly be due to oxidation for creating active eicosanoids (Whyte et al. 1993).

Evans et al. (1995) state that energy from saturates are important during fertilization and development, and Rainuzzo et al. (1997) mention the catabolization of DHA during development. However, few changes are seen in this study before 114 dd, when the cod are assumed to be going into starvation. This may be explained by the fact that patterns of usage differ between species and depend on such things as incubation time and temperature. Cod, which undergo a shorter period of incubation at 6°C than do halibut or haddock, may first use carbohydrates as a source of energy (Ohkubo and Matsubara 2001), as well as proteins and amino acids. In this case it's likely that until starvation is

reached, few changes may be seen in lipid profiles until these nutrients are used up. Instead, something else is being preferentially used to fuel maintenance and development.

The use of nutrients in developing eggs and larvae, apart from lipids, has been shown in several studies, and may help explain why lipids and FA appeared to be conserved in this study. In the common dentex (Gimenez et al. 2006), carbohydrates played a role in hatching success and endogenous larvae mortality. Increased amounts of ketose, glucose-6-phosphate, glucose and 6-deoxyhexose were associated with low quality, and it is suggested that these carbohydrates impaired energy catabolism and caused an accumulation of glycolysis metabolites in the larvae. Also associated with low quality was the amount of certain metabolic enzymes such as alkaline phosphatase. Alkaline phosphatase is involved with the catabolism of PL and dephosphorylation of phosvitin in the yolk. High amounts of this enzyme in larvae could cause the yolk to be consumed at a fast rate, resulting in higher levels of mortality in endogenous feeding larvae (Gimenez et al. 2006). These results indicate that it is essential to recognize the role of these other nutrients and enzymes in other species of marine fish.

FAA are also a possibility as a key source of energy for the developing embryo. This has been examined in both turbot (Rønnestad et al. 1992) and in the gilthead sea bream (Rønnestad et al. 1994), and seen in the cod by Fyhn and Serigstad (1987) where significant decreases in the FAA pool were observed during embryogenesis and larval development. The effect of FAA on cod egg and larval viability is the subject of the following chapter.

2.5 Conclusions

The results of this study show that lipid profiling may be useful in predicting future success of eggs. Eggs which came from batches with > 70% fertilization success showed higher levels of PL and 24:1, and lower levels of TAG, than eggs of lower success. Therefore the measurement of the levels of PL and TAGs in the lipid profile, along with 24:1, may reflect the potential of particular egg batches. High levels of PL provide energy and building blocks for membranes, while 24:1 can be important for neural development and myelation.

With the exception of the lipids and FA mentioned above, the variability of all other lipid and fatty acid levels, as well as lack of significant differences among batches of different fertilization and hatching success, may mean that lipid profiling may not be a generally reliable tool for predicting the future success of eggs and larvae in a commercial setting (Wiegand 1996). Lipid profiling requires access to laboratory equipment and expertise not routinely available in commercial hatcheries and, since it does not appear to significantly improve the ability to predict future egg viability, cannot be justified at this time. A good commercial broodstock diet likely assures that lipid biochemistry is not a factor by which egg quality will be affected in a commercial setting, though diet is indeed a variable of greater influence in wild stocks. It is also important to remember that other factors contribute to differences in egg quality – among them biochemical factors such as carbohydrates, enzymes, protein and amino acids, environmental factors such as temperature and water quality, and other factors such as stock differences and husbandry practices. Blastomere morphology is a much quicker method by which egg quality can be determined on site, and, while not 100% reliable, the strong correlation between

symmetry and hatching success shows that it is currently the most useful indicator of viability to hatch.

The conservation of lipids in the early stages of development shows that lipids are not the chief energy source for cod embryos. Instead, energy may be derived from carbohydrates, proteins and free amino acids. Because most lipids were not catabolized until halfway to yolk-sac absorption (114 dd), it is important that cod larvae be provided with exogenous feed before this stage to prevent starvation and allow conservation of essential FA such as DHA and EPA.

Further research needs to be done on the biochemical composition of cod eggs and the effect on egg quality and larval viability. We predict that an examination of amino acid profiles, alone or in combination with lipid profiling, will give further insight into egg quality and total energy. This combination of factors may be necessary as a composite indicator of the available energy for developing embryos. It is possible that factors outside of biochemistry, things such as husbandry practices among tanks or batches, are quite large compared to the variability in egg energy levels that potential differences among batches is obscured. In this case, egg hatching success will be defined more by incubation practices than by initial egg quality at fertilization.

3 Free amino acids in eggs and yolk-sac larvae of Atlantic cod

3.1 Introduction

This experiment investigated the levels of free amino acids (FAA) in eggs and larvae of Atlantic cod, *Gadus morhua*. FAA have shown an important role in energetics of marine fish eggs and larvae. While some FAA are incorporated into protein of the developing embryo (Rønnestad et al. 1992b, Rønnestad et al. 1993, Sivaloganathan et al. 1998), the decline in the FAA pool cannot wholly contribute to protein synthesis. Instead, the breaking down of FAA are used as an energy source either along with other nutrients, or as a separate resource. As well, some FAA are depleted at a significant rate during development, while others seem to be conserved (Fyhn and Serigstad 1987, Rønnestad et al. 1992a, Rønnestad et al. 1993, Finn and Fyhn 1995, Fyhn and Govani 1995, Rønnestad et al. 1994, Sivaloganathan et al. 1998).

The first part of the experiment examined newly spawned egg batches to determine if there is a relationship between levels of FAA and batch viability, and to find evidence of thresholds for particular FAA. Knowing whether FAA influence viability may then allow the selection of eggs with maximum potential, and thus maximize production in commercial settings.

The second part of this experiment examined the trends of FAA quantity from fertilization through to yolk-sac absorption (0 – 145 dd) in order to observe which are used – either as an energy substrate or for building of protein – and which are conserved. This can in turn create an understanding as to which FAA are important for energy and

development, and thus help develop broodstock and first-feeding diets that give success to eggs and larvae.

This experiment also examined the use of the EZ:faast^{TM} GC-FID Free (Physiological) Amino Acid Analysis Kit as a method for measuring FAA in fish eggs and larvae. FAA have been typically measured by extraction with 6% tri-chloroacetic acid (TCA) and analyses run on an automatic amino acid analyser such as the Chromaspek J180, Hilger Analytical (Fyhn and Serigstad 1987, Rønnestad et al. 1992a, Rønnestad et al. 1992b, Rønnestad et al. 1993, Rønnestad et al. 1994, Fyhn and Govoni 1995, Finn et al. 1995). The Amino Acid Analysis Kit used here was designed mainly for the analysis of blood or urine samples, and its application in analyzing fish samples, particularly eggs and larvae, is not known. While the kit does not measure taurine or arginine, it does measure a number of amino acids and their compounds which have not been looked at in previous studies.

3.2 Materials and methods

3.2.1 Sampling methods

Ten separate egg batches from the experiment in the previous chaper were used to analyse FAA content. Eggs were sampled for FAA analysis at the same time as lipid and FA samples were collected, with six sampling periods from 0 dd to 145 dd (see Materials and Methods section in Chapter 2 for description of rearing and sampling protocols). The batches were separated into two sampling groups – high fertilization success (> 70% of eggs fertilized) in six tanks and low fertilization success (< 70%) in another four tanks. Each group was sampled in triplicate. Triplicate samples (50 eggs/sample, 100 larvae/sample) were taken from each tank for each sampling stage. Egg samples were rinsed with filtered seawater, gently blotted dry, and then placed in 7 mL vials. Larvae were collected on glass-fibre filters, rinsed with filtered sea water, and placed in 7 mL vials. The vials were then filled with N_2(g), sealed with Teflon tape, and stored at -80°C until FAA analysis took place.

3.2.2 Free amino acid analysis

Egg and larval samples were ground up in 3 and 2 ml distilled water, respectively. Free amino acids were extracted and derivatized using an EZ:faast^{TM} GC-FID Free (Physiological) Amino Acid Analysis Kit. A total of 100 µl of sample was mixed with 100 µl of an internal standard, norvaline (0.2 mM) and n-propanol, and passed through a sorbent tip. It was then washed with 200 µl of n-propanol, and the sorbent material was then ejected in an eluting medium consisting of 3:2 sodium hydroxide/N-propanol. Fifty µl chloroform and 100 µl iso-octane were added to the solution to form the organic layer

containing the FAA. Finally, 1 N hydrochloric acid was added to lower the pH of the solution and complete the derivatization.

Samples were subsequently run on a Varian 3800 GC-FID to obtain peaks of FAA present, with the exception of taurine and arginine. Peak areas were quantified in comparison with a known quantity of internal standard to give quantitative values of FAA.

3.2.3 Statistical methods

Samples were sorted according to fertilized *vs* unfertilized eggs and fertilization success (% total eggs hatched) to determine statistical relationships between free amino acid profiles and the egg viability. Both essential and non-essential FAA were examined. All statistical tests were carried out using SigmaStat Version 13. Comparisons between fertilized eggs and unfertilized eggs were made using a paired t-test, and between groups of high fertilization success (>70%) and low fertilization success (<70%) comparisons were made using a Tukey's t-test. For samples which did not pass normality or equal variance tests, and did not pass after transformation, the median was tested using a Mann-Whitney Rank Sum test.

To examine changes through development, one-way ANOVAs were performed to look for changes in levels of FAA over time. A Dunn's test was used to perform pair-wise comparisons where significant differences were found. For those samples which did not pass normality or equal variance tests, and did not pass after transformation, a Kruskal-Wallis one-way ANOVA on ranks was performed.

3.3 Results

Eggs from batches with high fertilization success and those from batches with low fertilization success showed significant differences in quantities of sarcosine, thioproline, hydroxyproline, ornithine and hydroxylysine. However, when comparing successfully fertilized eggs to those which did not fertilize, significant differences were found between the majority of the FAA analysed, including total FAA (Table 3.1).

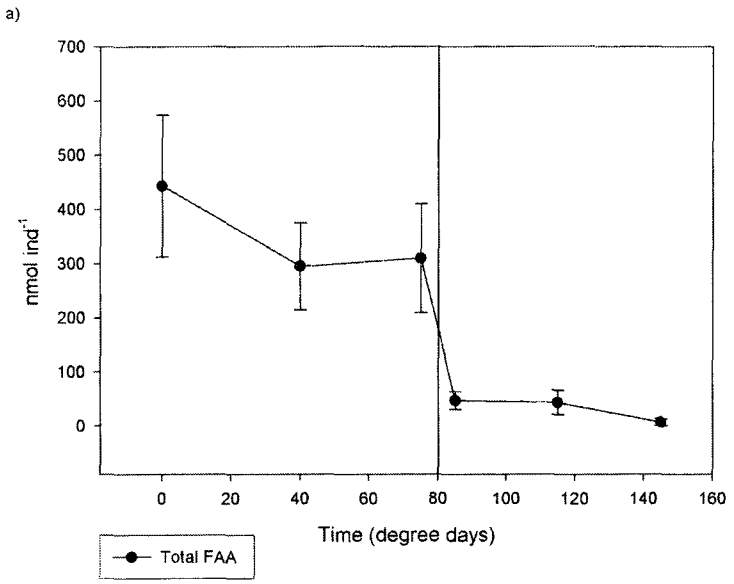
Appendix II includes a table of correlations that exist between FAA quantities and fertilization success, as well as some cell morphologies. Six FAA showed a positive correlation with fertilization success ($p = 0.008 - 0.031$), while others correlated negatively with cell symmetry, cell adhesions and cell clarity ($p = 0.008 - 0.050$). Cysteine was the only FAA to show a positive correlation, with cell margins ($r = 0.520$, $p = 0.008$). No FAA showed any significant correlation with hatching success.

No significant differences existed at any stage, the data are represented as pooled values of both high and low success groups. Total FAA drops significantly from 0 dd to 40 dd, and shows significantly lower value at hatch (Fig. 3.1). Alanine, glycine, valine, isoleucine, asparagine, threonine, serine, α-aminodipic acid, and methionine all experience significant differences in quantity during embryogenesis, while leucine, proline, histidine, allo-isoleucine and phenylalanine only show significant difference after hatch (Fig. 3.2 and 3.3). The remaining FAA show no significant changes throughout development. Lysine is the only essential FAA which showed no change over time.

Table 3.1: Quantities of FAA at fertilization (Day 0): A comparison of groups of high and low fertilization success and of fertilized and unfertilized Atlantic cod eggs. Values are nmol ind^{-1}, mean and standard deviation.

Free Amino Acids	High Success Eggs (n=6)	Low Success Eggs (n=4)	Fertilized Eggs (n=10)	Unfertilized eggs (n=9)
Alanine, ALA	22.8 ± 2.10	25.0 ± 3.36	23.7 ± 1.93 [a]	11.4 ± 2.28 [b]
Sarcosine, SAR	0.47 ± 0.13 [a]	0.25 ± 0.03 [b]	0.46 ± 0.12	0.37 ± 0.64
Glycine, GLY	7.05 ± 0.58	6.57 ± 0.83	9.51 ± 2.75 [a]	4.90 ± 1.30 [b]
amino-n-butyric acid, ABA	0.71 ± 0.12	0.99 ± 0.15	0.82 ± 0.10 [a]	0.50 ± 0.64 [b]
Valine, VAL	14.1 ± 1.55	13.8 ± 1.94	13.8 ± 1.19 [a]	7.70 ± 1.13 [b]
Aminoisobutyric acid, BAIB	0.46 ± 0.10	0.51 ± 0.06	0.47 ± 0.12 [a]	0.27 ± 0.05 [b]
Leucine, LEU	10.8 ± 0.70	11.5 ± 1.66	11.1 ± 1.65 [a]	5.65 ± 1.02 [b]
Isoleucine, ILE	12.1 ± 1.14	11.8 ± 1.66	11.8 ± 0.98 [a]	5.78 ± 1.10 [b]
Allo-isoleucine, AILE	1.43 ± 0.67	0.05 ± 0.05	2.40 ± 1.72	0. 67 ± 0.33
Asparagine, ASN	14.4 ± 2.13	17.5 ± 3.00	24.7 ± 8.85 [a]	6.94 ± 1.60 [b]
Threonine, THR	12.1 ± 2.87	20.7 ± 3.54	15.9 ± 2.42 [a]	7.17 ± 2.12 [b]
Serine, SER	47.3 ± 12.8	40.3 ± 17.5	68.5 ± 11.3 [a]	30.4 ± 10.0 [b]
Proline, PRO	4.02 ± 0.38	4.53 ± 0.67	4.25 ± 0.37 [a]	2.02 ± 0.44 [b]
Thioproline, TPR	1.30 ± 0.48 [a]	0.16 ± 0.04 [b]	1.96 ± 1.27	1.16 ± 0.52
Aspartic acid, ASP	1.23 ± 0.20	1.32 ± 0.16	1.22 ± 0.13 [a]	0.75 ± 0.13 [b]
Methionine, MET	2.46 ± 0.42	3.60 ± 0.49	2.93 ± 0.35 [a]	1.49 ± 0.35 [b]
Hydroxyproline, HYP	19.6 ± 6.12 [a]	4.98 ± 0.49 [b]	12.2 ± 3.40	14.8 ± 6.01
Glutamic acid, GLU	1.50 ± 0.47	3.35 ± 0.79	2.36 ± 0.49 [a]	1.15 ± 0.41 [b]
Phenylalanine, PHE	1.99 ± 0.28	2.79 ± 0.39	2.37 ± 0.24 [a]	1.22 ± 0.29 [b]
α-aminodipic acid, AAA	17.1 ± 1.86	20.4 ± 2.30	47.65 ± 28.9 [a]	16.4 ± 1.71 [b]
α-aminopimelic acid, APA	1.27 ± 0.38	2.26 ± 0.47	1.71 ± 0.31 [a]	0.90 ± 0.24 [b]
Glutimine, GLN	0.45 ± 0.18	1.10 ± 0.30	0.76 ± 0.18	1.04 ± 0.39
Ornithine, ORN	8.25 ± 2.54 [a]	3.61 ± 0.65 [b]	10.0 ± 4.37	6.10 ± 2.02
Glycine-proline, GPR	0.33 ± 0.33	1.27 ± 1.16	0.79 ± 0.59	1.24 ± 0.77
Lysine, LYS	0.31 ± 0.11	0.53 ± 0.13	0.41 ± 0.09	0.19 ± 0.07
Histidine, HIS	0.20 ± 0.09	0.40 ± 0.10	0.30 ± 0.07	0.15 ± 0.06
Hydroxylysine, HLY	18.4 ± 6.90 [a]	3.81 ± 1.12 [b]	20.3 ± 10.2	15.0 ± 5.55
Tyrosine, TYR	12.1 ± 2.86	17.7 ± 4.16	17.7 ± 3.64 [a]	6.34 ± 2.01 [b]
Proline-hydroxy proline, PHP	82.5 ± 30.8	18.1 ± 5.81	98.5 ± 52.0	60.0 ± 24.4
Tryptophan, TRP	2.41 ± 0.72	4.44 ± 0.83	3.33 ± 0.58 [a]	1.82 ± 0.50 [b]
Cystathionine, CTH	14.0 ± 5.32	3.49 ± 0.78	15.5 ± 7.49	11.4 ± 4.11
Cystine, C-C	1.92 ± 0.43	1.85 ± 0.32	1.80 ± 0.26	1.60 ± 0.35
TOTAL	335 ± 53.1	299 ± 40.0	443 ± 131 [a]	227 ± 55.5 [b]

*Treatments in the same row followed by different superscript letters are significantly different ($P<0.05$)



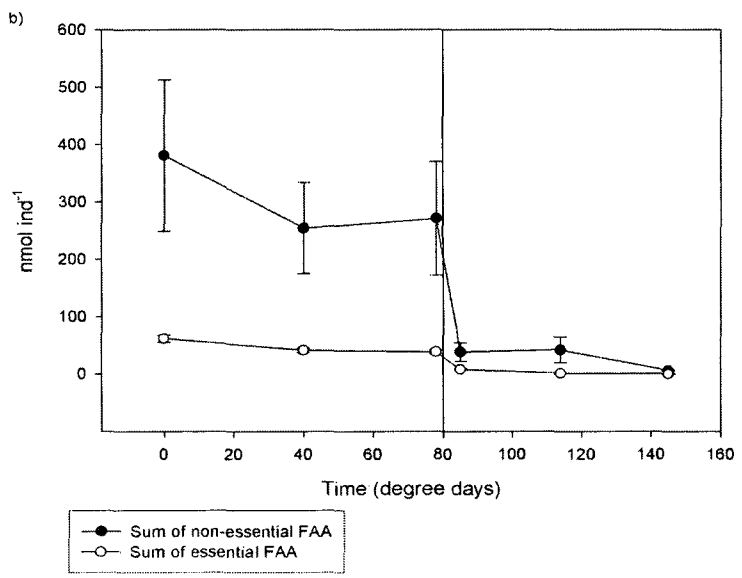


Figure 3.1: Total FAA (a) and sum of essential and non-essential FAA (b) from egg fertilization (0 dd) to larval yolk-sac absorption (145 dd). Values are mean ± standard error. Line indicates time of hatch. Values are for all Atlantic eggs/larvae from all groups in sample period.

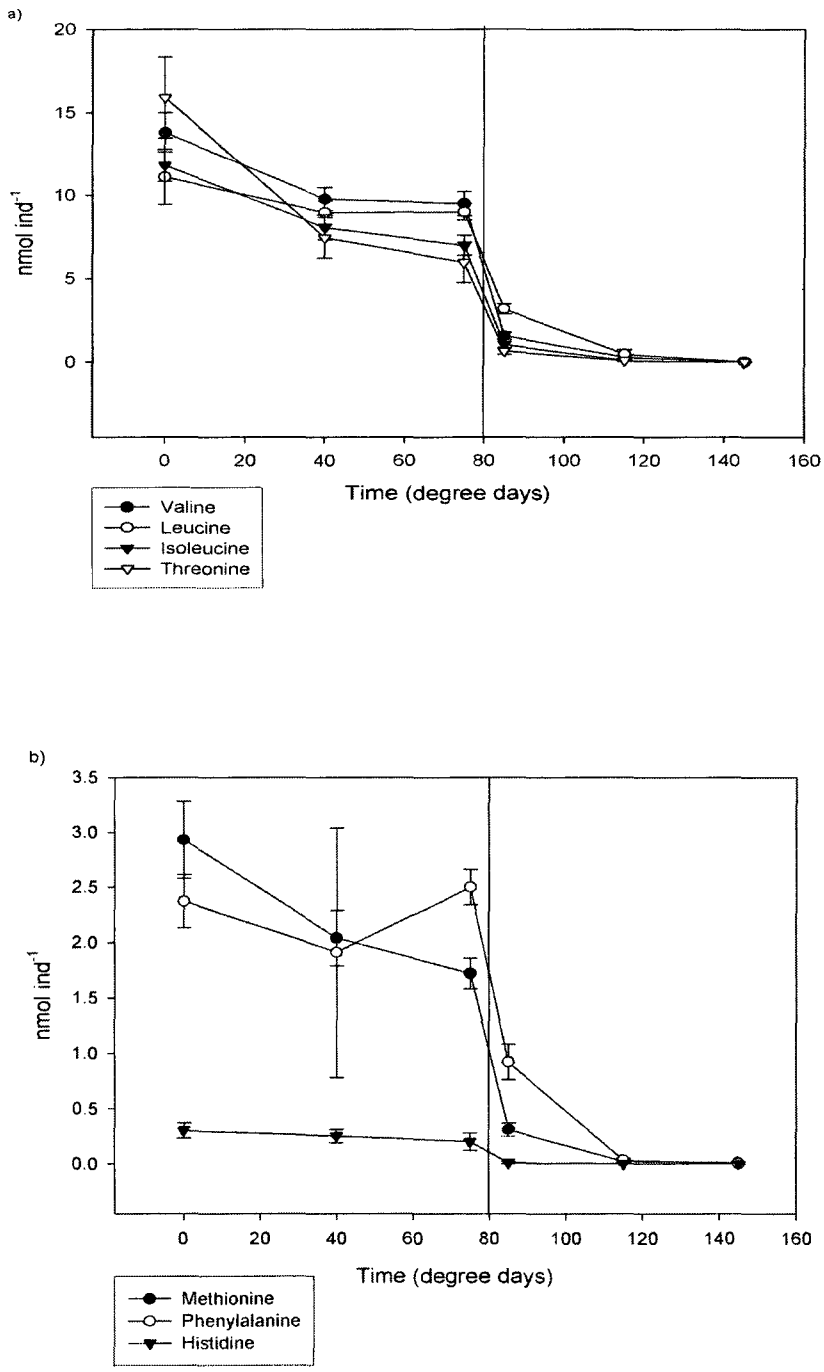


Figure 3.2: Essential FAA from egg fertilization (0 dd) to larval yolk-sac absorption (145 dd). The line indicates time of hatch. Values are mean ± standard error. Values are for all Atlantic cod eggs/larvae from all groups in sample period.

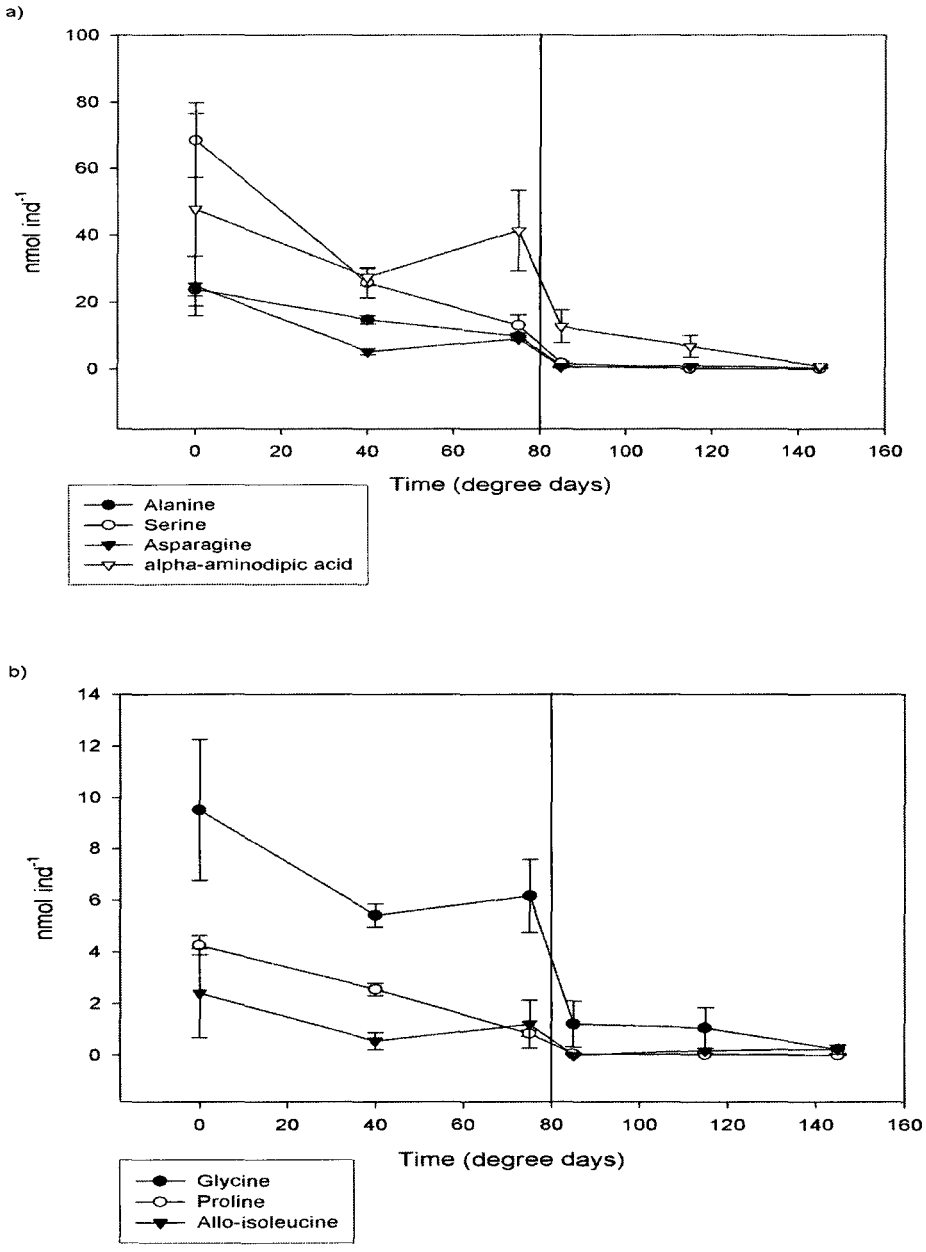


Figure 3.3: Non-essential FAA from egg fertilization (0 dd) to larval yolk-sac absorption (145 dd). The line indicates time of hatch. Values are mean ± standard error. Values are for all Atlantic cod eggs/larvae from all groups in sample period.

3.4 Discussion

3.4.1 Free amino acids in day 0 eggs

The EZ:faast^{TM} GC-FID Free (Physiological) Amino Acid Analysis Kit was successful in measuring levels of FAA in cod eggs and larvae. Total FAA quantities were comparable to those of other studies on marine fish eggs and larvae, where the TCA method was used (Fyhn and Serigstad 1987, Rønnestad et al. 1992a, Rønnestad et al. 1992b, Rønnestad Rønnestad et al. 1994, Fyhn and Govoni 1995). The kit is easy to use, and the quick preparation of samples makes it a useful tool for performing analysis in a commercial setting. While the kit could not measure taurine and arginine levels, it did provide data for a range of FAA broader than that provided by the TCA method.

Free amino acids have been shown to be used by cod, as well as other marine fish species, during embryogenesis as a source of energy and as building blocks for important protein growth (Fyhn and Serigstad 1987, Rønnestad et al. 1992a, Rønnestad et al. 1993, Finn and Fyhn 1995). Without these FAA available, it can be assumed that success of eggs and newly hatch larvae will be limited. The results of FAA analysis on Day 0 eggs show that some FAA may indeed be related to an egg's ability to be successfully fertilized. However, it is not certain that the difference in FAA is a cause for differences in fertilization, or a result of fertilization itself. A simple experiment to collect batches of eggs before exposure to sperm for FAA analysis preceding fertilization, and then to follow the batches through fertilization, would indicate whether FAA levels differ prior to fertilization, and can then be linked to resulting fertilization success.

The average total FAA, at 443 ± 131 nmol ind^{-1}, is more than twice the totals reported by Fyhn and Serigstad (1987) for Day 0 cod eggs. However, the array of FAA included in this study is larger, and while missing taurine and arginine, it includes eighteen FAA not reported in that study. These contribute to an increased total. When looking at only those which were studied by Fyhn and Serigstad, with the exclusion of taurine and arginine, the total is reduced by almost half and thus close to their value of 200 nmol ind^{-1}. This shows that there are a broad array of FAA which may play a role in embryogenesis and larval development, beyond those looked at in earlier papers, including papers examining other marine fish species (Rønnestad et al 1992a, Rønnestad et al. 1992b, Rønnestad et al. 1993, Finn and Fyhn 1995, Silvanoganathan et al. 1998, Halfyard and Parrish 2002).

The quantities of FAA in eggs which successfully fertilized, compared to those which did not, show significant differences in most FAA, including total FAA. Significant differences existed between quantities of five individual FAA in high success *vs* low success groups, and not in total FAA. While the eggs in this case were all those from each group that were successfully fertilized, it may be that all FAA need to meet a particular threshold to allow fertilization to occur. In the case of those FAA which did not show a significant difference, the threshold would have to be below the lowest level in the two groups and therefore did not influence the success of fertilization.

The correlations between FAA and fertilization success show a positive relationship. Only five of the FAA showed significant differences in the two groups, between egg batches of high and low fertilization success. These were sarcosine, thioproline, hydroxyproline, ornithine, and hydroxylysine. On average, the levels of these five in the low success group were less than half those of the high success group. There is little

information available about the exact roles of these amino acids in fish, however it is known that hydroxylated proline and lysine are important in the formation of collagen (Eastoe 1957), which is important in early life stages for the development of the skeletal system and fish skin. No significant correlations exist between FAA and hatching success, indicating that FAA do not influence hatching potential.

3.4.2 Changes in quantities of free amino acids from fertilization to yolk-sac absorption

While the majority of FAA measured showed a significant decrease in levels throughout development, there were eighteen which appeared to be conserved. Of all of these, lysine was the only essential FAA which was not metabolized. Lysine was already at a very low level at the time of fertilization, and this may be why it was conserved. Use of this amino acid for energy or protein synthesis would have resulted in a total depletion very quickly.

Total FAA showed a large decrease at the time of hatch, along with most individual FAA. While some FAA could be lost with the chorion upon hatch, it is more likely that the decrease is a result of the energy cost of the hatching process. At the time of yolk-sac absorption (10 dph, or 145 dd), the total FAA had decreased to 6.40 nmol ind^{-1}. These results indicate that FAA is an important energy substrate during the early development of cod, a finding that agrees with previous work on cod (Fyhn and Serigstad 1987, Finn and Fyhn 1995). When comparing FAA depletion to the relative stability of most FA during embryogenesis, it appears that FAA are a primary source of nutrients for both energy and tissue growth while lipids are conserved until post-hatch. This indicates that lipids and FAA together may be the important indicators to measure to define total

energy availability for the developing embryo. This has been found with many marine fish species, including Atlantic halibut (*Hippoglossus hippoglossus*), lemon sole (*Microstomus kitt*), turbot (*Scophthalmus maximus*), Atlantic spot (*Leiostomus xanthurus*) and menhaden (*Brevoortia tyrannus)* (Fyhn and Serigstad 1987, Rønnestad et al 1992a, Rønnestad et al 1992b, Rønnestad et al 1994, Finn and Fyhn 1995, Fyhn and Govoni 1995). Work in these species measuring oxygen uptake and ammonia excretion has shown that FAA are being depleted primarily as a source of energy during these early stages (Rønnestad et al 1992a, Rønnestad et al 1992b, Rønnestad et al 1994).

Previous research has found that some species of marine fish, including cod, depend on FAA as the primary source of energy during embryogenesis at approximately 75% (Fyhn and Serigstad 1987), while only about 23% (Finn et al. 1995) of lipids and FA are catabolized during this time and the majority only catabolize from hatch and onward (Finn and Fyhn 1995). While this is evident in fish whose yolk has no oil globule, those with oil globules, such as Atlantic spot, depend primarily on lipids and FA for fuel (Fyhn and Govoni 1995).

As examined in Chapter 2, a significant increase was seen at hatch of the monosaturate 18:0. Research by Zhu et al. (2003) on Atlantic halibut found that depleted FAA may be used as a precursor for lipid synthesis. Once deaminated, the carbon skeleton may then be used as a carbon source for lipid formation. Under hypoxia, fish embryos may experience the stimulation of fatty acid chain elongation. This can occur during the hatching process of pelagic embryos (Finn et al. 1991).

As well, there were no evident differences in the depletion of essential FAA over non-essentials, so they are not preferentially conserved. Since essential amino acids can only be provided through the diet, this means that the requirement of essential FAA by first feeding larvae should be emphasized in formulation of first feeding diets and enrichments. By getting high levels of these FAA into larvae, it is possible that the potential for subsequent survival and growth will be greatly improved through providing a high source of energy as well as building blocks for body protein.

3.5 Conclusions

The EZ:faast^{TM} GC-FID Free (Physiological) Amino Acid Analysis Kit was effective in measuring FAA. It is a quick method, with a preparation of approximately ten minutes per sample, and is very easy to use. This makes it a valuable tool for performing analysis in a commercial setting.

Results show that FAA play an important role in the nutrition of developing cod embryos and endogenous-feeding larvae, and FAA profiling may be useful for examining viability of early life cod. The majority of FAA significantly differed between fertilized and unfertilized eggs within batches. It is not certain whether this difference exists before fertilization occurs, and is a precursor to fertilization, or if an increase in levels of FAA occurs as a consequence of fertilization itself. In this case, since lipids did not show any difference, the FAA would have to be derived from the breaking down of proteins. This protein breakdown in the egg may occur at fertilization in order to provide energy for the developing embryo.

Differences between FAA in high and low fertilization success groups were found in sarcosine, thioproline, hydroxylysine, ornithine and hydroxylysine. It is recommended that this be tested with further study into the specific roles of these in embryo development and metabolism. Because of the differences that exist in Day 0 eggs, these particular FAA may be useful for indicating egg and larval success and should be examined further. Appendix II includes a table of correlations that exist between FAA quantities and fertilization success, as well as some cell morphologies. A number of FAA showed a positive correlation with fertilization success, while others correlated negatively with cell symmetry, cell adhesions, and cell clarity. Cysteine was the only FAA to show a positive correlation with cell margins (see Appendix II, Table 3). These correlations indicate that FAA have a significant relationship with embryo development. There were no significant correlations with hatching success, which indicates that levels of FAA do not influence hatching potential.

The catabolism of FAA in early development indicates that, while lipids and fatty acids are being conserved, FAA serve as a primary fuel and building blocks for protein synthesis. There did not appear to be any preferred conservation of essential FAA over non-essential. The FAA which were conserved was the essential lysine – already at low levels relative to most other FAA at Day 0 – and seventeen other non-essentials. This information can now contribute to developing diets for broodstock and first-feeding larvae which are high in the FAA needed for energy, and for supplementing those which are at extremely low levels in yolk-sac larvae.

4 Overall conclusions

The two most abundant components of marine fish eggs are lipids and amino acids. Both of these are utilized from the yolk-sac during embryogenesis and early larval development as sources of energy, as well as building blocks for the growth of tissues. The determination of how these biochemical factors affect egg and larval quality can serve as a means to separate poor eggs from highly successful eggs, allowing producers to select viable eggs that will result in maximum rates of fertilization, hatch, and larval survival. Levels of total lipids, lipid classes, fatty acids and free amino acids were followed in Atlantic cod eggs and larvae from fertilization to yolk-sac absorption, and correlated with fertilization and hatching success. A select number of lipid classes and free amino acids correlated with fertilization and hatching success, and significant differences were found between high and low success groups in some lipid classes and FAA. The phospholipids and the fatty acid 24:1 showed the most potential as markers of viability, with 24:1 correlating with both fertilization and hatching success. Lipids showed conservation throughout embryogenesis, with PL and the phospholipids 20:5ω3, 22:5ω3 and 22:6ω3 only decreasing from hatch to yolk-sac absorption. The majority of FAA was catabolized as a primary energy source during embryogenesis. Blastomere morphologies were also analyzed in fertilized eggs. Cell symmetry, uniformity, margins and adhesions showed positive correlations with hatching success ($p < 0.001 - 0.028$).

It is likely that a combination of these biochemical factors influence the success of cod eggs and larvae. No one lipid class, fatty acid, or free amino acid shows strong evidence

that it alone would serve as a reliable indicator of egg and larval viability. It is, however, evident that free amino acids are important as energy in the developing larvae, and that lipids are primarily conserved until after hatch. It is recommended that the role of the fatty acid 24:1, as well as the free amino acids showing significant differences among Day 0 egg groups, be investigated further to understand their roles in embryo and larval development.

Morphology may still be used as a fast and efficient indicator of embryo viability in a commercial setting, however the need still exists to find a more concrete tool for batch evaluation. It is suggested that this work be repeated with cod eggs prior to exposure to sperm, in order to evaluate the biochemical make-up of eggs before fertilization can occur, and then allow these batches to be fertilized and followed through to yolk-sac absorption. This will allow analysis of lipid, FA and FAA composition of the complete yolk-sac before egg cleavage can begin, which will rule out any changes that may occur at the beginning of gastrulation and give a clearer picture of differences that exist in the biochemical make-up of batches at spawn.

The EZ:faast^{TM} GC-FID Free (Physiological) Amino Acid Analysis Kit is a quick and effective method for measuring FAA, and has great potential for use in commercial aquaculture settings. It is recommended that this kit be tested with samples of a variety of fish and marine invertebrates at all stages of development, to examine its potential for experiments involving a broader range of models.

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Appendix I:

Supplementary lipid and fatty acid data

Table 1: Day 0 Atlantic cod eggs: Values are ug/g dry weight, mean ± standard error.

	High Fertilization Success (>70%)	Low Fertilization Success (< 70%)	Fertilized eggs	Unfertilized eggs
Lipids (ug/g)				
Hydrocarbons	1128 ± 426	1045 ± 565	1099 ± 336	999 ± 354
Steryl Esters/Wax Esters	1868 ± 686	958 ± 673	1556 ± 506	2300 ± 595
Ethyl Ketones	865 ± 628	0	568 ± 415	628 ± 337
Methyl Ketones	4378 ± 1471	2200 ± 1006	3631 ± 1031	4996 ± 2221
Triacylglycerols	1406 ± 489	7080 ± 2732	3351 ± 1069	4051 ± 2032
Sterols	5944 ± 1716	8907 ± 1491	6960 ± 1248	6625 ± 1275
Acetone Mobile Polar Lipids	4727 ± 1005	2798 ± 1100	4065 ± 767	3293 ± 839
Phospholipids	69425 ± 6468	57285 ± 7343	65263 ± 4975	52942 ± 4569
Total Lipids	96314 ± 10689	85619 ± 9635	92647 ± 7723	88627 ± 11247
Fatty acids (ug/g)				
14:0	901 ± 136	862 ± 128	888 ± 98	967 ± 164
16:0	15124 ± 2407	15496 ± 1991	15255 ± 1689	16540 ± 2617
16:1ω9?	1117 ± 150	1020 ± 137	1083 ± 108	1058 ± 163
16:1ω7	1546 ± 229	1349 ± 205	1477 ± 164	1568 ± 213
18:0	2208 ± 322	2564 ± 431	2333 ± 255	2330 ± 353
18:1ω9	8272 ± 1263	7416 ± 1090	7969 ± 896	7776 ± 1081
18:1ω7	2887 ± 398	2632 ± 382	2797 ± 288	2584 ± 361
18:2ω6	467 ± 90.7	468 ± 66.1	467 ± 62.4	538 ± 97.6
20:1ω9	1912 ± 348	1844 ± 213	1888 ± 235	1806 ± 269
20:5ω3	3859 ± 949	2357 ± 498	3329 ± 639	4270 ± 1081
22:5ω3	545 ± 95.4	317 ± 58.4	465 ± 67.2	445 ± 95.6
22:6ω3	9088 ± 2308	4523 ± 956	7376 ± 1523	8589 ± 2202
24:1	2064 ± 476	785 ± 140	1584 ± 319	1150 ± 234

Table 2: Quantities of lipids and fatty acids throughout Atlantic cod egg and larval development. Values are ug/g dry weight, mean ± standard error.

	Fertilization (Day 0)	Half-way to hatch (~40dd)	Pre-hatch (~78dd)	Post-hatch (~85dd)	Half-way to Yolk-sac absorption (~ 105dd)	Yolk-sac absorption (140dd)
Lipids (ug/g)						
Hydrocarbons	1099 ± 336	996 ± 391	2671 ± 886	821 ± 259	3770 ± 1502	3589 ± 1812
Steryl Esters/Wax Esters	1556 ± 506	2138 ± 658	1855 ± 702	1445 ± 651	798 ± 213	1075 ± 457
Ethyl Ketones	568 ± 415	992 ± 436	911 ± 370	771 ± 357	566 ± 469	661 ± 384
Methyl Ketones	3631 ± 1031	1678 ± 478	3710 ± 1163	24678 ± 14484	14636 ± 8468	8034 ± 2678
Triacylglycerols	3351 ± 1069	743 ± 266	921 ± 694	22427 ± 15372	4508 ± 2381	4014 ± 2556
Sterols	6960 ± 1248	5405 ± 1003	5131 ± 1713	11868 ± 2066	8507 ± 2007	6888 ± 1825
Acetone Mobile Polar Lipids	4065 ± 767	2165 ± 574	2117 ± 707	2605 ± 958	2959 ± 853	5054 ± 1584
Phospholipids	65263 ± 4975	47708 ± 4316	59719 ± 7537	70358 ± 7703	55513 ± 11296	50054 ± 5670
Total Lipids	92647 ± 7723	65537 ± 4881	84076 ± 9868	174654 ± 44841	118696 ± 20271	114483 ± 15463
Fatty acids (ug/g)						
14:0	887 ± 97.5	744 ± 77.7	658 ± 98.9	1569 ± 470	575 ± 134	674 ± 104
16:0	15255 ± 1689	14126 ± 1571	11787 ± 1774	33213 ± 9564	18793 ± 4296	26493 ± 4414
16:1ω9?	1083 ± 108	916 ± 102	897 ± 147	2574 ± 773	1250 ± 207	1624 ± 247
16:1ω7	1477 ± 164	1233 ± 105	1158 ± 173	3095 ± 886	1116 ± 222	1825 ± 282
18:0	2333 ± 255	2974 ± 591	2469 ± 360	9215 ± 2714	7049 ± 1342	10901 ± 1866
18:1ω9	7969 ± 896	6253 ± 626	5836 ± 840	16460 ± 5405	6740 ± 1281	10255 ± 1634
18:1ω7	2797 ± 288	2218 ± 246	2026 ± 302	6431 ± 2106	2766 ± 501	4146 ± 652
18:2ω6	467 ± 62.5	343 ± 40.7	338 ± 58.8	911 ± 277	341 ± 78.0	412 ± 81
20:1ω9	1888 ± 235	1547 ± 169	1377 ± 222	4436 ± 1164	1541 ± 265	2465 ± 381
20:5ω3	3329 ± 639	2704 ± 400	3016 ± 922	6604 ± 1544	1558 ± 369	1742 ± 531
22:5ω3	465 ± 67.2	327 ± 60.1	348 ± 78.5	771 ± 171	504 ± 190	157 ± 34.6
22:6ω3	7376 ±	5327 ±	6351 ±	14263 ±	4336 ±	2993 ±

	1523	818	1804	3441	1132	551
24:1	1584 ± 318	1013 ± 207	1461 ± 495	2469 ± 802	1228 ± 275	1991 ± 415

Table 3: Fatty acid ratios and sums of Day 0 Atlantic cod eggs. Sum values are ug/g dry weight, mean ± standard error.

	Fertilized Eggs	Unfertilized Eggs	High Success Eggs	Low Success Eggs
ω3/ω6	10.2 ± 1.01	13.0 ± 2.95	11.4 ± 5.67	8.60 ± 4.72
AA:EPA	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
∑ essentials	1.32 ± 0.26	1.50 ± 0.39	1.64 ± 1.81	0.75 ± 0.56
DHA:EPA	1.88 ± 0.43	1.87 ± 0.53	2.11 ± 0.20	1.77 ± 0.09
P:S	0.70 ± 0.62	0.71 ± 0.62	0.84 ± 0.74	0.48 ± 0.27

Table 4: Correlations of Day 0 lipid and fatty acid values (µg ind-1) with fertilization success, hatching success, and egg morphology. Values are r-values. Significant correlations ($p < 0.05$) are in bold.

Lipids	Fertilization Success	Hatching Success	Symmetry	Uniformity	Adhesions	Margins	Clarity	Cell Number
Hydrocarbons	+ 0.059	- 0.171	- 0.329	- 0.317	- 0.194	- 0.259	- 0.152	- 0.089
Ethyl Ketones	+ 0.200	+ 0.276	+ 0.348	+ 0.007	+ 0.251	+ 0.066	- 0.429	+ 0.405
Methyl Ketones	+ 0.311	+ 0.297	+ 0.413	+ 0.346	+ 0.302	+ 0.289	+ 0.130	+ 0.373
Triacylglycerols	- 0.547	- 0.050	- 0.026	- 0.430	+ 0.081	- 0.142	+ 0.272	- 0.485
Sterols	- 0.139	+ 0.350	+ 0. 197	+ 0.115	+ 0.257	+ 0.123	+ 0.211	- 0.061
Acetone Mobile Polar Lipids	+ 0.218	+0.142	+ 0.178	- 0.243	+ 0.102	- 0.013	- 0.265	+ 0.135
Phospholipids	+ 0.488	**+ 0.640**	+ 0.441	+ 0.406	+ 0.322	+ 0.282	+ 0.162	+ 0.142
Total Lipids	+ 0.396	**+ 0.740**	+ 0.568	+ 0.414	+ 0.513	+ 0.310	+ 0.303	+ 0.197
Fatty Acids								
14:0	+ 0.049	**+ 0.664**	+ 0.535	+ 0.367	+ 0.482	+ 0.247	+ 0.469	+ 0.138
16:0	+ 0.114	**+ 0.660**	+ 0.522	+ 0.437	+ 0.427	+ 0.351	+ 0.407	+ 0.227
16:1w9?	+ 0.265	+ 0.595	+ 0.470	+ 0.440	+ 0.429	+ 0.381	+ 0.243	+ 0.185
16:1w7	+ 0.183	**+ 0.689**	+ 0.542	+ 0.433	+ 0.495	+ 0.338	+ 0.481	+ 0.202
18:0	+ 0.239	+ 0.597	+ 0.494	+ 0.506	+ 0.393	+ 0.394	+ 0.467	+ 0.262
18:1w9	+ 0.260	**+ 0.642**	+ 0.494	+ 0.474	+ 0.414	+ 0.353	+ 0.393	+ 0.255
18:1w7	+ 0.257	+ 0.596	+ 0.456	+ 0.441	+ 0.360	+ 0.347	+ 0.367	+ 0.206
20:1w9	+ 0.097	+ 0. 532	+ 0.398	+ 0.421	+ 0.411	+ 0.222	+ 0.489	+ 0.241
20:5w3	+ 0.436	+ 0.369	+ 0.314	+ 0. 076	+ 0.389	+ 0.183	+ 0.167	- 0.201
22:5w3	+ 0.456	+ 0.595	+ 0.530	+ 0. 076	+ 0.520	+ 0.206	+ 0.136	- 0.057
22:6w3	+ 0.491	+ 0.329	+ 0.264	+ 0. 076	+ 0.338	+ 0.170	+ 0.046	- 0.180
24:1	**+ 0.671**	**+ 0.682**	**+ 0.688**	+ 0. 076	**+ 0.699**	+ 0.325	+ 0.027	+ 0.517

Appendix II:

Mole Percent Data for Free Amino Acids

Table 1: Percent mole values of free amino acids throughout development of Atlantic cod eggs and larvae.. Values are % ± standard deviation.

	Day 0, Fertilized	Halfway to hatch	Pre-hatch	Post-hatch	Halfway to yolk-sac absorption	Yolk-sac absorption
Alanine, ALA	7.00 ± 2.38	7.48 ± 2.02	5.81 ± 1.13	2.61 ± 2.30	0.97 ± 0.86	1.26 ± 0.54
Sarcosine, SAR	0.18 ± 0.19	0.16 ± 0.14	0.17 ± 0.07	0.21 ± 0.22	0.57 ± 1.11	0.86 ± 1.17
Glycine, GLY	2.07 ± 0.77	2.69 ± 0.56	2.80 ± 0.38	2.00 ± 0.69	3.52 ± 2.14	4.60 ± 1.50
amino-n-butyric acid, ABA	0.27 ± 0.17	0.48 ± 0.40	0.50 ± 0.19	1.11 ± 1.09	0.28 ± 0.47	0.10 ± 0.19
Valine, VAL	4.30 ± 0.78	5.27 ± 1.66	5.95 ± 1.29	7.44 ± 4.70	2.80 ± 2.49	1.65 ± 1.48
Aminoisobutyric acid, BAIB	0.16 ± 0.06	0.18 ± 0.09	0.26 ± 0.15	0.23 ± 0.17	0.47 ± 0.62	0.96 ± 1.10
Leucine, LEU	3.41 ± 1.02	4.62 ± 1.04	6.37 ± 1.67	13.9 ± 10.9	2.00 ± 3.70	0.26 ± 0.56
Isoleucine, ILE	0.23 ± 0.30	0.57 ± 1.40	0.14 ± 0.21	0	2.59 ± 2.97	4.09 ± 2.07
Allo-isoleucine, AILE	3.52 ± 0.92	4.19 ± 1.11	4.34 ± 1.00	3.57 ± 2.09	0.56 ± 1.08	0.03 ± 0.07
Asparagine, ASN	5.12 ± 1.69	4.12 ± 6.69	2.65 ± 0.73	1.61 ± 1.61	4.96 ± 5.12	4.34 ± 2.12
Threonine, THR	9.26 ± 9.87	3.26 ± 2.24	2.80 ± 1.43	0.97 ± 1.30	0.33 ± 0.48	0.30 ± 0.45
Serine, SER	11.4 ± 11.5	9.87 ± 5.71	7.53 ± 4.36	2.34 ± 4.74	0.05 ± 0.08	0
Proline, PRO	2.34 ± 2.11	1.06 ± 0.49	0.46 ± 0.14	0.23 ± 0.21	0.11 ± 0.22	0.02 ± 0.06
Thioproline, TPR	0.29 ± 0.19	0.20 ± 0.14	0.38 ± 0.13	0.60 ± 0.57	1.64 ± 1.36	2.57 ± 1.66
Aspartic acid, ASP	0.83 ± 0.93	1.31 ± 1.41	0.99 ± 0.57	5.87 ± 5.70	10.8 ± 10.3	11.2 ± 8.89
Methionine, MET	0.93 ±0.34	0.99 ± 0.52	1.16 ± 0.25	1.18 ± 0.77	0.01 ± 0.03	0.33 ± 0.66
Hydroxyproline, HYP	4.48 ± 2.21	3.23 ± 1.69	4.38 ± 1.61	3.30 ± 3.01	7.09 ± 6.71	6.57 ± 3.58
Glutamic acid, GLU	0.74 ± 0.63	0.44 ± 0.33	0.62 ± 0.41	0.14 ± 0.16	0	0.15 ± 0.40
Phenylalanine, PHE	0.71 ± 0.30	1.63 ± 2.37	1.85 ± 0.54	4.14 ± 4.27	0.04 ± 0.09	0
α-aminodipic acid, AAA	10.5 ± 8.22	14.8 ± 9.92	17.8 ± 5.62	24.3 ± 23.8	17.2 ± 13.8	13.0 ± 8.08
α-aminopimelic acid, APA	0.47 ± 0.40	0.44 ± 0.44	0.31 ± 0.32	0.08 ± 0.15	0.11 ± 0.29	0.09 ± 0.23
Glutimine, GLN	0.24 ± 0.26	0.24 ± 0.34	0.30 ± 0.43	0.17 ± 0.22	0	0.08 ± 0.21
Ornithine, ORN	1.95 ± 0.66	2.05 ± 1.43	2.63 ± 0.98	2.04 ± 1.25	3.13 ± 1.69	4.22 ± 0.88
Glycine-proline, GPR	0.40 ± 0.77	0.48 ± 1.20	0.18 ± 0.33	0.15 ± 0.25	0.17 ± 0.49	0.50 ± 0.56
Lysine, LYS	0.11 ± 0.10	0.24 ± 0.39	0.19 ± 0.19	0.01 ± 0.02	0	0
Histidine, HIS	0.08 ± 0.07	0.36 ± 0.77	0.10 ± 0.10	0.01 ± 0.04	0	0
Hydroxylysine, HLY	3.80 ± 2.23	4.19 ± 2.50	4.23 ± 1.11	1.18 ± 1.14	4.95 ± 3.87	4.57 ± 2.62
Tyrosine, TYR	3.88 ± 2.44	3.85 ± 2.57	2.51 ± 1.72	4.20 ± 2.63	9.89 ± 9.42	6.54 ± 4.99
Proline-hydroxy proline, PHP	17.1 ± 9.88	13.7 ± 8.94	15.7 ± 5.23	2.28 ± 4.19	3.15 ± 6.86	7.82 ± 11.6
Tryptophan, TRP	0.90 ± 0.63	1.08 ± 0.71	1.58 ± 1.00	1.02 ± 0.72	1.30 ± 0.80	1.92 ± 1.09
Cystathionine, CTH	2.79 ± 1.65	3.09 ± 2.97	2.52 ± 0.80	2.44 ± 2.09	13.2 ± 17.8	4.14 ± 2.96
Cystine, C-C	0.57 ± 0.39	0.81 ± 0.74	0.73 ± 0.40	0.10 ± 0.15	0.77 ± 1.32	0.32 ± 0.26

Table 2: Mole percent values of free amino acids in Day 0 Atlantic cod eggs. Values are mean ± standard deviation.

	High Success Eggs	Low Success Eggs	Day 0, Fertilized	Day 0, Unfertilized
Alanine, ALA	6.84 ± 3.13	7.23 ± 0.68	7.00 ± 2.38	5.36 ± 3.19
Sarcosine, SAR	0.22 ± 0.24	0.13 ± 0.04	0.18 ± 0.19	0.30 ± 0.26
Glycine, GLY	1.90 ± 0.97	2.32 ± 0.23	2.07 ± 0.77	2.09 ± 0.80
amino-n-butyric acid, ABA	0.31 ± 0.20	0.21 ± 0.08	0.27 ± 0.17	0.24 ± 0.26
Valine, VAL	4.19 ± 0.89	4.48 ± 0.68	4.30 ± 0.78	5.25 ± 2.78
Aminoisobutyric acid, BAIB	0.17 ± 0.04	0.15 ± 0.08	0.16 ± 0.06	0.17 ± 0.11
Leucine, LEU	3.34 ± 1.28	3.50 ± 0.63	3.41 ± 1.02	2.95 ± 1.91
Isoleucine, ILE	0.09 ± 0.18	0.44 ± 0.34	0.23 ± 0.30	0.48 ± 0.60
Allo-isoleucine, AILE	3.34 ± 1.17	3.78 ± 0.31	3.52 ± 0.92	2.83 ± 1.46
Asparagine, ASN	5.22 ± 1.77	4.96 ± 1.81	5.12 ± 1.69	4.09 ± 2.53
Threonine, THR	12.9 ± 11.6	3.77 ± 1.50	9.26 ± 9.87	3.05 ± 3.92
Serine, SER	9.87 ± 14.6	13.7 ± 5.03	11.4 ± 11.5	6.43 ± 7.89
Proline, PRO	3.03 ± 2.56	1.30 ± 0.19	2.34 ± 2.11	1.54 ± 2.28
Thioproline, TPR	0.22 ± 0.19	0.39 ± 0.15	0.29 ± 0.19	0.41 ± 0.28
Aspartic acid, ASP	1.07 ± 1.13	0.46 ± 0.37	0.83 ± 0.93	0.88 ± 0.95
Methionine, MET	1.00 ± 0.40	0.81 ± 0.25	0.93 ±0.34	0.71 ± 0.52
Hydroxyproline, HYP	3.61 ± 2.28	5.79 ± 1.43	4.48 ± 2.21	5.83 ± 3.08
Glutamic acid, GLU	0.92 ± 0.73	0.48 ± 0.40	0.74 ± 0.63	0.32 ± 0.39
Phenylalanine, PHE	0.73 ± 0.39	0.67 ± 0.11	0.71 ± 0.30	0.86 ± 1.29
α-aminodipic acid, AAA	12.3 ± 10.4	7.85 ± 2.37	10.5 ± 8.22	13.5 ± 7.37
α-aminopimelic acid, APA	0.53 ± 0.45	0.38 ± 0.36	0.47 ± 0.40	0.31 ± 0.26
Glutimine, GLN	0.31 ± 0.32	0.14 ± 0.10	0.24 ± 0.26	0.32 ± 0.33
Ornithine, ORN	1.74 ± 0.69	2.27 ± 0.54	1.95 ± 0.66	2.48 ± 0.91
Glycine-proline, GPR	0.63 ± 0.95	0.07 ± 0.13	0.40 ± 0.77	0.24 ± 0.44
Lysine, LYS	0.12 ± 0.11	0.10 ± 0.11	0.11 ± 0.10	0.05 ± 0.07
Histidine, HIS	0.09 ± 0.08	0.05 ± 0.04	0.08 ± 0.07	0.37 ± 1.00
Hydroxylysine, HLY	3.13 ± 2.18	4.79 ± 2.19	3.80 ± 2.23	6.44 ± 2.78
Tyrosine, TYR	4.11 ± 2.69	3.53 ± 2.35	3.88 ± 2.44	3.67 ± 3.86
Proline-hydroxy proline, PHP	14.1 ± 10.5	21.5 ± 8.15	17.1 ± 9.88	19.7 ± 12.7
Tryptophan, TRP	1.00 ± 0.60	0.74 ± 0.73	0.90 ± 0.63	0.74 ± 0.88
Cystathionine, CTH	2.31 ± 1.73	3.51 ± 1.43	2.79 ± 1.65	4.12 ± 1.94
Cystine, C-C	0.60 ± 0.43	0.52 ± 0.38	0.57 ± 0.39	1.18 ± 1.15

Table 3: Correlations of Day 0 free amino values (nmol ind^{-1}) with fertilization success, hatching success, and egg morphology in Atlantic cod. Values are r-values. Significant correlations ($p < 0.05$) are in bold.

FAA	Fertilization Success	Hatching Success	Symmetry	Uniformity	Adhesions	Margins	Clarity	Cell Number
Alanine, ALA	+ 0.306	+ 0.035	- 0.071	+ 0.147	- 0.041	+ 0.287	- 0.049	+ 0.106
Sarcosine, SAR	- 0.011	- 0.331	**- 0.404**	-0.300	**- 0.412**	- 0.171	- 0.187	- 0.169
Glycine, GLY	+ 0.028	- 0.335	**- 0.438**	- 0.339	- 0.366	- 0.249	- 0.225	- 0.177
amino-n-butyric acid, ABA	**+ 0.515**	- 0.007	- 0.438	+ 0.268	- 0.177	+ 0.250	- 0.267	+ 0.394
Valine, VAL	+ 0.182	- 0.046	- 0.157	- 0.038	- 0.092	+ 0.167	- 0.120	- 0.009
Aminoisobutyric acid, BAIB	+ 0.306	+ 0.125	- 0.049	+ 0.161	- 0.157	+ 0.190	- 0.142	+ 0.277
Leucine, LEU	+ 0.196	+ 0.027	- 0.045	+ 0.090	+ 0.005	+ 0.234	+ 0.057	- 0.010
Isoleucine, ILE	+ 0.175	- 0.003	- 0.059	+ 0.037	- 0.066	+ 0.253	- 0.055	- 0.033
Allo-isoleucine, AILE	- 0.009	- 0.362	**- 0.446**	- 0.354	- 0.392	- 0.280	- 0.228	- 0.195
Asparagine, ASN	+ 0.059	- 0.314	**- 0.427**	- 0.337	- 0.332	- 0.258	- 0.262	- 0.162
Threonine, THR	**+ 0.462**	+ 0.265	- 0.149	+ 0.175	+ 0.263	+ 0.260	- 0.155	+ 0.191
Serine, SER	**+ 0.433**	+ 0.214	+ 0.085	+ 0.072	+ 0.189	+ 0.182	- 0.199	+ 0.085
Proline, PRO	+ 0.244	+ 0.120	+ 0.028	+ 0.127	+ 0.100	+ 0.289	- 0.010	+ 0.062
Thioproline, TPR	+ 0.055	- 0.344	**- 0.443**	- 0.306	- 0.381	- 0.259	- 0.290	- 0.131
Aspartic acid, ASP	+ 0.197	- 0.183	- 0.238	- 0.094	- 0.276	- 0.196	- 0.321	- 0.061
Methionine, MET	+ 0.390	- 0.153	- 0.345	- 0.043	- 0.209	- 0.051	**- 0.409**	+ 0.146
Hydroxyproline, HYP	+ 0.009	- 0.355	**- 0.445**	- 0.338	- 0.390	- 0.264	- 0.246	- 0.173
Glutamic acid, GLU	**+ 0.503**	+ 0.299	+ 0.146	+ 0.065	+ 0.299	+ 0.151	- 0.327	+ 0.093
Phenylalanine, PHE	**+ 0.445**	+ 0.165	- 0.005	+ 0.176	+ 0.111	+ 0.277	- 0.080	+ 0.182
α-aminodipic acid, AAA	- 0.248	- 0.154	-0.166	- 0.363	- 0.143	- 0.171	+ 0.198	- 0.344
α-aminopimelic acid, APA	+ 0.293	- 0.205	- 0.365	- 0.100	- 0.246	- 0.143	**- 0.398**	+ 0.108
Glutimine, GLN	+ 0.222	- 0.264	-0.390	- 0.159	- 0.313	- 0.193	**- 0.467**	+ 0.034
Ornithine, ORN	+ 0.020	- 0.346	**- 0.435**	- 0.310	- 0.389	- 0.239	- 0.238	- 0.152
Glycine-proline, GPR	+ 0.203	- 0.271	- 0.393	- 0.160	- 0.317	- 0.201	**- 0.396**	+ 0.033
Lysine, LYS	+ 0.250	- 0.262	**- 0.407**	- 0.175	- 0.296	- 0.189	**- 0.261**	+ 0.035
Histidine, HIS	+ 0.310	- 0.139	- 0.283	- 0.052	- 0.188	- 0.085	- 0.355	+ 0.116
Hydroxylysine, HLY	+ 0.016	- 0.357	**- 0.440**	- 0.313	**- 0.399**	- 0.249	- 0.256	- 0.155

Tyrosine, TYR	+ 0.158	- 0.268	**- 0.407**	- 0.346	- 0.284	- 0.214	- 0.365	- 0.167
Proline-hydroxy proline, PHP	- 0.027	- 0.356	**- 0.430**	- 0.338	- 0.394	- 0.248	- 0.207	- 0.141
Tryptophan, TRP	**+ 0.488**	+ 0.203	+ 0.011	+ 0.094	+ 0.219	+ 0.182	- 0.313	- 0.202
Cystathionine, CTH	- 0.011	- 0.356	**- 0.432**	- 0.320	**- 0.399**	- 0.237	- 0.225	- 0.172
Cystine, C-C	+ 0.189	+ 0.245	+ 0.250	+ 0.181	+ 0.043	**+ 0.520**	+ 0.002	+ 0.014
TOTAL	+ 0.022	- 0.334	**-0.430**	- 0.344	- 0.370	- 0.238	- 0.225	- 0.189