INCORPORATION OF A KRILL PROTEIN HYDROLYSATE INTO THE FEEDING REGIME OF ATLANTIC COD (*Gadus morhua*) LARVAE: EFFECT ON GROWTH AND SURVIVAL

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Incorporation of a Krill Protein Hydrolysate into the Feeding Regime of Atlantic Cod (*Gadus morhua*) Larvae: Effect on Growth and Survival

By

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Abstract

Atlantic cod (*Gadus morhua*) is being developed as a species for commercial scale aquaculture production. Years of study concerning the nutritional requirements of marine fish larvae have focused on the omega-3 (ω-3) fatty acid and lipid requirements, overshadowing the role and importance of protein and free amino acids in the larval diet. This study incorporated a Krill Protein Hydrolysate (Krill Protein) into a fatty acid/lipid-rich *Artemia* enrichment regime and examined the contribution that amino acids make to the growth and survival of Atlantic cod larvae.

Atlantic cod larvae were fed *Artemia* enriched in AlgaMac 3010, DHA Selco and a Krill Protein Hydrolysate in eight different feeding regimes. The eight feeding treatments also included an unenriched *Artemia* treatment, and all were carried out in triplicate during the *Artemia* feeding stage (a 20 day period).

During the *Artemia* enrichment, the Krill Protein Hydrolysate incorporated the highest proportion of phospholipid (25.5% total lipid), the most arachidonic acid (7.7% fatty acids) and DHA Selco and Krill Protein contributed similar proportions of total lipid (5.6%; 5.5%). Unenriched *Artemia* contained the highest concentrations of all free amino acids (FAA) studied. FAA incorporated by the *Artemia* enriched with Krill Protein and AlgaMac 3010, respectively, expressed as a percent of FAA concentrations in unenriched *Artemia* were: alanine (54% of unenriched; 50% of unenriched), leucine (39%; 66%), serine (44%; 56%), isoleucine (40%; 67%), lysine (32%; 59%), and valine (44%; 62%).

Growth during the experiment, measured as total length (mm), was significantly improved in the larvae fed DHA Selco/Krill Protein enriched *Artemia* on alternating days

(P=0.010). The DHA Selco/Krill Protein treatment produced the best specific growth rate (2.55), in fact, all treatments containing Krill Protein produced significantly higher SGRs than that found in the unenriched treatment ($P<0.05$). Alanine, leucine, serine, isoleucine, lysine and valine concentrations, measured as mole %, were significantly higher in larvae fed *Artemia* enriched with AlgaMac 3010/Krill Protein and those fed DHA Selco/Krill Protein than the FAA levels in the unenriched treatment. Further, larvae fed solely AlgaMac 3010 enriched *Artemia* had significantly higher levels of alanine than larvae fed unenriched *Artemia*. *Artemia* enriched in DHA Selco and the Krill Protein Hydrolysate contained high concentrations of total lipid (TL), total fatty acids (TFA) and triacylglycerol (TAG).

These nutritional components probably contributed to the increased growth rates found in larvae fed DHA Selco/Krill Protein enriched *Artemia* on alternating days. *Artemia* enriched in AlgaMac 3010 contained higher levels of phospholipid than all other treatments, possibly contributing to the significantly improved survival rate in the larvae fed AlgaMac 3010/Krill protein enriched *Artemia* in combination (44%) as opposed to those fed Krill Protein enriched *Artemia* alone.

A suggested *Artemia* diet feeding regime for Atlantic cod larvae using DHA Selco (lipid), AlgaMac 3010 (lipid) and a Krill Protein Hydrolysate (protein) in a 1:1 (lipid:protein) ratio would be: Day 1 - AlgaMac 3010 enriched *Artemia*, Day 2 - Krill Protein enriched *Artemia*, Day 3 - DHA Selco enriched *Artemia*, Day 4 - Krill Protein enriched *Artemia*, and continue to repeat this cycle.

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Table of Contents

Chapter 3 – Growth, Survival, Fatty Acid and Free Amino Acid Composition of Atlantic Cod Larvae Fed Enriched and Unenriched *Artemia*

Chapter 4 – Summary of Experiments and Recommendations for Enrichment Regimes for First Feeding Atlantic Cod Larvae

List of Tables

List of Figures

acids in the larvae ($P<0.05$). Alg = AlgaMac 3010, DHA = DHA Selco, Krill Protein = Krill Protein Hydrolysate.

List of Abbreviations

AA	Amino Acid
Alg	AlgaMac 3010
ANOVA	Analysis of Variance
ARA	Arachidonic acid (20:4n-6)
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
FAA	Free Amino Acid
HCl	Hydrochloric Acid
HUFA	Highly Unsaturated Fatty Acids (4-6 double bonds)
Krill Protein	Krill Protein Hydrolysate
Li^+	Lithium
MUFA	Monounsaturated Fatty Acids
Na^+	Sodium
PL	Phospholipid
PUFA	Polyunsaturated Fatty Acids (≥2 double bonds)
SGR	Specific Growth Rate
TAG	Triacylglycerol
TFA	Total Fatty Acids
TLC/FID	Thin Layer Chromatography with Flame Ionization Detection
TL	Total Lipid

Chapter 1

General Introduction and Overview

1.1 Introduction

Since 1992, when a moratorium was imposed on the Atlantic Cod fishery off the east coast of Canada, there has been keen interest in developing Atlantic cod as a species for commercial aquaculture production. Successful rearing protocols have been developed for the intensive production of Atlantic cod (*Gadus morhua*) larvae but improvements can be made in regard to the nutritional quality of the live food items. *Artemia* (brine shrimp) and rotifers are the cultured live food organisms of choice for first feeding marine fish larvae because they are the appropriate size and they are easily mass-produced. Due to the mass culture conditions of these organisms, they tend to lack the nutritional components necessary for optimal growth and proper development of the larvae. As a result, the live food organisms are routinely enriched with various enrichment products developed to provide high amounts of the polyunsaturated fatty acids and ω-3 (omega-3) fatty acids needed for proper growth and development during the larval stage.

Highly unsaturated fatty acids, arachidonic acid (ARA, 20:4ω-6), eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) have already been proven to be a dietary requirement for marine fish. Dietary lipids are an important source of essential fatty acids for the normal growth and development of first feeding larvae. Regardless of tissue or species, triacylglycerol (TAG) is the predominant

form of reserve lipid and is always metabolized before phospholipid (PL) during starvation (Tocher et al., 1989). It seems that TAG is used to satisfy demands for energy and PL is conserved, as it is a structural component of cell membranes (Rainuzzo et al., 1997).

Chu and Ozkizilcik (1995) concluded that the fatty acid composition of the fish larvae was also modified significantly by the diet. They noticed substantial changes in certain fatty acid components where these components either increased or decreased to a level similar to that of the diet, in this case, *Artemia.*

Webster and Lovell (1990) found that the larvae of striped bass may be influenced by the EPA content of the *Artemia* fed to them. Data from their study indicate that normally functioning striped bass larvae have lipids that contain about 11% EPA, compared to below 5% found in larvae with reduced growth and survival rates. This indicates that striped bass larvae, like turbot, are not able to elongate and desaturate linolenic acid into EPA.

Furuita et al. (1999) conducted experiments on growth, survival and salinity tolerance in Japanese flounder larvae after being fed *Artemia* nauplii enriched with unsaturated fatty acids. Growth and survival of the larvae were improved by increasing the DHA or EPA content of the diet, although there was no clear difference between the two fatty acids.

Navarro et al. (1999) studied lipid conversions during enrichment of *Artemia* and found that EPA was the most extensively incorporated fatty acid followed by DHA. The amount of each substrate fatty acid recovered in *Artemia* lipid was directly related to its

relative mass in the enrichment medium. Although EPA comprised 37.4% of the mass of the substrate fatty acid mixture, it accounted for 47.6% of the total incorporated mass of fatty acids. Conversely, DHA represented 21.9% of the total incorporated fatty acids, whereas it comprised 29% of the original fatty acid mixture. At the end of the enrichment period, more than 50% of each incorporated radio labeled fatty acid was recovered in TAG (Navarro et al., 1999). They concluded that the transformation of ethyl esters to other lipid classes during the enrichment process does occur and is not simply due to a retention of undigested ethyl esters in the gut but reflects a definite assimilation of the exogenous fatty acids into tissue lipids. The study demonstrated that *Artemia* nauplii have the ability to retroconvert incorporated DHA to EPA during enrichment and subsequent starvation.

DHA was absorbed at a higher rate when the dietary phospholipid level was 10% as compared to 5% but there was no increase in absorption rate at higher phospholipid levels; mixing phospholipids with DHA sodium salts maximized absorption of DHA phospholipids in *Artemia* (Harel and Place, 1998; Harel et al., 1999).

Garcia et al. (2008) found the lipid class composition of the enriched *Artemia* differed from the lipid class composition of the enrichment media. They observed that *Artemia* modified the fatty acid composition of the enrichment products as well as the lipid classes. The DC DHA Selco enrichment used in the experiment contained higher ARA levels than that found in the AlgaMac 2000 enrichment but the *Artemia*, after enrichment, contained similar levels of this fatty acid. They concluded that *Artemia*

containing a DHA/EPA/ARA ratio of 7/2/1 result in good larval performance and that prey items should contain proportionally high DHA and ARA levels.

Lipids and fatty acids are essential to the developing larvae of marine fish species, but what about the importance of protein in the larval diet? Protein and amino acids act as an energy source for fish and are necessary for proper growth and survival. In the intensive culture of marine finfish larvae, the importance of amino acids and protein content of live food have been largely ignored due to the focus on ω-3 fatty acids. Growth optimization in fish larvae is closely linked to the supply of dietary protein of appropriate quality and quantity; more than 50% of larval composition is protein (dry matter) (Conceição et al., 2003).

Morais et al. (2004) studied the digestive capacity of Senegalese sole larvae and found that they have a high capacity for digesting live prey items even at young stages. They conducted TCA (trichloroacetic acid) extractions on the *Artemia* labeled with protein hydrolysate and found, on average, 82% of the radio label was incorporated into the trichloroacetic acid precipitate (mostly protein) and 18% was incorporated as soluble fractions. This study resulted in Sole larvae showing considerable capacity to digest and absorb *Artemia* proteins and amino acids, with digestibility varying between 77% and 83% of the total label fed.

Halibut larvae also rapidly absorbed free amino acids (FAA) when tube-fed with amino acid solutions containing ^{14}C tracers. An average of 71% of alanine, glutamate, arginine or lysine was being absorbed from the gut in 30 minutes of tube feeding and

only a 6% loss of all FAA solutions were seen after an 8 hour evacuation period (Applebaum and Rønnestad, 2004).

Rust et al. (1993) tested the functionality of first feeding larvae by force feeding larvae at different ages and measuring nutrient absorption rates. Fish larvae that lack a stomach at first-feeding initially absorb free amino acids more efficiently than amino acids in polymerised form (Rust et al., 1993).

Aragão et al. (2004) estimated amino acid requirements during early ontogeny of gilthead seabream and Senegalese sole. Valine was found to be the first limiting amino acid (AA) for the 5 day old sole larvae feeding on *Artemia* nauplii. *Artemia* metanauplii enriched for 24 hours with microalgae followed by 24 hours in Super Selco seemed to be deficient in histidine and sulphur AA when fed to 26 day old sole larvae. At all stages of development there were at least 20%, in some cases 50%, unavoidable losses of AA intake due to dietary AA imbalances. Aragão et al. (2004) suggests that a balanced dietary AA profile will not improve larval growth because losses due to energy dissipation or for other metabolic purposes will be more than the losses induced by dietary imbalances.

Free amino acids accounted for 20% of the total amino acid pool of the Dentex egg with serine, alanine and lysine being predominant (Tulli and Tibaldi, 1997). They found that all amino acid levels declined with the exception of taurine, which stayed constant for the first 30 days after hatch.

The free amino acids that were in highest abundance in newly spawned pelagic eggs, included leucine, valine, isoleucine, lysine, alanine, and serine (Rønnestad et al.,

1996). The free amino acid pool of pelagic eggs from marine fish species is established during final oocyte maturation. In pelagic eggs with an oil globule, the FAA pool disappears prior to hatch, on the other hand, FAA depletion continues beyond hatch in species that do not contain an oil globule, like Atlantic cod (Finn et al., 1995).

Dabrowski and Rusiecki (1983) looked at the free amino acid content of various zooplankton used as prey items for fish and found that *Artemia* nauplii were abundant in proline, alanine, glycine and serine.

Næss et al. (1995) studied Atlantic halibut larvae fed different combinations of *Artemia* and wild zooplankton, and found that the free amino acid concentration was significantly higher in wild zooplankton than in unenriched *Artemia*. They used the Super Selco enrichment product by INVE to enrich the *Artemia,* boosting the AA concentration from 46 μmol/g wet weight to 57 μmol/g wet weight. Super Selco especially boosted the alanine content as well as considerably improved the lipid content (1.3% to 3% of wet weight) making the *Artemia* comparable to the highest level found in the wild zooplankton. In the halibut larvae, the fish fed the enriched *Artemia* had the largest myotome height and demonstrated the best survival rate by day 19.

Larvae can only store AA in the form of proteins and there will be AA loss when there are imbalances between dietary and larval AA profiles (Conceição et al., 1997). Conceição et al. studied protein turnover in larval turbot fed natural zooplankton or *Artemia* and estimated that the dietary amino acid intake may be up to 10 times the larvae FAA pool. As well, they found that protein synthesis and degradation may replenish and remove up to 20 and 10 times the size of the FAA pool on a daily basis, respectively.

This thesis looked at incorporating a Krill Protein Hydrolysate into the *Artemia* enrichment regime and compared the growth and survival results with those found when lipid-rich enrichment products were used. The effect of the lipid/polyunsaturated fatty acids in conjunction with the protein/amino acids in enriched *Artemia* on the growth and survival of Atlantic cod larvae were studied.

Routine and proven commercial enrichment products, DHA Selco and AlgaMac 3010, were used as the lipid-rich enrichment products for the experiment. A Krill Protein Hydrolysate was studied as a possible additive to the *Artemia* enrichment regime in an intensive marine finfish hatchery setting. Ratios of DHA:EPA:ARA are considered to be important in the enrichment protocols but here we built on that concept and looked at which ratios of free amino acids to lipids and essential fatty acids would give the best growth and survival results in cod larvae. The final aim was to recommend a first feeding regime that could be considered for the intensive production of Atlantic cod or other marine fish species requiring *Artemia* at some stage of development.

1.2 Atlantic Cod Biology and Culture

Atlantic Cod (*Gadus morhua*) is one of 59 species of the family Gadidae and occurs in the cool waters of northern seas. Cod on average weigh 2-3 kg and are about 60-70 cm long, having three dorsal fins along the back and two anal fins. Cod are usually distinguished by the elongated hair-like projection called a barbel on the chin and they are generally gray or green in colour. Atlantic cod occur from inshore shallow water of about 5 m, to the edge of the continental shelf in water as deep as 600 m. They are

distributed from Frobisher Bay in the north to Cape Hatteras in the south, becoming more abundant along the coast of Labrador and off Newfoundland (DFO, 2007).

The female Atlantic cod becomes sexually mature at 6 years of age with a range from 5 to 8 years, depending on the stock. Cod start spawning in March and continue into June depending on the stock and the water temperature; usually around 2.5 to 4° C. Female cod 80 cm long can produce about two million eggs while cod about 130 cm long can produce over eleven million eggs. The eggs are buoyant, round and 1-2 mm in diameter and they float in the surface water, which is at about 30‰ salinity. The fertilized eggs rise to the surface where they hatch and the resulting larvae are at the mercy of the currents where the mortality rates are very high. In nature, only 1 egg in every million will survive to be an adult cod (DFO, 2007).

The intensive culture of Atlantic cod larvae in tanks greatly increases the chances that a cod larva will reach adulthood. Survival rates for cod larvae from egg to juvenile range from 10% to 30% and higher depending on broodstock and egg quality. Broodstock spawn naturally in captivity and their spawning periods can be manipulated so that different groups of broodstock spawn at different times throughout the year. Once the eggs are fertilized they are allowed to incubate in conical tanks until they hatch, about two weeks later depending on water temperature. The newly hatched larvae are moved to larger tanks where first feeding with rotifers and marine microalgae can begin. Once the cod larvae are feeding heavily on rotifers and are large enough for a larger prey item, *Artemia* are introduced. The larvae readily consume the *Artemia* and within a matter of days this is their only food source. Enriched *Artemia* are then fed to the larvae until the

onset of metamorphosis, at which time dry starter feeds are introduced to the fish and by the end of metamorphosis the juvenile cod are completely weaned to a dry diet.

1.3 Objectives

This thesis is a study of the nutritional value of *Artemia* fed to Atlantic cod larvae during the period between the rotifer feeding stage, and the onset of metamorphosis and weaning to dry diet. Growth and survival of cod larvae were studied based on the lipid and amino acid content and composition of the enriched *Artemia* used in the feeding treatments.

Chapter 2 discusses the lipid, fatty acid and amino acid composition of the enriched *Artemia* diets in relation to the enrichment product used during enrichment. Chapter 3 discusses the Atlantic cod larvae and the effects that enriched and unenriched *Artemia* diets had on growth and survival of the larvae. Chapter 4 discusses what was found in the overall experiment in relation to the nutritional content of live food and the successful rearing of cod larvae. Then a conclusion is made as to how protein and amino acids contribute to growth and survival in larvae and how best to incorporate amino acids into enrichment protocols for *Artemia.*

Chapter 2

Lipid, Fatty Acid and Amino Acid Composition of Enriched and Unenriched Live Food (*Artemia*) Diets

2.1 Introduction

Rotifers and *Artemia* have been the first feeding organisms of choice for large-scale production of marine larvae, even though they lack many of the nutritional components required by the developing larvae. *Artemia* have the unique ability to form dormant cysts, which are easily stored in cans for long-term use and shipment. These dormant cysts can have their outer shell removed by a simple decapsulation process (see Appendix A, page 74), which allows the egg to hatch out over a 24 hour period in warm seawater. The resulting nauplii can then be fed to larvae or enriched with emulsions rich in lipid, fatty acids and/or protein and then fed to marine finfish larvae. Only Instar II *Artemia* should be enriched as newly hatched Instar I *Artemia* do not actively digest food. The *Artemia* usually has developed to Instar II at about 8 hours after hatch and is able to filter small food particles (1 – 50 μm) out of the water by the 2nd antennae and ingest these particles into the functional digestive tract (Van Stappen, 1996).

Polyunsaturated fatty acids like docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) are widely considered essential to the proper growth and development of marine larval fish. Fish cell membranes, as well as fish tissues, being particularly rich in DHA, have a correspondingly high dietary requirement for the ω-3 PUFA's. Even though it is present in small amounts, ARA does fulfill a

membrane structural role in fish but plays a much more important role as a precursor to eicosanoid production. Studies have shown that a diet high in polyunsaturated fatty acids like DHA, EPA and ARA are important for proper larval development. However, EPA is present in low amounts in *Artemia* nauplii and DHA is practically absent. For this reason, the nauplii need to be enriched before they can be used for feeding marine larvae (Navarro et al., 1999). The degree of success in modifying the fatty acid profile of the nauplii has been shown to be influenced by the enrichment diet used, the conditions of the enrichment and the strain of *Artemia* being used (Han et al., 2000). The method used for storing the enriched *Artemia* between feedings is also very important because *Artemia* metabolize DHA following enrichment (Danielsen et al., 1995; Evjemo et al., 1996). According to Rainuzzo et al. (1997), the lipid requirements of the larvae, especially for the ω-3 HUFA, may be lower during the *Artemia* stage if the larvae have already been supplied with the necessary fatty acids at the rotifer stage.

Since amino acids are the building blocks for protein synthesis and are essential for specific physiological functions, *Artemia* were enriched with a hydrolyzed krill protein to increase the amount of protein and amino acids available to the first feeding larvae. Fish larvae seem to have a higher requirement than older fish for protein but little is known about the specific amino acid requirements of fish larvae after the start of exogenous feeding (Dabrowski, 1986). During this time, it seems that the not yet fully developed larval digestive system is unable to digest complex proteins (Dabrowski, 1986). However, it has been found that 50% of the total amino acids in marine pelagic eggs are in their free form (Fyhn, 1989). These free amino acids are important energy

sources for larval fish as they are present in high quantities in the natural environment in zooplankton and phytoplankton (Rønnestad et al., 1992, 1994; Finn et al., 1995; Helland et al., 2003). Alanine, leucine, serine, isoleucine, lysine and valine together accounted for 75% of the disappearing free amino acids (FAA) (Fyhn and Serigstad, 1987). These were the six FAA's that Fyhn discovered to be mostly used as an energy source early in larval development and as a result are the free amino acids that are the focus of this study.

2.2. Materials and Methods

2.2.1 Enrichment Media Preparation

1. **DHA Selco® (INVE, Dendermond, Belgium)** – This was mixed in filtered water with a handheld blender at a concentration of 2 grams of DHA Selco per million *Artemia*.
2. **Spray-Dried Krill Hydrolysate (SD-KH2) – Specialty Marine Products Limited. Vancouver, B.C.**

Fresh krill are captured and go through a bio-process including hydrolysis which breaks down the long-chain proteins into shorter-chain peptides. According to company literature, the protein fraction of this Krill Protein Hydrolysate contains long and short chain polypeptides and free amino acids. This Krill Protein Hydrolysate also contains a wide range of fatty acids with a HUFA content of about 6% ω-6 and 35% ω–3. This krill protein enrichment is made using *Euphausia superba* with a typical crude protein of 60-65%. Particles size composition of the krill protein hydrolysate powder is as follows:

>1000 μm	= 10%
600-800 μm	= 10%
60-80 μm	= 15%

20-40 μm = 65%

Enrichment Mixture Method for the Krill Protein Hydrolysate (SD-KH2)

This enrichment was mixed according to the manufacturer's recommendation. Twenty-eight grams of the krill hydrolysate powder was mixed with 30 g of high quality fish oil (capelin oil used here) to create a thick brown/red paste. Seven grams of this paste was taken and mixed in a high-speed blender with 160 ml of water. Fourteen mls of the resulting milky liquid was added to 4 L of water and this volume was then fed to the *Artemia* in 2 feedings (4 pm and 12 am).

3. **AlgaMac 3010® (Aquafauna-BioMarine Inc., Hawthorne, CA, USA)** – This was mixed in filtered water with a handheld blender at a concentration of 2 g per million *Artemia.*

2.2.2 *Artemia* Enrichment Procedure

Artemia were decapsulated and placed in an *Artemia* hatching cone (300 L conical tank) overnight. Newly hatched *Artemia* nauplii were then rinsed and placed in the appropriate enrichment media. The enrichment was carried out in conical containers using 250 animals per ml of water.

Artemia enrichments were set-up in the afternoon, with enrichment given at 4 pm and 12 am. *Artemia* were washed in the morning and fed to the larvae according to feeding treatment. Any *Artemia* remaining from the morning feeding were held at 8-10°C for the rest of the day and fed to the larvae at 12 pm and 4 pm. The unenriched and enriched *Artemia* were separated into 4 different treatments:

1) Unenriched. 2) AlgaMac 3010. 3) DHA Selco. 4) Krill Protein Hydrolysate.

2.2.3 *Artemia* Lipid and Fatty Acid Extraction Method

Artemia were placed in each enrichment medium in triplicate at 4 pm and left to enrich overnight until 9 am (17 hr). The unenriched *Artemia* treatment was held in warm seawater with no enrichment during the enrichment period. *Artemia* samples from each enrichment cone were removed, washed and concentrated into vials with no water, placed directly in chloroform and stored at -20^{o} C until processed. Lipids were extracted in chloroform/methanol according to Parrish (1998), using a modified Folch procedure (Folch et al., 1957). Lipid classes were determined by thin layer chromatography with flame ionization detection (TLC/FID) in an Iatroscan (Parrish, 1987). Extracts were spotted on silica gel coated Chromarods and a three stage development system was used to separate lipid classes.

2.2.4 Amino Acid Sampling (*Artemia*)

a) Acid Hydrolysis

The sample (0.5-2 mg) was hydrolyzed in 1 ml of 6 N HCl with 0.05% phenol, using Corning 16 x 100 mm culture tubes with Teflon lined screw caps. The tubes were purged for 5 min. with nitrogen before capping. Samples were hydrolyzed for 24 hr at 110^{o}C. The HCl was removed under vacuum and the dried sample reconstituted with pH 2.2 sodium citrate buffer (0.2 M Na^{+}) prior to analysis (Blackburn, 1978; Ozols, 1990).

b) Amino Acid Analysis – Physiological Free Amino Acids

i. Deproteinization

Samples were deproteinized with 10% sulfosalicylic acid (SSA) using 1 part SSA to 4 parts sample and 3 parts lithium citrate buffer pH 2.2, 0.3 N Li^+ for a final dilution of 1:2. Samples were vortexed, let stand for 20 min. at 4 °C and then spun at 12,000 g for 5 min. The supernatant was removed and filtered through a 0.2 μm nylon filter.

ii. Analysis

Deproteinized samples were analyzed on a Beckman 121 MB Amino Acid Analyzer using Benson D-X8, 0.25 Cation Xchange Resin and a single column, three buffer lithium method as per Beckman 121 MB –TB –017 application notes. Quantitation of the results was achieved using a Hewlett Packard Computing Integrator Model 3395A.

2.2.5 Statistical Analysis

One-way ANOVA tested for significant differences within and between treatments for all data sets and was followed by Tukey's multiple comparison tests. A significance level of $P<0.05$ was set for all comparisons. Survival percentages were converted to arcsine values for the one-way ANOVA. Error bars on all graphs are standard deviations.

2.3 Results

2.3.1 Total Lipid, Lipid Class and Fatty Acid Composition of the Enrichment Media

Table 2.1 shows the concentrations of TL, TAG and PL in the enrichment media as compared to the concentrations in the *Artemia* after enrichment. The DHA Selco and

Krill Protein enrichments contributed the highest percent of TL to the enriched *Artemia* (5.6% & 5.5%) while AlgaMac 3010 contributed 3.0%. The greatest incorporation of PL came from the Krill Protein Hydrolysate, incorporating 25.5% during the enrichment period (Table 2.1). The AlgaMac 3010 and DHA Selco enrichment products incorporated approximately the same amount of PL during the *Artemia* enrichment, 13.3% and 13.9%, respectively. TAG concentrations in the enriched *Artemia* were contributed to equally by the Krill Protein Hydrolysate and DHA Selco treatments, with 3.9% and 3.6% being incorporated into the *Artemia,* respectively. Only 1.2% of the TAG in the AlgaMac 3010 enrichment product was incorporated into the *Artemia.*

Table 2.1 compares the amount of ARA, EPA, DHA present in the enrichment media with the concentrations found in the enriched *Artemia*. This table shows that a relatively small portion (<10%) of the ARA, EPA, DHA content of the Krill Protein Hydrolysate, DHA Selco and AlgaMac 3010 media was eventually incorporated into the *Artemia* during the enrichment period.

Even though the Krill Protein enrichment contained the lowest amount of DHA, 10 times lower than the DHA concentration in DHA Selco, it incorporated a proportion of DHA in the *Artemia* equal to that of DHA Selco (Table 2.1: Krill- 0.9%; DHA Selco - 0.9%). The *Artemia* incorporated only 0.35% of the DHA found in AlgaMac 3010. The enriched *Artemia* incorporated equal amounts of EPA from all the enrichment products: DHA Selco, 3.5%; Krill Protein, 3.5%; AlgaMac 3010, 3.3%. During the enrichment period, the *Artemia* incorporated the most ARA from the Krill Protein enrichment

(7.7%). DHA Selco and AlgaMac 3010 enrichment products resulted in the incorporation of 4.1% and 1.7%, respectively, of their ARA content into the *Artemia* diet.

Table 2.1: Summary of total lipid (TL), triacylgylcerol (TAG), phospholipid (PL) and fatty acid (ARA, EPA, DHA) concentrations (mg g^{-1}) in the enrichment media, enriched *Artemia* and the percentage incorporated.

	DHA Selco Enrichment (mg/g)	DHA Selco *Artemia* (mg/g)	DHA Selco (% incorporated)
TL	646.3	36.2	5.6
TAG	533.6	19.1	3.6
PL	25.1	3.5	13.9
ARA	8.1	0.3	4.0
EPA	33.5	1.2	3.5
DHA	100.2	0.9	0.9

	Krill Protein Enrichment (mg/g)	Krill Protein *Artemia* (mg/g)	Krill Protein (% incorporated)
TL	489.9	27.1	5.5
TAG	361.7	14.0	3.9
PL	22.0	5.6	25.5
ARA	2.7	0.2	7.7
EPA	19.1	0.7	3.5
DHA	10.8	0.1	0.9

	AlgaMac 3010 Enrichment (mg/g)	AlgaMac 3010 *Artemia* (mg/g)	AlgaMac 3010 (% incorporated)
TL	426.8	12.9	3.0
TAG	341.9	4.0	1.2
PL	20.6	2.7	13.3
ARA	8.3	0.1	1.7
EPA	7.4	0.3	3.3
DHA	14.1	0.1	0.4

2.3.2 Free Amino Acid Composition of the Enrichment Media

Six free amino acids (FAA) were selected based on evidence from Rønnestad et al. (1996) that show alanine, lysine, serine, leucine, isoleucine and valine being abundant in pelagic eggs and accounting for 75% of the disappearing FAA early in larval development (Fyhn and Serigstad, 1987). These selected FAAs were measured in the enrichment media used to enrich *Artemia* for first feeding cod larvae. Table 2.2 shows that the Krill Protein Hydrolysate contained substantially more of all the selected free amino acids than the AlgaMac 3010 enrichment product. DHA Selco is an oil emulsion and could not be analyzed for free amino acids because oil could not be placed in the protein extraction column, which is a necessary step in analyzing free amino acids.

Table 2.2 shows the amount of FAA incorporated into the enriched *Artemia* as a percentage of that found in the enrichment media alone. The Krill Protein enriched *Artemia* incorporated 19.4% of the Krill Protein enrichment serine level, even though the Krill Protein contained low amounts of serine (5.7 μmoles g^{-1}) relative to the other FAAs. Conversely, the Krill Protein Hydrolysate enrichment contained a high concentration of alanine (20.6 μmoles g^{-1}) but *Artemia* incorporated the lowest percentage (10.4%) of all FAA studied.

Except for alanine, all FAA levels in the AlgaMac 3010 enriched *Artemia* were much greater (approx. 100% - 500% higher) than the concentrations found in AlgaMac 3010 enrichment. Since the amounts of FAAs in the unenriched *Artemia* were higher than the concentrations in both the Krill Protein and AlgaMac 3010 enriched *Artemia*, the unenriched *Artemia* could be used as a reference for FAA uptake during the enrichment

period. The unenriched *Artemia* contained up to 67% more of each FAA than *Artemia* enriched in Krill Protein or AlgaMac 3010. The concentration of FAA in Krill Protein and AlgaMac 3010 enriched *Artemia* as a percentage of the concentrations found in unenriched *Artemia* were respectively: alanine (54% and 50% of unenriched), leucine (39% and 66% of unenriched), serine (44% and 56% of unenriched), isoleucine (40% and 67% of unenriched), lysine (32% and 59% of unenriched) and valine (44% and 62% of unenriched).

Table 2.2: Summary of free amino acid (FAA) concentrations (μmoles g^{-1} wet wt) in the enrichment media, enrichment manufacturer's data, enriched *Artemia* and the percentage of FAA incorporated. DHA Selco was not analyzed because it is an oil emulsion.

	Krill Protein Hydrolysate (μmoles g^{-1})	Krill Protein Hydrolysate (% dry weight) Manufacturer's data	Krill Protein *Artemia* (μmoles g^{-1})	FAA (% incorporated)
Alanine	20.6	3.12	2.14	10.4
Leucine	15.2	3.80	2.13	14.1
Serine	5.7	2.12	1.10	19.4
Isoleucine	7.5	2.29	1.20	16.0
Lysine	15.6	3.18	2.87	18.4
Valine	9.9	2.69	1.11	11.3

	AlgaMac 3010 (μmoles g^{-1})	Algamac 3010 (mg g^{-1}) Manufacturer's data	AlgaMac 3010 *Artemia* (μmoles g^{-1})	FAA (% incorporated)
Alanine	2.33	7.5	2.32	99.6
Leucine	0.21	7.0	1.21	574.6
Serine	0.26	4.6	0.86	330.8
Isoleucine	0.18	4.0	0.66	365.9
Lysine	0.30	5.3	1.74	579.6
Valine	0.66	6.1	0.74	112.1

2.3.3 Total Lipid, Lipid Classes and Fatty Acid Composition of the Diets (*Artemia)*

Table 2.3 summarizes the concentrations of TL, total fatty acids (TFA), TAG and PL in the enriched and unenriched *Artemia* with the statistical data. TL concentrations in DHA Selco enriched *Artemia* were significantly greater than those of the AlgaMac 3010 enriched *Artemia* (P= 0.024). The Krill Protein enriched *Artemia* and the unenriched *Artemia* both had TL levels close to that of DHA Selco enriched *Artemia* but were not significantly different from the AlgaMac 3010 treatment (Table 2.3, Figure 2.1).

Artemia enriched with AlgaMac 3010 also had significantly lower levels of both TFA (P= 0.036) and TAG (P= 0.036) than the DHA Selco enriched *Artemia.* AlgaMac 3010 enriched *Artemia* had much lower concentrations of TAG than in the other 3 treatments but only significantly different from the DHA Selco treatment (P= 0.036). Krill Protein enriched *Artemia* and the unenriched *Artemia* treatments contained high TFA concentrations but were not significantly higher than AlgaMac 3010.

Table 2.3 shows that PL was found to be significantly higher in the AlgaMac 3010 enriched *Artemia* than all other treatments, whereas PL concentrations in DHA Selco enriched *Artemia* were the same as those in unenriched *Artemia.* Krill Protein enriched *Artemia* had PL levels not significantly different from DHA Selco enriched *Artemia* but had significantly higher PL levels than unenriched *Artemia.*

Please refer to Appendix A (Tables A1-A5) for further details of the Tukey's Multiple Comparison Test results.

Table 2.3: Total lipid, total fatty acid, triacylglycerol and phospholipid concentrations (mg g^{-1} ± st. dev.) in *Artemia* enriched with DHA Selco, Krill Protein, AlgaMac 3010, and unenriched *Artemia* as a control.

	Total Lipid	Total Fatty Acid	Triacylglycerol	Phospholipid
Unenriched	24.2 ± 2.92^{ab}	17.5 ± 1.54^{ab}	8.97 ± 0.75^{ab}	1.56 ± 0.29^{a}
DHA Selco	36.2 ± 11.61^{a}	26.9 ± 10.9^{a}	18.3 ± 8.30^{a}	2.50 ± 0.59^{ab}
Krill Protein	27.4 ± 2.49^{ab}	21.2 ± 2.45^{ab}	13.4 ± 1.58^{ab}	3.99 ± 1.0^{b}
AlgaMac 3010	12.9 ± 0.50^{b}	7.25 ± 0.09^{b}	3.57 ± 0.11^{b}	6.80 ± 0.48^{c}

* Different superscripts indicate a significant difference ($P<0.05$).

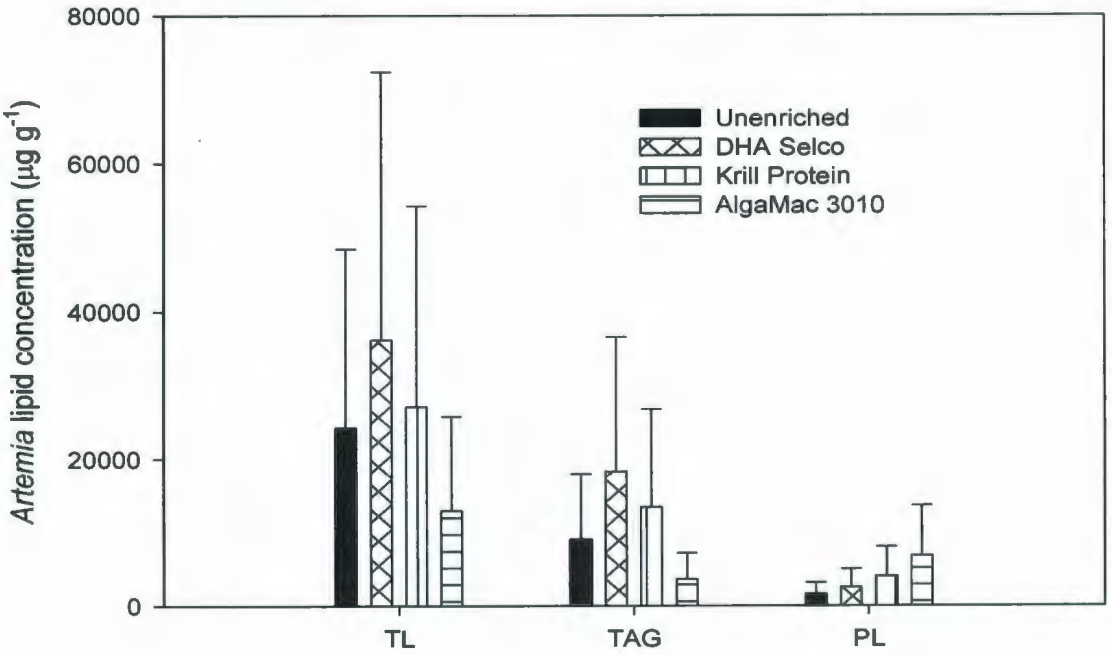


Figure 2.1: Total lipid (TL), triacylglycerols (TAG) and phospholipid (PL) concentrations (µg g^{-1}) in enriched and unenriched *Artemia*.

Table 2.4: Total lipid, selected lipid class and fatty acid composition of the *Artemia*

	Enrichment Treatments			
	Unenriched	**DHA Selco**	**Krill Protein**	**Algamac 3010**
Total lipid (mg g^{-1} dw)	24.2 ± 2.9	36.2 ± 9.4	27.1 ± 2.0	12.9 ± 0.3
Lipid Class (% total lipids)				
Triacylglycerols	9.0 ± 0.27	0.6 ± 1.0	49.4 ± 0.4	7.5 ± 0.05
Sterols	1.7 ± 0.3	2.5 ± 0.9	1.6 ± 0.1	1.3 ± 0.0
Phospholipids	6.2 ± 0.05	6.9 ± 3.7	39.9 ± 0.0	2.7 ± 0.7
Fatty Acid (% total fatty acids)				
14:0	2.4 ± 0.1	1.3 ± 1.0	0.8 ± 0.1	1.4 ± 0.0
16:0	15.6 ± 0.1	15.1 ± 0.1	15.9 ± 0.1	15.7 ± 0.0
18:0	3.1 ± 0.36	3.6 ± 1.3	2.6 ± 0.24	0.88 ± 0.0
∑SFA[a]	**22.8 ± 1.0**	**24.5 ± 3.6**	**22.9 ± 0.7**	**22.1 ± 0.3**
16:1ω7	3.2 ± 0.1	4.1 ± 0.1	4.1 ± 0.0	3.6 ± 0.0
18:1ω9	18.4 ± 0.2	19.0 ± 0.2	25.5 ± 0.5	15.3 ± 0.0
∑MUFA[b]	**22.2 ± 0.4**	**24.8 ± 1.0**	**30.2 ± 0.5**	**20.2 ± 0.1**
18:2ω6	3.8 ± 0.1	4.5 ± 0.2	7.5 ± 0.1	3.6 ± 0.6
20:4ω6(ARA)	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	2.0 ± 0.0
20:5ω3(EPA)	2.5 ± 0.4	4.3 ± 0.4	3.1 ± 0.0	3.4 ± 0.0
22:6ω3(DHA)	0.2 ± 0.0	3.2 ± 0.3	0.3 ± 0.0	6.9 ± 0.0
∑PUFA[c]	**4.4 ± 0.7**	**13.4 ± 1.4**	**11.9 ± 0.3**	**13.9 ± 0.2**

[a] includes ai-15:0, 15:0, i-17:0,17:0, and 20:0
[b] includes 18:1ω-5, 20:1ω-9, 20:1ω-7, and 24:1
[c] includes 16:2ω-4, 16:4ω-3, 20:2ω-6, 20:3ω-6, and 20:4ω-3.

Artemia were enriched in a Krill Protein Hydrolysate and two commercial products with high levels of total lipid and/or ω–3 fatty acids (Table 2.4: DHA Selco and AlgaMac 3010). DHA concentrations were higher in the DHA Selco enriched *Artemia* than in the Krill Protein enriched *Artemia* (Table 2.5) (P= 0.014). AlgaMac 3010 contained fairly high proportions of DHA as well, 6.9% of TFA, as compared to 3.2% in DHA Selco. EPA and ARA levels, as percent of total fatty acid (TFA), were highest in both the DHA Selco (4.3%, 1.2%) and AlgaMac 3010 (3.4%, 1.95%) enriched *Artemia*. Krill Protein enriched *Artemia* had the lowest levels of EPA (3.1% of TFA) and ARA (0.99% of TFA). The unenriched *Artemia* were significantly different from the DHA Selco enriched *Artemia* in concentrations of ARA, EPA, DHA (P= 0.009) but were not significantly different from all other treatments.

Figure 2.2 shows that the DHA Selco enriched *Artemia* contained a higher concentration of ARA, EPA and DHA than the other enrichment treatments, significantly higher than the unenriched and Krill Protein enriched *Artemia*. AlgaMac 3010 enriched *Artemia* contained low concentrations of EPA but higher levels of DHA, whereas the Krill protein enriched *Artemia* contained high concentrations of EPA and low concentrations of DHA. No significant difference was found between the AlgaMac 3010, Krill Protein and unenriched treatments.

Table 2.5: Comparison of TFA, ARA, EPA and DHA concentrations (mg g^{-1} wet weight) and proportion (%) in enriched and unenriched *Artemia*. Data are mean ± st. dev.

Treatment	TFA (mg g^{-1})	ARA (mg g^{-1})	% ARA (TFA)
Unenriched	17.5 ± 1.3^{a}	0.19 ± 0.0^{a}	1.09 ± 0.1^{a}
DHA Selco	26.9 ± 8.9^{ab}	0.32 ± 0.1^{b}	1.20 ± 0.8^{a}
Krill Protein	21.2 ± 2.0^{a}	0.22 ± 0.02^{a}	0.99 ± 0.2^{a}
AlgaMac 3010	7.20 ± 0.1^{ac}	0.14 ± 0.02^{ab}	1.95 ± 0.2^{a}

Treatment	TFA (mg g^{-1})	EPA (mg g^{-1})	% EPA (TFA)
Unenriched	17.5 ± 1.3^{a}	0.43 ± 0.01^{a}	2.48 ± 0.2^{a}
DHA Selco	26.9 ± 8.9^{ab}	1.16 ± 0.47^{b}	4.32 ± 0.3^{b}
Krill Protein	21.2 ± 2.0^{a}	0.67 ± 0.06^{ab}	3.14 ± 0.4^{ac}
AlgaMac 3010	7.20 ± 0.1^{ac}	0.25 ± 0.02^{a}	3.36 ± 0.2^{ab}

Treatment	TFA (mg g^{-1})	DHA (mg g^{-1})	% DHA (TFA)
Unenriched	17.5 ± 1.3^{a}	0.04 ± 0.01^{a}	0.21 ± 0.0^{a}
DHA Selco	26.9 ± 8.9^{ab}	0.85 ± 0.32^{b}	3.17 ± 0.3^{b}
Krill Protein	21.2 ± 2.0^{a}	0.10 ± 0.01^{a}	0.39 ± 0.0^{a}
AlgaMac 3010	7.20 ± 0.1^{ac}	0.50 ± 0.00^{ab}	6.91 ± 0.0^{c}

Different superscript letters indicate a significant difference at $P<0.05$.

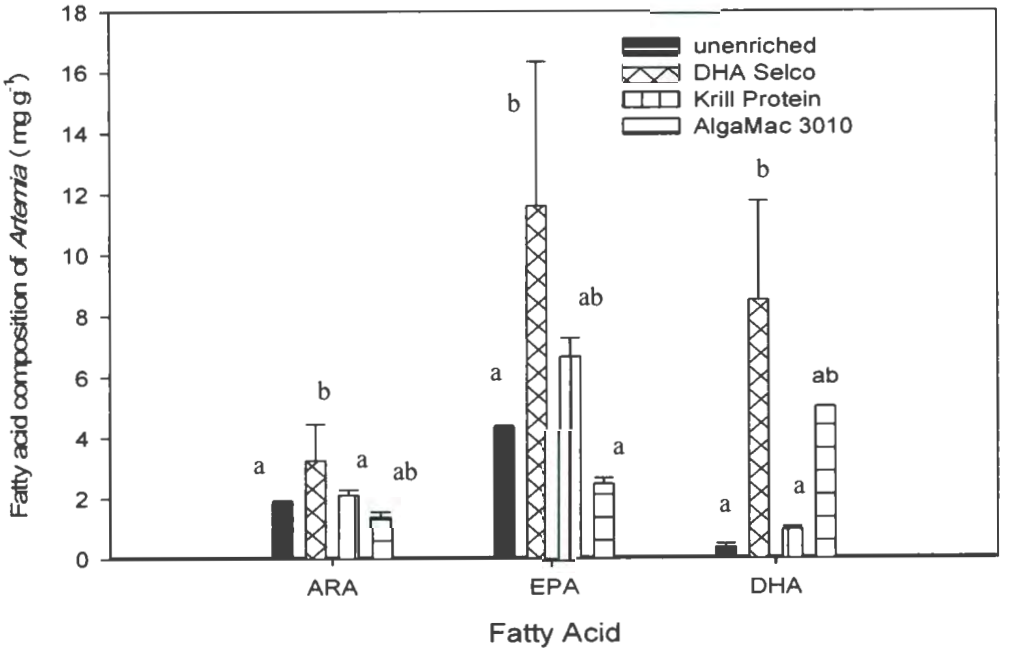


Figure 2.2: Comparison of arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) concentrations (mg g^{-1}) in *Artemia* enriched with DHA Selco, a Krill Protein Hydrolysate and AlgaMac 3010. Unenriched *Artemia* was a control. Different superscript letters indicate a significant difference ($P<0.05$).

2.3.4 Free Amino Acid Composition of the Diets (*Artemia)*

DHA Selco enriched *Artemia* and the unenriched *Artemia* contained higher levels of all amino acids measured (Figure 2.3; Table 2.6). Alanine levels were significantly higher in the DHA Selco treatment than in the Krill Protein treatment (P= 0.010) and the unenriched *Artemia* had significantly more alanine than the AlgaMac 3010 treatment (P= 0.047) (Table 2.6). Serine was also found in high levels in the DHA Selco treatment and the unenriched *Artemia*, significantly higher than both the Krill Protein (P= 0.001; P= 0.005) and AlgaMac 3010 enriched *Artemia* (P= 0.001; P= 0.006). Unenriched *Artemia* had the highest amounts of leucine, isoleucine, lysine and valine, significantly higher in all instances than the AlgaMac 3010 (P= 0.033). DHA Selco enriched *Artemia* contained high levels of these FAA's, just slightly lower than that found in unenriched *Artemia.* The Krill Protein enriched *Artemia* contained approximately half of the amount of leucine, isoleucine and valine than that found in the unenriched *Artemia.* In the matter of valine, the unenriched *Artemia* had significantly more than the AlgaMac 3010 (P= 0.006) enriched *Artemia.*

Analysis of Variance (one-way ANOVA) was also conducted on total non-essential and essential amino acid data for enriched and unenriched *Artemia* but no significant differences were found among treatments for these amino acids. The non-essential amino acids considered were: serine, glutamic acid, alanine, proline. The essential amino acids were: lysine, arginine, valine, isoleucine, and leucine.

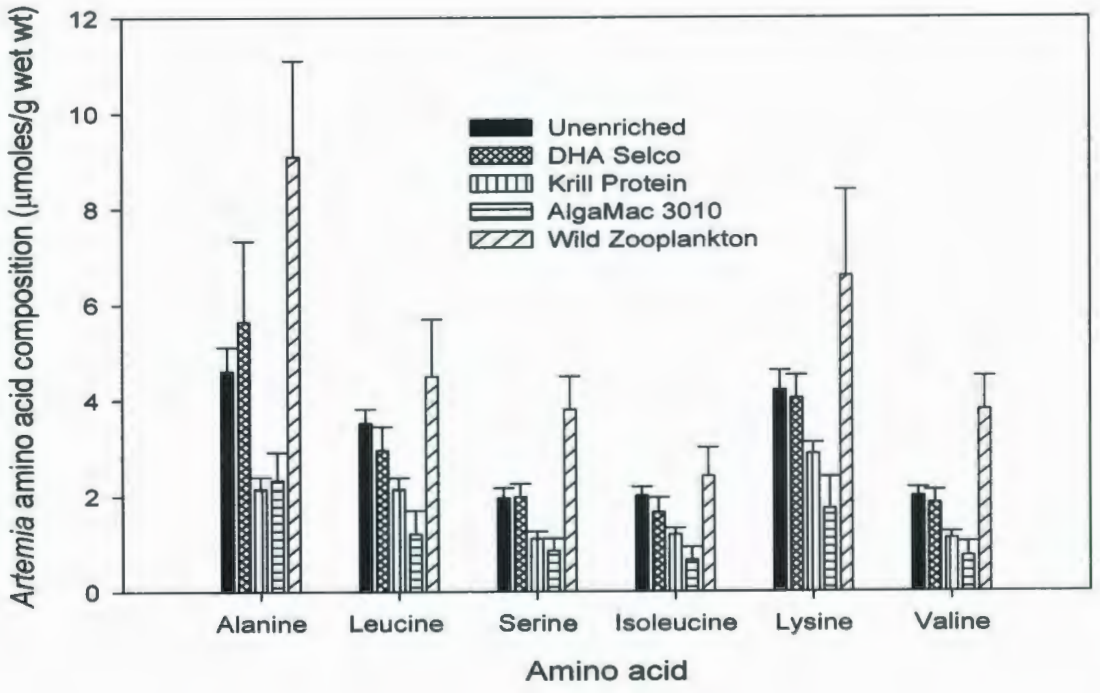


Figure 2.3: Comparison of free amino acid (FAA) concentrations (µmoles g^{-1} wet wt) in *Artemia* enriched with DHA Selco, a Krill Protein Hydrolysate and AlgaMac 3010. Unenriched *Artemia* was a control and wild zooplankton data from Næss et al. (1995).

Table 2.6: Comparison of selected free amino acid concentrations (μmoles gram^{-1} wet wt) in *Artemia* enriched with DHA Selco, Krill Protein, AlgaMac 3010. Unenriched *Artemia* was a control.

	Alanine	**Leucine**	**Serine**
Unenriched	4.61 ± 0.50^{ab}	3.52 ± 0.30^{a}	1.96 ± 0.20^{a}
DHA Selco	5.65 ± 1.68^{a}	2.94 ± 0.50^{ab}	1.98 ± 0.26^{a}
Krill Protein	2.14 ± 0.25^{b}	2.13 ± 0.24^{ab}	1.10 ± 0.16^{b}
AlgaMac 3010	2.32 ± 0.58^{cb}	1.21 ± 0.49^{b}	0.86 ± 0.25^{b}

	Isoleucine	**Lysine**	**Valine**
Unenriched	2.00 ± 0.17^{a}	4.21 ± 0.42^{a}	1.99 ± 0.17^{a}
DHA Selco	1.66 ± 0.30^{ab}	4.04 ± 0.48^{ab}	1.85 ± 0.26^{ab}
Krill Protein	1.20 ± 0.13^{ab}	2.87 ± 0.23^{ab}	1.11 ± 0.14^{ab}
AlgaMac 3010	0.66 ± 0.27^{b}	1.74 ± 0.65^{b}	0.74 ± 0.30^{b}

2.4. Discussion

2.4.1 Lipids and Fatty Acids

It is widely accepted that marine larvae require the ω-3 highly unsaturated fatty acids (HUFA) ARA, EPA, and DHA for normal development and survival. For years the focus on these ω-3 fatty acids has, for the most part, overshadowed the study of the role that amino acids and proteins play in the diet of first feeding marine larvae. This study incorporated a protein-rich hydrolyzed krill protein into the *Artemia* enrichment regime for feeding to Atlantic cod larvae and compared these results with the results of two commercially used lipid-rich *Artemia* enrichment products.

Artemia nauplii contain almost no DHA, and EPA is present in low amounts (Figure 2.2), so *Artemia* have to be enriched with these fatty acids before they are presented to the developing larvae. Through enrichment with DHA Selco, which is high in DHA, the amount of DHA found in the enriched *Artemia* was about eight times higher than that found in the unenriched *Artemia*. DHA Selco enriched *Artemia* also showed more than twice the amount of EPA than that in the unenriched *Artemia*. It could be assumed that enriched *Artemia* are merely bioencapsulated food items containing exactly the nutrient content of the enrichment product used. McEvoy et al. (1996) found that this is certainly not the case; they showed that *Artemia* enriched with an enrichment containing 75% total lipid as ethyl esters did not contain ethyl esters after an 18 hr enrichment but showed a marked increase in their content of TAG. This suggested that *Artemia* metabolize the enrichment they are immersed in, and incorporate the lipids and FAs into their own energy reserves. The DHA Selco enriched *Artemia* in this study did

show an increase in TAG over that found in the unenriched *Artemia* nauplii. However, AlgaMac 3010 enriched *Artemia* also showed a marked increase in DHA but the TAG level in this treatment was actually lower than that found in the unenriched *Artemia* (Figures 2.1, 2.2). Navarro et al. (1999) clearly demonstrated that *Artemia* have the ability to retroconvert DHA to EPA during enrichment and subsequent starvation. This may explain the large amount of EPA found in the DHA Selco enriched *Artemia,* as seen in Figure 2.2. ARA is a required precursor in the production of eicosanoids and thus an important HUFA (Castell et al., 1994; Sargent et al., 1999). ARA was found to be highest in DHA Selco enriched *Artemia*, which also produced the highest levels of DHA and EPA.

Navarro et al. (1999) showed that the ethyl esters of polyunsaturated fatty acids (PUFA), the form in which PUFA are commonly present in commercial enrichments, are rapidly transformed into other lipid classes such as TAG and PL. The DHA Selco enriched *Artemia* contained higher levels of TL, TFA and TAG than the unenriched, Krill Protein and AlgaMac 3010 enriched *Artemia*. Zooplankton have a relatively low total lipid content, about 9-12% dry weight, and a high DHA content, as much as 40% of the total fatty acids present (Evjemo and Olsen, 1997). Zooplankton, a natural food source for marine larvae, should give the best results for normal development and survival, with its low total lipid content and high DHA content. DHA Selco enriched *Artemia* contained high levels of ARA, EPA, DHA, TL, TFA and TAG; would *Artemia* enriched in DHA Selco produce optimal results when it comes to proper growth and development in first feeding cod larvae? Other enrichment products, like, AlgaMac 3010, produced high PL

concentrations in enriched *Artemia* and boosted DHA concentrations by about 4.5 times those found in the unenriched *Artemia*.

According to Rainuzzo et al. (1997), TAG is used to satisfy energy demands and is always metabolized before PL, which is conserved. In the AlgaMac 3010 enriched *Artemia*, PL was conserved and if any TAG had been taken up during the enrichment it was used to satisfy energy demands before sampling had occurred. TAG is also the primary storage molecule in teleost fish and we can expect lipid mobilization if there is depletion of TL and TAG in the fish tissue (Sheridan, 1988).

2.4.2 Free Amino Acids

Free amino acids (FAA) make up a substantial portion of the total amino acids found in pelagic egg species, between 40-60% of the dry mass of the egg. By the time the yolk sac has been absorbed by the newly hatched larvae, just before first feeding, the FAA pool will have been mostly depleted (Fyhn, 1989). The rapid use of this FAA pool by the newly hatched larvae suggests that FAAs are crucial components of the larval diet when they begin exogenous feeding. Rønnestad and Næss (1993) also support this conclusion; Atlantic halibut larvae derived about 60% of their energy requirements during the first 30 days of exogenous feeding from amino acids in the diet. From this high percentage they concluded that amino acids, preferably in their free form, should comprise a large part of the larval diet. Alanine, leucine, serine, isoleucine, lysine and valine were chosen as the focus of the amino acid analysis in this study due to the fact that Fyhn and Serigstad (1987) found that these FAA accounted for 75% of the FAA

being used as an energy source in first feeding larvae. Fyhn (1989) also found threonine, arginine and aspartic acid to be important FAAs. Taurine was also considered in this study due to the fact that Halfyard (2002) found a strong relationship between the free amino acid taurine and growth and survival in first feeding wolffish larvae. However, although taurine is found in high levels in demersal egg species, such as wolffish, it is found in very low amounts in pelagic eggs (Rønnestad et al., 1999), thus we did not include this free amino acid in our study on Atlantic cod larvae.

Artemia increases its FAA content in response to increased salinity and certain strains of *Artemia* contain much less FAA than do other strains (Helland, 1995). The results in Figure 2.6 show that all FAAs measured were higher in the DHA Selco enriched and the unenriched *Artemia*. The most surprising result was the high FAA concentrations found in the unenriched *Artemia*. Unenriched *Artemia* had significantly more leucine, isoleucine and lysine than AlgaMac 3010 enriched *Artemia* and significantly more alanine, serine and valine than Krill Protein enriched *Artemia*. The high amounts of FAA in the unenriched *Artemia* may be attributed to the original nutritional quality of the *Artemia* cysts or the amount of other nutritional components in the enrichments. Since all *Artemia* were hatched from the same batch of *Artemia* over the duration of the experiment, the reduction in FAA during the enrichment process must be explained. If all the *Artemia* nauplii started out with the same amounts of FAA, then the nutritional composition of the enriched *Artemia* must have changed due to the *Artemia's* own metabolism during enrichment and the type of enrichment used. The unenriched *Artemia* were allowed to sit for the same amount of time as the other enrichments. During

this period with no food it may be concluded that the FAAs were conserved during this holding period, equating to starvation conditions or reduced access to food.

Another reason for the increased FAA concentrations found in unenriched *Artemia* could be that other nutritional components, fatty acids, amino acids, other lipids etc, all have a mass and take up space once eaten / absorbed by the *Artemia.* The high proportions of free amino acids in the unenriched *Artemia* may be explained by considering the absence of other components in the unenriched *Artemia*; like lipids and fatty acids. During the enrichment process, the enrichment product is being taken up by the *Artemia* and the amount of space available to further store amino acids or fatty acids becomes restricted by the body size of the *Artemia*. Carvalho et al. (2003) stated that live food organisms, such as rotifers and *Artemia*, contain only 3-7% of soluble nitrogen as free amino acids. The molecular weight profile of free amino acids should be considered when proposing a feeding regime for fish larvae (Carvalho et al., 2003), as faster absorption of free amino acids can create imbalances and negatively affect larval performance (Rønnestad et al., 2000).

The DHA Selco diet also contained high levels of all studied FAA's, unexpectedly more than that found in the Krill Protein enriched *Artemia*. The Krill Protein Hydrolysate showed lower amounts of FAA than that of both the DHA Selco enriched *Artemia* and the unenriched *Artemia*. Krill Protein may still be a suitable enrichment for a marine larval diet as the larvae should be offered a high, but balanced, amount of FAA (Fyhn, 1989; Rønnestad et al., 1999). Zooplankton have a high protein : lipid ratio compared to live feed (Olsen et al., 1999) so it seems natural that we would

take measures to ensure that there are high levels of HUFAs/lipids as well as proper ratios of free amino acids : protein to create a balanced lipid : protein ratio in the live food diet. Incorporation of a Krill Protein Hydrolysate into current enrichment regimes for *Artemia* should create a more suitable nutritional content, closer to that of wild zooplankton, and balance the lipid:protein ratio produced in the enriched live food. Chapter 3 will consider the incorporation rates of lipids, fatty acids and amino acids into the Atlantic cod larvae.

Chapter 3

Growth, Survival, Fatty Acid and Amino Acid Composition of Atlantic Cod Larvae Fed Enriched and Unenriched *Artemia*

3.0 Introduction

The intensive culture of marine fish larvae often requires large concentrations of live food organisms for feeding. The rotifer and *Artemia* are suitable for mass production in marine hatcheries but lack the nutritional quality of copepods naturally occurring in the ocean. Marine fish larvae require the long chained polyunsaturated fatty acids, ARA, EPA and DHA for their normal growth and development but are unable to elongate 18:3ω-3 to EPA and DHA (Tocher et al., 1989). Marine fish have low or no delta-5 desaturase activity and are incapable of using dietary precursors to make these longer chain fatty acids. As a result, essential fatty acids are required as part of the marine finfish diet due to their important role as components of membrane phospholipids (Hirofumi et al., 1999). It is then essential to enrich or add nutrients to the live food organisms fed to first feeding larvae in order to deliver the necessary dietary elements for proper development.

The specifics of what amounts and ratios of fatty acids to amino acids and lipids to protein required by the larvae of marine fish are still largely unknown. DHA, which is usually found in high amounts in the eggs of marine fish species, is quickly reduced during larval development due to the larval requirement of DHA. However, it is unknown whether DHA is utilized as an energy source or if it is converted to other physiologically important substances during development (Watanabe, 1993). According to Watanabe,

marine larvae require 0.5% to 2.0% of these highly unsaturated fatty acids (HUFA) depending on species and growth stage. In regards to cod eggs, the initial value of 0.61 grams of DHA per 100 grams of eggs decreased by about 20% during development (Watanabe, 1993). The EPA content gradually increased when the larvae started eating rotifers cultured in *Nannochloropsis* rich in EPA.

It has been proposed that a good indication of the amino acid (AA) requirements of larval fish is to use the profile found in the fish carcass or muscle (Watanabe and Kiron, 1994). A first approximation to what extent AA requirements are met by the prey organisms used to feed first feeding larvae can be done by comparing AA profiles in *Artemia* with that found in the larvae (Conceição et al., 2003). Helland et al. (2003) found that the major essential amino acids in *Artemia* nauplii were lysine (7%), arginine (5%), valine (3%) and leucine (3%); glycine, the major non-essential amino acid found in the zooplankton, made up only 5% of the total free amino acids (FAA) in *Artemia* nauplii. The FAA pool of pelagic eggs is dominated by neutral amino acids like leucine, valine, isoleucine, alanine and serine (Rønnestad et al., 1999). Tonheim et al. (2000) demonstrated that the free methionine levels in *Artemia* can be increased by as much as 60 times, through enrichment with a liposome technique. Can the manipulation of the FAA pool of live food have an impact in compensating for AA imbalances in larval fish? If methionine levels can be enhanced in *Artemia* through enrichment, then other FAAs could possibly be incorporated into the live food through enrichment.

Studies indicate that nitrogen quotients are very high during the first weeks of exogenous feeding, which suggests that energy metabolism is geared towards embryonic

development in cod. Alanine, serine, leucine, isoleucine, lysine and valine, in that order, quantitatively dominated the FAA pool and together accounted for 75% of the decrease (Fyhn and Serigstad, 1987). Conceição et al. (2002) studied metabolic budgets for lysine in herring larvae and found that 63% of the lysine was retained in the body and only 22% was oxidized.

Halfyard (2002) found taurine to be an important amino acid in the diet of the wolffish (*Anarhichas lupus)*, whereas Fyhn and Serigstad (1987) reported that taurine remained at a constant level during the embryonic development of cod. This is due to the fact that taurine is an amino acid analogue that is not incorporated into protein and is found to dominate demersal eggs like those of the wolffish. In species like Atlantic cod, which lack an oil globule, the FAA depletion continues beyond hatch (Rønnestad et al., 1999). The digestive tract of marine fish larvae is morphologically and functionally incomplete when they initiate exogenous feeding; so larvae, especially those from pelagic eggs, may require a supply of FAA after depletion of their own reserves (Fyhn et al., 1989).

To further support the requirement for FAA in the larval diet, Fyhn and Serigstad (1987) also reported alanine, leucine and serine as being, quantitatively, the dominating FAAs during the first days after fertilization. Alanine and serine together accounted for 40% of the decrease in the FAA pool during egg development, while alanine and leucine accounted for 40% of the decrease in FAA during the yolksac larvae stage (Fyhn and Serigstad, 1987). Isoleucine, lysine and valine were also present in relatively high amounts. There is strong evidence that these six FAAs dominate the FAA pool during the

egg and yolk sac larvae stages and account for 75% of the disappearing FAA. In *Dentex* eggs, a marine species with pelagic eggs, FAAs accounted for 20% of the total amino acid pool, with serine, alanine and lysine being predominant. The FAA fraction underwent a considerable reduction once the larvae hatched, resembling the pattern reported for other marine species having pelagic eggs (Tulli and Tibaldi, 1997).

In this experiment, the intention was to incorporate krill protein hydrolysate enriched *Artemia* into the existing lipid-rich diet of cod larvae and study the effects on growth and survival.

3.2 Materials and Methods

3.2.1 Larval Rearing

Atlantic cod larvae were taken from a single egg batch and raised to day 39 on AlgaMac 3010 enriched rotifers in the same larval rearing tank. On day 39 post hatch these fish were moved to twenty-four, 30 L aquaria. Sixty-one fish were randomly placed into each aquarium where they remained for the duration of the experiment. For the first three days, the larvae received unenriched *Artemia* nauplii to ensure that all larvae were fully weaned from rotifers to *Artemia* before beginning the experiment. *Artemia* were added to each aquarium 3 times per day at 9 am, 12 pm and 4:30 pm, in amounts to keep the *Artemia* concentration in the tank water at 2000 L^{-1}. The larvae were fed enriched and unenriched *Artemia* in 8 different combinations in triplicate, as listed below:

1. Unenriched *Artemia* only
2. AlgaMac 3010 enriched *Artemia* only
3. DHA Selco enriched *Artemia* only
4. Krill Protein Hydrolysate enriched *Artemia* only
5. AlgaMac 3010 / DHA Selco enriched *Artemia* - alternating days
6. AlgaMac 3010 / Krill Protein enriched *Artemia* – alternating days
7. DHA Selco / Krill Protein enriched *Artemia* – alternating days
8. DHA Selco / Krill Protein / AlgaMac 3010 enriched *Artemia* – alternating days

The experiment was ended when mortalities due to the onset of metamorphosis began occurring; metamorphosis occurs when 50% of the larvae are 12 mm in total length.

3.2.2 Growth and Survival

At the end of the experiment, surviving larvae were counted to determine survival rate for the overall experiment. Total length and body depth were measured at the beginning and at the end of the experiment. Total length was defined as the length in mm from the tip of the snout to the end of the tail fin. Body depth was defined as the width of the larvae just posterior to the anus not including the fin fold. Measurements were completed quickly using a dissecting microscope and Petri-dish. Wet weight of each larva was determined by first removing excess water from the larvae and placing the larvae on a sensitive digital scale.

Specific Growth Rate (SGR) was calculated using this equation:

$$SGR = Ln\ (TL_2 / TL_1) \div (t_2 - t_1)$$

The time (t) of the experiment was measured in days and the increase in size of the larvae was measured as total length (TL) in millimeters. Thus the specific growth rate is expressed as percent per day (% day^{-1}).

3.2.3 Lipid and Fatty Acid Extraction Method

Cod larvae were taken for lipid analyses from the initial feeding tank prior to the experiment (before *Artemia* were introduced as a prey item), along with triplicate samples from all eight feeding treatments at the end of the experiment (at the onset of metamorphosis). Larval samples were placed directly in chloroform and stored at –20°C until processed. Lipids were extracted in chloroform/methanol according to Parrish (1998), using a modified Folch procedure (Folch et al., 1957). Lipid classes were determined by thin layer chromatography with flame ionization detection in an Iatroscan (Parrish, 1987). Extracts were spotted on silica gel coated Chromarods and a three stage development system was used to separate lipid classes.

3.2.4 Amino Acid Sampling Method

a) Acid Hydrolysis

The sample (0.5-2 mg) was hydrolyzed in 1 ml of 6N HCl with 0.05% phenol, using corning 16 x 100 mm culture tubes with Teflon lined screw caps. The tubes were purged for 5 min. with nitrogen before capping. Samples were hydrolyzed for 24 hr periods (48 hr and 72 hr) at 110°C or for 4 hrs at 145°C . The HCl was removed under vacuum and

the dried sample reconstituted with pH 2.2 sodium citrate buffer 0.2 M Na^+ prior to analysis (Blackburn, 1978; Ozols, 1990).

b) Amino Acid Analysis – Physiological Free Amino Acids

i. Deproteinization

Samples were deproteinized with 10% sulfoslicylic acid (SSA) using 1 part SSA to 4 parts sample and 3 parts lithium citrate buffer pH 2.2, 0.3 N Li^+ for a final dilution of 1:2. Samples were vortexed, let stand 20 mins. at 4 °C and spun at 12,000 g for 5 mins, the supernatant was removed and filtered through a 0.2 µm nylon filter.

ii. Analysis

Deproteinized samples were analyzed on a Beckman 121 MB Amino Acid Analyzer using Benson D-X8, 0.25 Cation Xchange Resin and a single column, three buffer lithium method as per Beckman 121 MB –TB –017 application notes. Quantitation of the results was achieved using a Hewlett Packard Computing Integrator Model 3395A.

3.2.5 Statistical Analysis

One-way ANOVA's tested for significant differences within and between treatments for all data sets and were followed by Tukey's multiple comparison tests. Specific growth rate data were tested using SigmaStat 2.03 for normality and equal variance. Because the normality test failed, a one way ANOVA on ranks was performed followed by a Dunn's test. A significance level of P=0.05 was used unless otherwise noted. Minitab 15 was used to conduct a randomization test to compare treatments giving

the highest and lowest mean survivals. Error bars on all graphs were calculated using standard deviations.

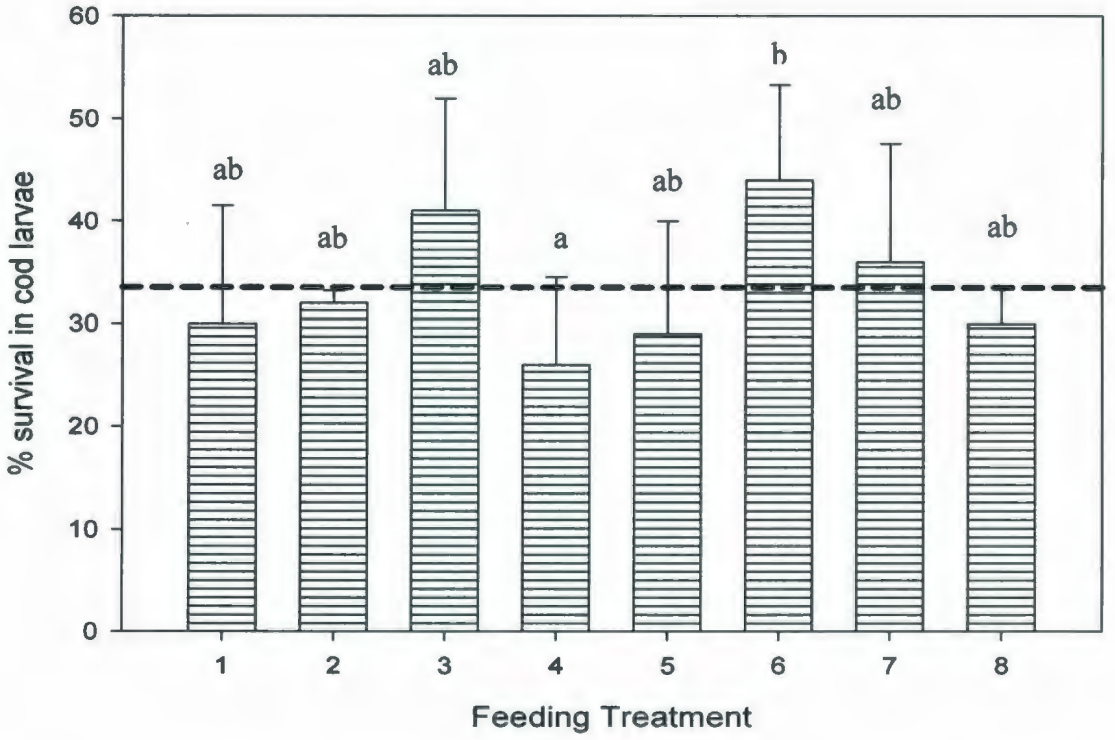
3.3 Results

3.3.1 Larval Growth and Survival

Larvae fed AlgaMac 3010/Krill Protein enriched *Artemia* on alternating days produced significantly higher survival (44%, $P<0.001$) than larvae fed Krill Protein enriched *Artemia* alone (Figure 3.1). Separately, AlgaMac 3010 resulted in a 32% survival rate and the Krill Protein treatment had a 26% survival rate. Cod larvae fed all three types of enriched *Artemia* resulted in an average survival rate of 30%. Survival rates were also good with the feeding of DHA Selco enriched *Artemia* (41%) but the only significantly higher survival rate was observed with the AlgaMac 3010/Krill Protein enriched *Artemia* fed in combination (44%). The larvae fed *Artemia* enriched with Krill Protein alone produced a significantly lower survival rate and was even lower than the survival rate found in the larvae fed unenriched *Artemia*. Krill Protein enriched *Artemia* fed on alternating days with DHA Selco enriched *Artemia* created an average survival rate of 36% (Krill Protein 26% & DHA Selco 41%).

The best growth, shown in Figure 3.2 as total length and Figure 3.3 as specific growth rate (SGR - mm day^{-1}), was observed when DHA Selco and Krill Protein enriched *Artemia* were fed to the larvae on alternating days. Growth, expressed as the increase in total length from the start of the experiment to the end of the experiment, was significantly greater ($P=0.010$) in the larvae fed Krill Protein and DHA Selco enriched *Artemia* on alternating days. Actually, all larvae fed Krill Protein alone or in combination

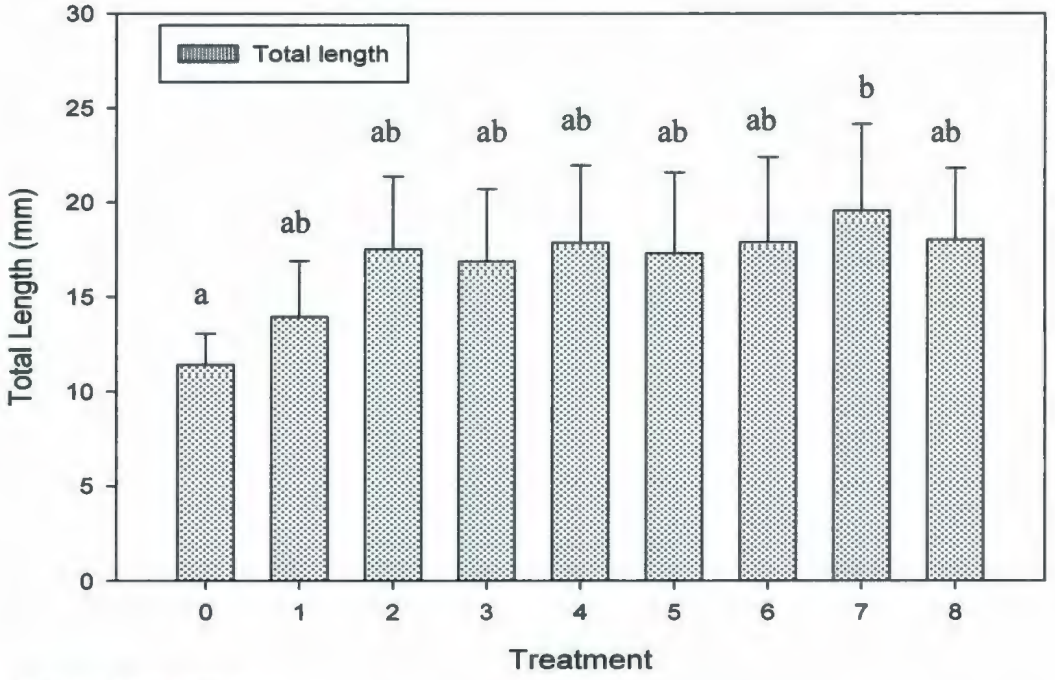
with other *Artemia* enrichments produced good growth rates. Figure 3.2, shows that all treatments involving Krill Protein enriched *Artemia* produced significantly higher SGRs than that found in the unenriched treatment. The larvae fed *Artemia* enriched in DHA Selco alone were the only treatment that did not produce SGRs that were significantly different from that found in the unenriched treatment. The larvae fed unenriched *Artemia* produced significantly lower SGRs than all other treatments, with the exception of the DHA Selco treatment.



Feeding Treatments

1. Unenriched *Artemia*
2. AlgaMac 3010
3. DHA Selco
4. Krill Protein
5. AlgaMac/DHA Selco
6. AlgaMac/Krill Protein
7. DHA Selco/Krill Protein
8. AlgaMac/DHA Selco/Krill Protein

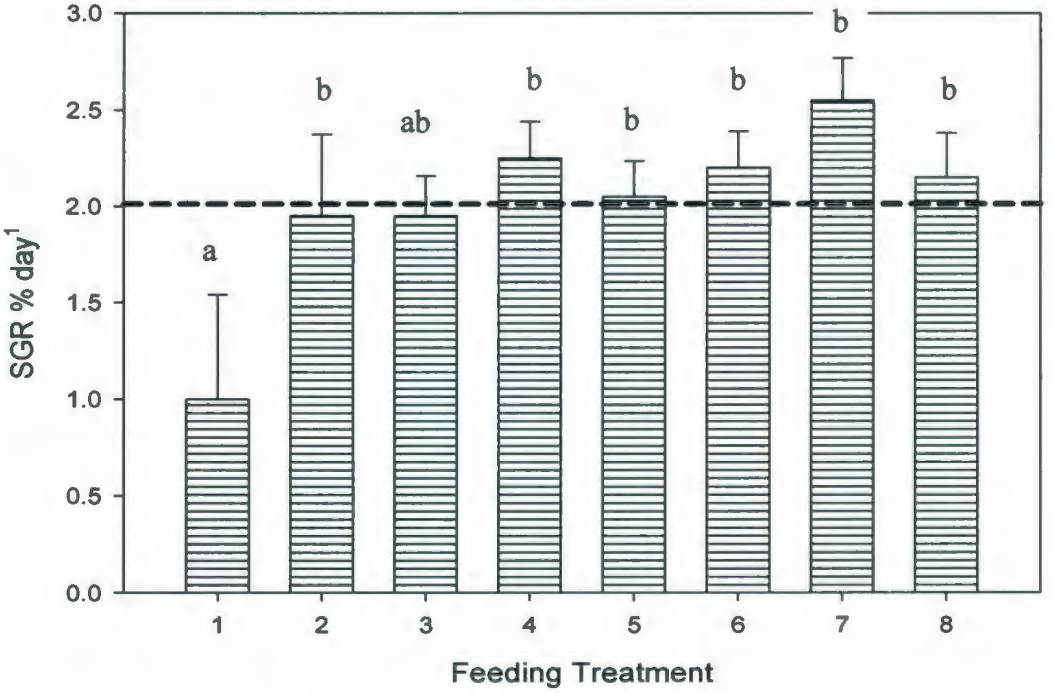
Figure 3.1: Comparison of survival rates in Atlantic cod larvae fed enriched and unenriched *Artemia* in eight different feeding regimes. Broken line indicates overall mean survival (33.5%). Significant differences are denoted by different letters ($P<0.001$). Data are means ± sd.



Feeding Treatments

0. Total length at start
1. Unenriched *Artemia*
2. AlgaMac 3010
3. DHA Selco
4. Krill Protein
5. AlgaMac/DHA Selco
6. AlgaMac/Krill Protein
7. DHA Selco/Krill Protein
8. AlgaMac/DHA Selco/Krill Protein

Figure 3.2: Comparison of growth, as a measurement of total length (mm), of Atlantic cod larvae fed enriched and unenriched *Artemia* in eight different feeding regimes. Treatment 0 is the total length measurement from a sample of larvae at the start of the experiment. The remaining treatments were measured at the end of the experiment; at the onset of metamorphosis. Different letters indicate significant differences ($P<0.05$).



Feeding Treatments

1. Unenriched *Artemia*
2. AlgaMac 3010
3. DHA Selco
4. Krill Protein
5. AlgaMac/DHA Selco
6. AlgaMac/Krill Protein
7. DHA Selco/Krill Protein
8. AlgaMac/DHA Selco/Krill Protein

Figure 3.3: Comparison of specific growth rates (SGR % day^{-1}) of Atlantic cod larvae fed enriched and unenriched *Artemia* in eight different feeding regimes. Broken line indicates overall mean specific growth rate (2.01 % day 1). Significant differences are denoted by different letters ($P<0.05$).

3.3.2 Total Lipid, Triacylglycerol and Phospholipid Composition of Atlantic Cod Larvae

There were no significant differences in TL, PL or TAG concentrations in the Atlantic cod larvae fed enriched and unenriched *Artemia* but Figure 3.4 shows a trend that cod larvae fed Krill Protein or AlgaMac 3010 enriched *Artemia* in combination or alone had higher levels of TL, TAG and PL. The unenriched *Artemia* produced the lowest levels in all three lipid classes studied. Krill Protein enriched *Artemia* alone and AlgaMac 3010 enriched *Artemia* alone produced equally high amounts of TL in the larvae. The second highest amount of TL was produced by the AlgaMac 3010 /Krill Protein enriched *Artemia* combination, fed on alternating days.

The AlgaMac 3010 enriched *Artemia* produced the highest levels of PL in the larvae by the end of the experiment. The AlgaMac 3010/DHA Selco/Krill Protein combination gave the next highest concentrations of PL in the larvae followed by Krill Protein alone and DHA Selco/Krill Protein fed in combination.

Krill Protein enriched *Artemia* produced the highest concentration of TAG in the larvae, followed by the AlgaMac 3010 treatment. AlgaMac 3010/Krill Protein enriched *Artemia* fed on alternating days, DHA Selco enriched *Artemia* alone and treatments fed all three diet types in combination, produced equal TAG levels in the larvae.

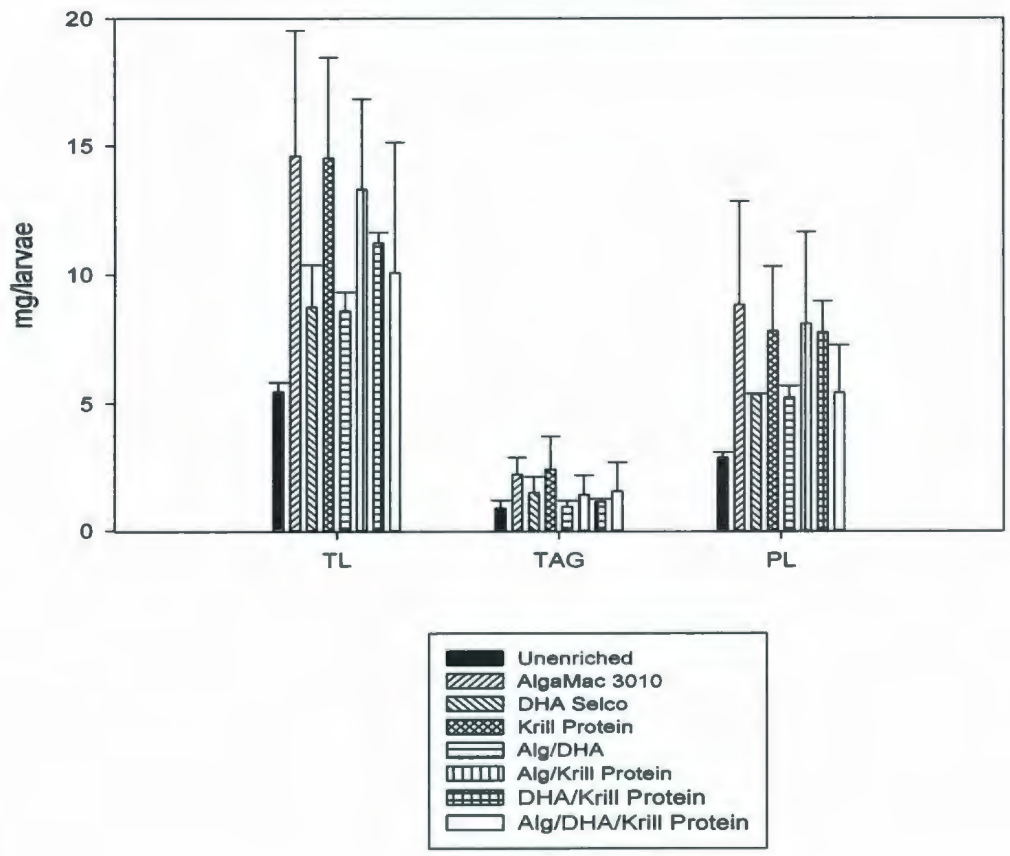


Figure 3.4: Comparison of total lipid (TL), triacylglycerol (TAG) and phospholipid (PL) concentrations (mg larva^{-1}) in Atlantic cod larvae fed enriched and unenriched *Artemia* using eight different feeding regimes. No significant differences were observed in TL, TAG, or PL concentrations among treatments ($P<0.05$). Alg=AlgaMac 3010, DHA=DHA Selco, Krill Protein=Krill Protein Hydrolysate.

3.3.3 ARA, EPA, DHA Composition of Atlantic Cod Larvae

The larvae fed AlgaMac 3010 enriched *Artemia* contained the highest levels of ARA and DHA of all the treatments and the Krill Protein Hydrolysate enriched *Artemia* produced the highest levels of EPA and TFA in the larvae. The total fatty acid level was also high in the AlgaMac 3010 treatment, the next highest was DHA Selco/Krill Protein and AlgaMac 3010/Krill Protein combinations, in descending order. A significant difference was found in the DHA level of larvae fed unenriched *Artemia* (low) and those fed AlgaMac 3010 (high) enriched *Artemia* (P=0.009). The EPA level found in larvae fed Krill Protein enriched *Artemia* was significantly higher than that in the larvae fed unenriched *Artemia* (P=0.042) (Figure 3.5). However, there were no significant differences among treatments for ARA or TFA levels.

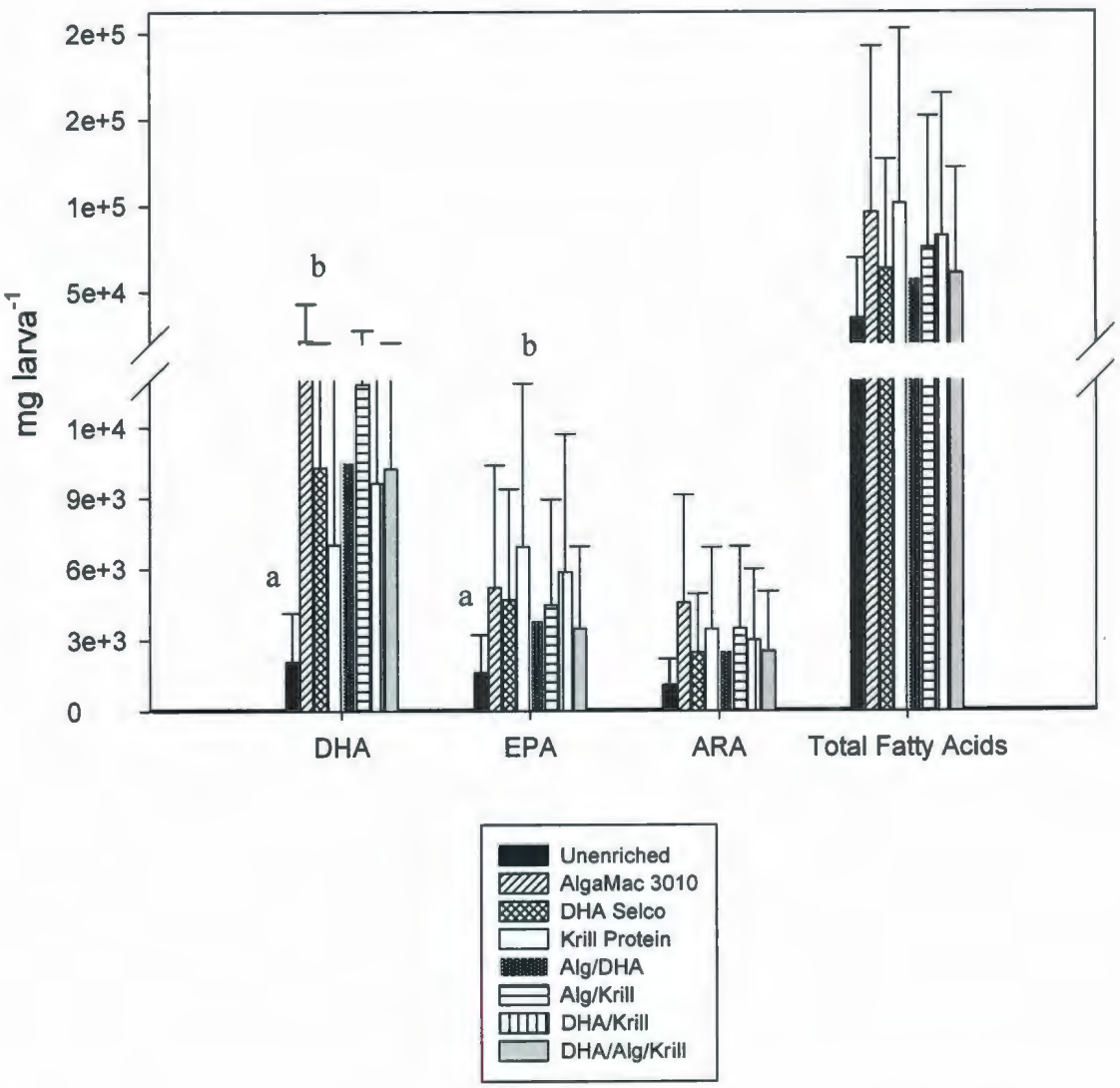
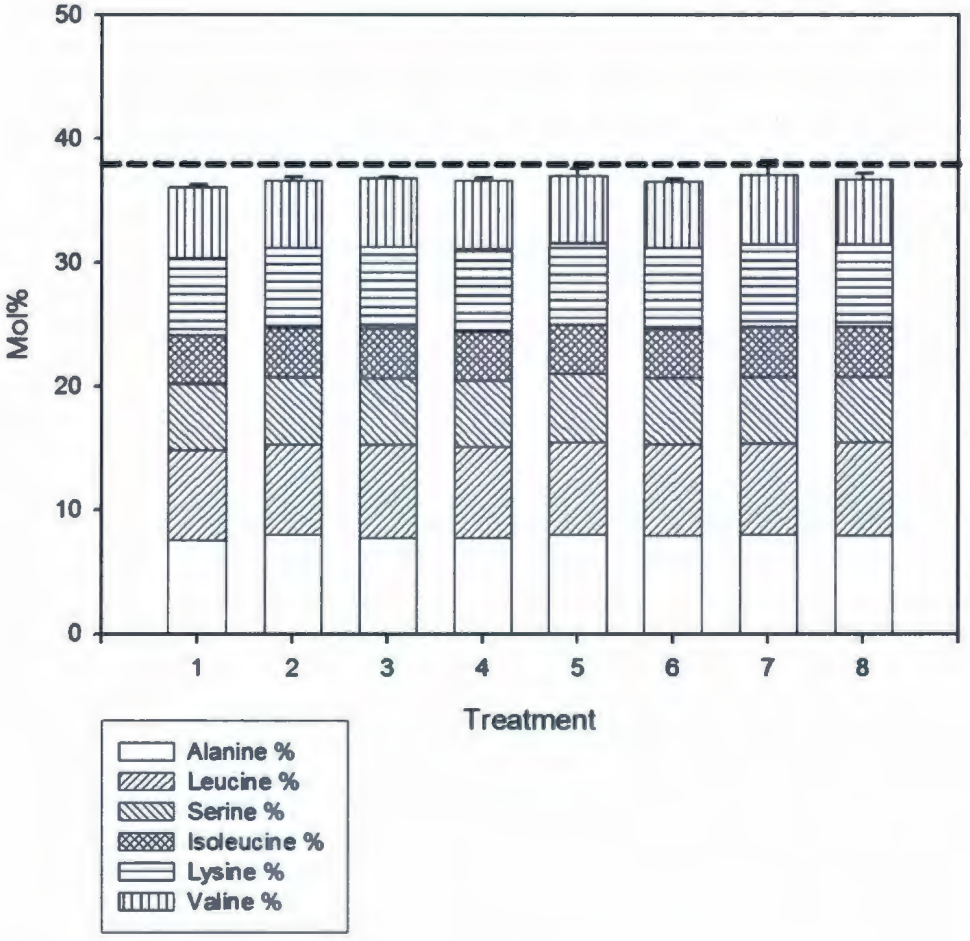


Figure 3.5: Comparison of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (ARA) and total fatty acid (TFA) levels (mg larva^{-1}) in Atlantic cod larvae fed enriched and unenriched *Artemia* using eight different feeding regimes. Treatments denoted by different letters are significantly different ($P<0.05$). Alg=AlgaMac 3010, DHA = DHA Selco, Krill Protein= Krill Protein Hydrolysate.

3.3.4 Selected Free Amino Acids in Atlantic Cod Larvae

Figure 3.6 shows that concentrations of selected free amino acids; alanine, leucine, serine, isoleucine, lysine and valine, in cod larvae, expressed as mole percent of the total amino acids, made up approximately 37% of the total in all treatments. The broken line depicts the mol% concentration of the six amino acids contained in the larvae at the start of the experiment. All larvae, except those fed AlgaMac/DHA Selco and DHA Selco/Krill Protein enriched *Artemia,* contained significantly less of these free amino acids then the larvae at the start of the experiment ($P<0.05$).

Lysine was found to be significantly higher in larvae fed DHA Selco/Krill Protein and AlgaMac/DHA Selco/Krill Protein enriched *Artemia* than those fed unenriched *Artemia* ($P<0.05$).



Feeding Treatments

1. Unenriched *Artemia*
2. AlgaMac 3010
3. DHA Selco
4. Krill Protein
5. AlgaMac/DHA Selco
6. AlgaMac/Krill Protein
7. DHA Selco/Krill Protein
8. AlgaMac/DHA Selco/Krill Protein

Figure 3.6: Comparison of selected free amino acid concentrations in mol% of the total amino acids. The broken line indicates the mol% of these free amino acids present in the larvae at the start of the experiment (38%). All treatments, except 5 and 7, were significantly different from the starting value of 38% ($P<0.05$). The mol% of lysine in treatments 7 and 8 were significantly different from the % lysine in the unenriched treatment ($P<0.05$). Error bars are standard deviations for the sum of the six selected amino acids.

3.4 Discussion

First feeding Atlantic cod larvae require certain dietary components for proper growth, development and survival in intensive culture systems. These dietary components have to be injected into the live food organisms fed to the larvae at the appropriate life history stage. This study focused on growth, survival and nutritional composition of *Artemia* and Atlantic cod larvae during the *Artemia* feeding stage. Of importance here as well, are the effects of incorporating an enrichment product high in protein and free amino acids into an enrichment regime that was previously injecting high levels of ω-3 fatty acids and lipids.

3.4.1 Growth and Survival in Atlantic Cod Larvae

First feeding cod larvae fed *Artemia* enriched in AlgaMac 3010/Krill Protein on alternating days gave the best survival rate during the experiment. DHA Selco enriched *Artemia* also resulted in good larval survival. This suggests that adding protein to an enrichment regime already high in fatty acids may contribute to improved survival in cod larvae. Larvae fed *Artemia* enriched in DHA Selco/Krill Protein on alternating days and those that were fed AlgaMac/DHA Selco/Krill Protein on alternating days also contained significantly higher levels of lysine than the unenriched treatment.

It is interesting to look at the DHA:EPA:ARA ratio associated with the treatments producing high survival. The ratios for AlgaMac 3010 enriched *Artemia* and Krill Protein enriched *Artemia* are 6:3.5:1 and 1:7:2 respectively, with a combined ratio of 2.3:3.5:1 (DHA:EPA:ARA). The ratio for the DHA Selco enriched *Artemia*, which also gave good

survival, was 2.7:3.7:1. Garcia et al. (2008) found that a ratio of DHA:EPA:ARA of both 0.3:3.7:1 and 7:2.4:1 resulted in a high survival rate in cod larvae; very similar to the ratio producing good survival in this study. A ratio of approximately 2.5:3.5:1 DHA:EPA:ARA, found in both treatments with high survival rates, seemed to be an optimal ratio for survival in cod larvae.

Growth, as measured by specific growth rate, was best in the larvae fed a combination of DHA Selco/Krill Protein enriched *Artemia.* Larvae fed Krill Protein alone also produced good growth rates. Growth, as a measure of total length (mm) was significantly improved in the larval treatment fed DHA Selco / Krill Protein enriched *Artemia* in combination. Lysine was found to be significantly higher in larvae fed this combination of enriched *Artemia*, suggesting this essential amino acid, in particular, may be contributing to improved growth in cod larvae. In herring larvae, significantly more lysine was retained in the body after amino acid assimilation than glutamate, suggesting that the larvae were preferentially using glutamate over lysine as an energy substrate (Conceição et al., 2002). A similar situation could be occurring in cod larvae, where lysine is not being used intensively as an energy source but is being retained in the body. It seemed that Krill Protein also helped to boost both growth and survival in cod larvae and that, elements of AlgaMac 3010 contributed to improved survival. Nutritional components of DHA Selco fed in the appropriate ratio with Krill Protein translated into much better growth in cod larvae than DHA Selco enriched *Artemia* fed alone. Figures 2.2 and 3.3 suggested that higher levels of DHA, EPA and ARA found in DHA Selco enriched *Artemia* are important contributors to survival in Atlantic cod larvae.

DHA Selco enriched *Artemia*, especially, had high levels of EPA present which may have been a factor in survival, as Webster et al. (1990) found that survival was significantly improved in striped sea bass with increased levels of EPA. Japanese flounder larvae, after a salinity test, had significantly improved survival rates when fed *Artemia* containing high levels of DHA, not EPA; whereas both DHA and EPA were found to significantly improve growth (Furuita et al., 1999). Morais et al. (2004) found no relationship between highly unsaturated fatty acid concentration and growth and survival in Senegalese sole larvae.

According to Rønnestad et al. (1999), growth is primarily an increase in body muscle mass by protein synthesis and since fish larvae have very high growth rates, they have a high dietary requirement for amino acids. Amino acids in their free form were found, in this case, to contribute to improved growth and survival rates in cod larvae. Results depicted in Figures 2.3 and 3.3 show a relationship between increased FAA levels and better survival rates where DHA Selco enriched *Artemia* and unenriched *Artemia* contained high levels of all the FAAs studied but the larval treatment fed DHA Selco enriched *Artemia* in combination, produced a better survival rate. Larvae fed the AlgaMac 3010/Krill Protein enriched *Artemia* on alternating days produced the best survival rate suggesting that all enrichments may be cumulatively contributing to the FAA pool available for growth and as a result improving survival rates.

DHA Selco enriched *Artemia* contained the highest levels of alanine and serine, 5.65 and 1.98 μmoles g^{-1} wet wt, respectively. Leucine, isoleucine, lysine and valine levels were higher, though, in the unenriched *Artemia*. Conversely, the larvae fed

unenriched *Artemia* contained significantly lower levels of all free amino acids studied. Krill Protein enriched *Artemia* contained the third highest levels of FAAs but resulted in better SGR than both the DHA Selco and unenriched *Artemia* treatments. DHA Selco/Krill Protein enriched *Artemia* produced the best growth rate, as well as producing significantly more lysine in the larval FAA content than the unenriched treatment, suggesting that there may be a favourable ratio of fatty acids to amino acids required in the diet.

As was previously stated, Fyhn and Serigstad (1987) found that alanine, leucine, serine, isoleucine, lysine and valine, together, accounted for 75% of the disappearing FAAs and were used as an energy source early in larval development. In this case, six free amino acids were measured as mol percent composition in the larvae and were found to be significantly lower at the end of the experiment than at the start in all but two treatments. This finding is in line with Fyhn and Serigstad's findings that these FAAs are most likely an important source of energy and growth during early larval development in Atlantic cod larvae.

The DHA Selco enriched *Artemia* contained the highest amounts of total lipid and triacylglycerol after enrichment which may have contributed, in part, to the improved larval growth observed in that treatment. AlgaMac 3010 enriched *Artemia* were high in phospholipid which could be contributing to the improved survival rate in the AlgaMac 3010/Krill Protein treatment. The *Artemia* enriched with DHA Selco and *Artemia* enriched with Krill Protein were both found to have high total lipid concentrations and resulted in improved growth rates when fed to cod larvae. Due to the high total lipid

content of the *Artemia* and improved growth of the larvae, it could be concluded that total lipid concentrations are important in achieving optimal growth in Atlantic cod larvae.

With regards to discussing optimal ratios of free amino acids, fatty acids and total lipid, larvae fed AlgaMac 3010, DHA Selco and Krill Protein enriched *Artemia* on alternating days would be the best feeding treatment to look at because this treatment received all three enrichment diets. This treatment produced good growth (SGR = 2.15) but poor survival (30%, same as unenriched *Artemia*) and contained two lipid-rich and one protein-rich component. Compared with the growth rate produced by the DHA Selco/Krill Protein treatment, one lipid-rich component and one protein-rich component, the growth was only slightly improved. According to this example, a lipid to protein ratio of 1:1 may be better than 2:1.

Further, lipid : protein ratios, AlgaMac 3010 and Krill Protein enriched *Artemia* fed on alternating days produced the best survival rate but the treatment fed all three enriched *Artemia* on alternating days produced a poor survival rate. This suggests that the ratio of feeding one protein- rich component with two lipid-rich components is not an optimal lipid : protein ratio for improved survival rates in Atlantic cod larvae. As a result of the improved survival rate in the AlgaMac/Krill Protein treatment as well as the improved growth rate in the DHA Selco/Krill Protein treatment, a suggested feeding regime involving AlgaMac 3010, DHA Selco and Krill Protein enriched *Artemia* fed in a 1:1 lipid : protein ratio would look like this: Day 1 – lipid (AlgaMac 3010 - survival), Day 2- protein (Krill Protein - growth/survival), Day 3 – lipid (DHA Selco - growth), Day 4 – protein (Krill Protein - growth/survival), repeat days 1-4 on a continuous cycle.

3.4.2 Larval Composition

TAG is the primary storage molecule for lipids in higher bony fishes (Sheridan, 1988). TAG levels or lipid storage, were highest in the larvae fed Krill Protein enriched *Artemia* and were also high in the AlgaMac 3010 larval treatment (although the *Artemia* had low levels of TAG). The larval treatments fed these enrichments produced the higher survival rates, suggesting that larvae with lipid reserves are probably less affected by stress and are healthier overall. However, the larvae had much lower amounts of TAG relative to the content of total lipid that was originally measured in the enriched *Artemia*. Due to the improved SGR in the DHA Selco/Krill Protein treatment and the higher levels of TAG in these two enriched *Artemia* groups, it could be concluded that TAG, in this instance, may be an important lipid source for larval growth. Lysine was found to be significantly higher in the larvae fed DHA Selco/Krill Protein enriched *Artemia* in combination. In addition, the *Artemia* enriched in DHA Selco contained fairly high levels of lysine, suggesting that lysine in the enriched *Artemia* translated into significant improvements in larval growth and a higher mol% of lysine in larval composition.

Total lipid was greatest in the DHA Selco enriched *Artemia*, followed by Krill Protein enriched *Artemia*, unenriched *Artemia* and AlgaMac 3010 enriched *Artemia*; in that order (Figure 2.1). Total lipid in the larvae at the end of the experimental period was higher in the Krill Protein fed larvae, as well as the AlgaMac 3010 fed larvae. This suggests that, in the case of lipids, the larvae are not taking on the nutritional composition of the *Artemia* in all instances.

Chu and Ozkizilcik (1995) found that the fatty acid (FA) composition of striped bass larvae was significantly influenced by the *Artemia* diet. As well, the FA composition of Atlantic Halibut showed development towards the composition of the *Artemia* they were fed (Næss et al., 1995). However, halibut larvae fed wild zooplankton maintained a FA composition closer to that of the prefed larvae (Næss et al., 1995). Figure 3.4 shows that the phospholipid level in the larvae fed AlgaMac 3010 enriched *Artemia* remained relatively high, as compared to the amount originally found in the *Artemia.* This suggests that in cod larvae, as well as in other species, larval body composition develops towards the composition of the diet.

However, in many instances the larvae were different in nutritional composition than the *Artemia* that they were fed. The larvae fed the AlgaMac 3010 enriched *Artemia* contained the highest levels of DHA and ARA but the AlgaMac 3010 enriched *Artemia* contained the lowest levels of ARA, and less DHA than DHA Selco enriched *Artemia.* The larvae fed the Krill Protein enriched *Artemia* contained the highest levels of total fatty acids, but the Krill Protein enriched *Artemia* actually contained low amounts of DHA, EPA and ARA. Total lipid and TAG present in the enriched *Artemia* have been found to be hydrolyzed by the larvae in the lumen of the gut by pancreatic lipases to free fatty acids (Sheridan, 1988). This study supports this finding.

Chapter 4

Summary of Experiments and Recommendations for Enrichment Regimes for First Feeding Atlantic Cod Larvae

4.1 Summary

Artemia are readily accepted by first feeding cod larvae but they lack the nutritional components for normal growth and development. In intensive culture systems, it is important to seek out and implement protocols that will ensure the best growth and survival rates possible. In this experiment, a krill protein hydrolysate was used to enrich *Artemia* for their subsequent incorporation as a food item for the larvae. Enrichments high in lipids and ω-3 fatty acids are already used to inject ARA, EPA, DHA, lipids, phospholipids and triacylglycerols into *Artemia* for feeding to marine larvae with good success. Since amino acids and proteins are the building blocks for all stages of growth in fish, it would seem that by not injecting a protein source into these *Artemia*, the larvae would not be receiving a nutritionally complete food source.

Artemia enriched with the Krill Protein Hydrolysate when fed in conjunction with *Artemia* enriched in DHA Selco resulted in a significantly higher specific growth rate than that of the unenriched treatment. Krill Protein enriched *Artemia* were fed to four different treatments alone or in combination with other enriched *Artemia.* In all treatments fed Krill Protein, a higher specific growth rate was observed. AlgaMac 3010 enriched *Artemia* fed in conjunction with Krill Protein enriched *Artemia* produced a significantly higher survival rate than Krill Protein enriched *Artemia* fed alone.

AlgaMac 3010 enriched Artemia had higher levels of PL and a substantial amount of DHA (2nd highest amount after DHA Selco). PL and DHA from the AlgaMac 3010 enriched *Artemia* may be contributing to the high survival rate found in the AlgaMac 3010/Krill Protein treatment because the treatment where Krill Protein enriched *Artemia* was fed alone produced a significantly lower survival rate. Krill Protein enriched *Artemia* contained fairly high levels of EPA, TAG and TL. Free lysine was found to be significantly higher in the larvae fed DHA Selco/Krill Protein or AlgaMac 3010/DHA Selco/Krill Protein enriched *Artemia* on alternating days as compared to the mol% found in the larvae of the unenriched treatment. This suggests that EPA, TAG and TL, as well as FAA are proportionally contributing to growth.

PL levels in the *Artemia* were similar to the larvae with no major increases or decreases being observed. The highly unsaturated fatty acids ARA, EPA and DHA, as a percent of total fatty acids, increased in the larvae for all treatments, but the total fatty acid level increased overall in the larvae. TAG levels in the larvae were similar to that found in the *Artemia*. Free amino acids in the larvae were found to be significantly lower in all but two treatments as compared to the start of the experiment. Larvae from two treatments had significantly more lysine than in the unenriched treatment.

Overall, the study provided some clear insight into the dietary components contributing to growth and survival in Atlantic cod larvae by feeding various types of enriched *Artemia* including an element of protein.

4.2 Recommended Enrichment and Feeding Regime

Larvae fed AlgaMac 3010, DHA Selco and Krill Protein enriched *Artemia* on alternating days throughout the experimental period produced a good SGR but resulted in poor survival. The DHA Selco/Krill Protein enriched *Artemia* combination produced the best specific growth rate and Krill Protein enriched *Artemia* improved SGRs in all treatments where it was fed. AlgaMac 3010 enriched *Artemia* contributed to improved survival, but Krill Protein enriched *Artemia* fed alone did not.

The 1:1 lipid to protein ratio (DHA Selco/Krill Protein) produced better results than the 2:1 lipid: protein ratio (AlgaMac/DHA Selco/Krill Protein), but the three enrichments studied contributed individually to the improved growth and survival; then a feeding regime using this ratio should be employed. Using this premise, the three different enriched *Artemia* should be fed according to a 1:1 lipid to protein ratio. So, instead of a simple incorporation of the protein by feeding Krill Protein enriched *Artemia* once every 3 days, feed the Krill Protein enriched *Artemia* every second day and use AlgaMac 3010 and DHA Selco enriched *Artemia* alternately.

Recommended feeding regime (After rotifer feeding period and until weaning of the larvae to a dry diet***):***

- Day 1 – AlgaMac 3010 enriched *Artemia* (lipid source #1);
- Day 2 – Krill Protein enriched *Artemia* (protein source);
- Day 3 – DHA Selco enriched *Artemia* (lipid source #2);
- Day 4 – Krill Protein enriched *Artemia* (protein source);
- Continue repetition of the 1-4 day feeding cycle.

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Appendix A

Artemia Decapsulation Procedure

HYDRATION STEP

· Hydrate cysts by placing them for 1 h in water (< 100 gm/l), with aeration, at 25°C.

DECAPSULATION STEP

· Collect cysts on a 125 µm mesh sieve, rinse, and transfer to the hypochlorite solution.

· The hypochlorite solution can be made up (in advance) of either liquid bleach NaOCl (fresh product; activity normally =11-13% w/w) or bleaching powder Ca $(OCl)_2$ (activity normally ± 70%) in the following proportions:

* 0.5 g active hypochlorite product (activity normally labeled on the package, otherwise to be determined by titration) per g of cysts; for procedure see further;

* an alkaline product to keep the pH>10; per g of cysts use:

¨ 0.15 g technical grade NaOH when using liquid bleach;

¨ either 0.67 $NaCO_3$ or 0.4 g CaO for bleaching powder; dissolve the bleaching powder before adding the alkaline product; use only the supernatants of this solution;

¨ seawater to make up the final solution to 14 ml per g of cysts.

· Cool the solution to 15-20°C (i.e. by placing the decapsulation container in a bath filled with ice water). Add the hydrated cysts and keep them in suspension (i.e. with an aeration tube) for 5-15 min. Check the temperature regularly, since the reaction is exothermic; never exceed 40°C (if needed add ice to decapsulation solution). You can assess how well the process is working by checking for decapsulation under a jewelers eyepiece or microscope.

WASHING STEP

· When cysts turn grey (with powder bleach) or orange (with liquid bleach), or when microscopic examination shows almost complete dissolution of the cyst shell (= after 3-15 min.), cysts should be removed from the decapsulation suspension and rinsed with water on a 125 µm screen until no chlorine smell is detected. It is crucial not to leave the embryos in the decapsulation solution longer than strictly necessary, since this will affect their viability.

DEACTIVATION STEP

· Deactivate all traces of hypochlorite by dipping the cysts (< 1 min.) either in 0.1 N HCl or in a 0.1% $Na2S_2O^3$ solution, then rinse again with water. Hypochlorite residues can be detected by putting some decapsulated cysts in a small amount of starch-iodine indicator (= starch, KI, H_2SO_4 and water). When the reagent turns blue, washing and deactivation has to be continued. Alternatively, a DPD chlorine test method may be used.

USE

· Incubate the cysts for hatching, or store in the refrigerator (0-4°C) for a few days before hatching incubation. For long term storage cysts need to be dehydrated in saturated brine solution (1 g of dry cysts per 10 ml of brine of 300 g NaCl.l-1). The brine has to be renewed after 24h.

Table A.1: Summary of ANOVA results for Lipid and Fatty Acid content of enriched and unenriched *Artemia*. Significance level $P < 0.05$.

Lipid/ Fatty Acid	d.f.	F	P
Total Lipid	3	5.197	0.034
Triacylglycerols	3	4.763	0.041
Phospholipid	3	33.94	0.000
ARA	3	2.519	0.142
EPA	3	4.283	0.052
DHA	3	9.475	0.007
Total FA	3	4.421	0.048

Table A.2: Summary of ANOVA results for Free Amino Acid content in enriched and unenriched *Artemia*. Significance level $P<0.05$.

Free Amino Acid	d.f.	F	P
Alanine	7	10.343	0.004
Serine	7	20.810	0.000
Valine	7	21.184	0.000
Isoleucine	7	19.472	0.001
Lysine	7	18.192	0.001
Leucine	7	18.960	0.001

Table A3: Summary of Tukey's Multiple Comparison tests for the *Artemia* enrichment treatments within the fatty acids measured. Significance level $P < 0.05$.

DHA	Krill Protein	AlgaMac 3010	DHA Selco
Krill Protein			
AlgaMac 3010	0.246		
DHA Selco	0.014	0.346	
Unenriched	0.985	0.166	0.009
EPA			
Krill Protein			
AlgaMac 3010	0.496		
DHA Selco	0.294	0.057	
Unenriched	0.796	0.910	0.090
ARA			
Krill Protein			
AlgaMac 3010	0.786		
DHA Selco	0.362	0.141	
Unenriched	0.253	0.904	0.253

Table A4: Summary of Tukey's multiple comparison tests for the *Artemia* enrichment treatments within lipids and fatty acids measured. Significance level $P < 0.05$.

Total Lipid	Krill Protein	AlgaMac 3010	DHA Selco
Krill Protein			
AlgaMac 3010	0.167		
DHA Selco	0.394	0.024	
Unenriched	0.945	0.310	0.203
Total Fatty Acid			
Krill Protein			
AlgaMac 3010	0.137		
DHA Selco	0.679	0.036	
Unenriched	0.867	0.326	0.306
Triacylglycerol			
Krill Protein			
AlgaMac 3010	0.169		
DHA Selco	0.581	0.036	
Unenriched	0.646	0.588	0.141
Phospholipid			
Krill Protein			
AlgaMac 3010	0.004		
DHA Selco	0.103	0.000	
Unenriched	0.010	0.000	0.386

Table A5: Summary of Tukey's multiple comparison tests for *Artemia* enrichment treatments within the free amino acids measured. Significance level $P < 0.05$.

Alanine	Krill Protein	AlgaMac 3010	DHA Selco
Krill Protein			
AlgaMac 3010	0.553		
DHA Selco	0.010	0.065	
Unenriched	0.007	0.047	0.995
Serine			
Krill Protein			
AlgaMac 3010	0.999		
DHA Selco	0.001	0.001	
Unenriched	0.005	0.006	0.556
Valine			
Krill Protein			
AlgaMac 3010	0.864		
DHA Selco	0.001	0.001	
Unenriched	0.016	0.006	0.260
Isoleucine			
Krill Protein			
AlgaMac 3010	0.328		
DHA Selco	0.003	0.000	
Unenriched	0.135	0.011	0.081
Leucine			
Krill Protein			
AlgaMac 3010	0.359		
DHA Selco	0.003	0.000	
Unenriched	0.136	0.012	0.084
Lysine			
Krill Protein			
AlgaMac 3010	0.969		
DHA Selco	0.001	0.001	
Unenriched	0.061	0.033	0.069

Table A6: Summary of survival rates and specific growth rates (SGR) for Atlantic cod larvae fed different combinations of enriched and unenriched *Artemia*. Feeding regime describes the type(s) of enriched *Artemia* fed to the larvae.

Treatment	Survival (%)	SGR (% day^{-1})
Unenriched only	30	1.00
AlgaMac 3010 only	32	1.95
DHA Selco only	41	1.95
Krill Protein Hydrolysate only	26	2.25
AlgaMac/DHA Selco	29	2.05
AlgaMac/Krill Protein	44	2.20
DHA Selco/Krill Protein	36	2.55
DHA/AlgaMac/Krill Protein	30	2.15

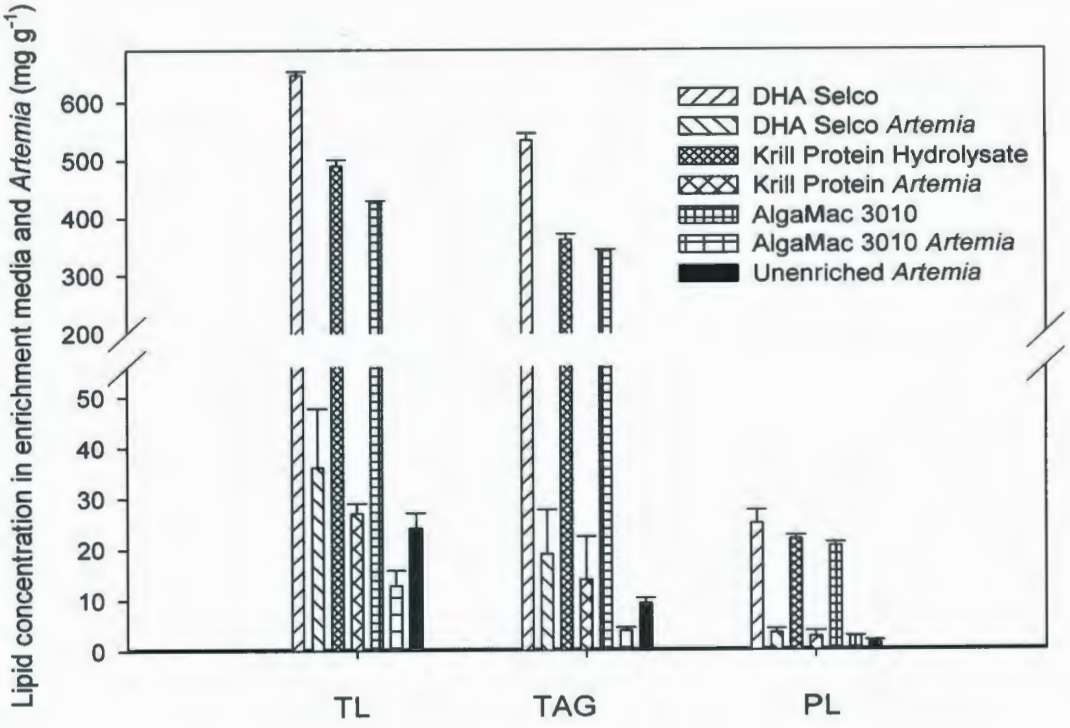

Figure 1A: Total lipid (TL), triacylglycerol (TAG) and phospholipid (PL) concentrations (mg g^{-1}) in *Artemia* enriched in DHA Selco, a Krill Protein Hydrolysate and AlgaMac 3010. Unenriched *Artemia* was a control.

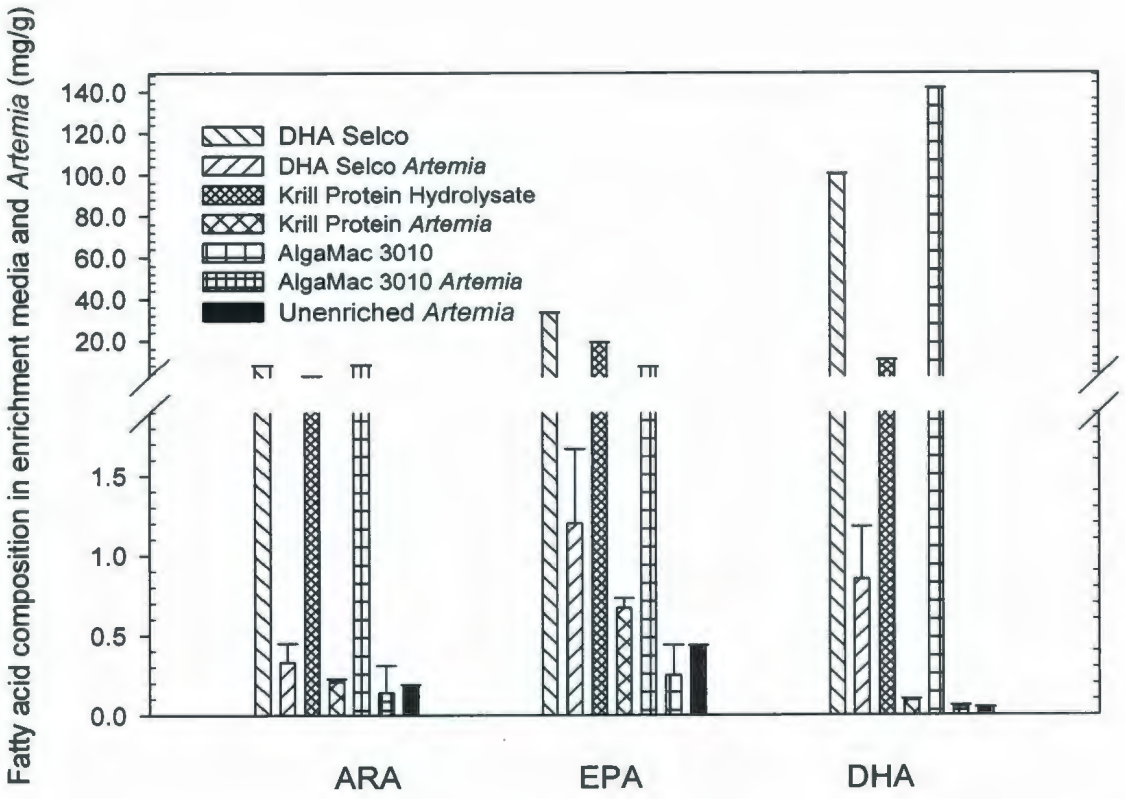

Figure 2A: Comparison of fatty acid concentrations (mg g^{-1} of ARA, EPA and DHA) in the enrichment media and the enriched *Artemia.* See Table 2.1 for percentage of fatty acid incorporation in *Artemia* during enrichment.

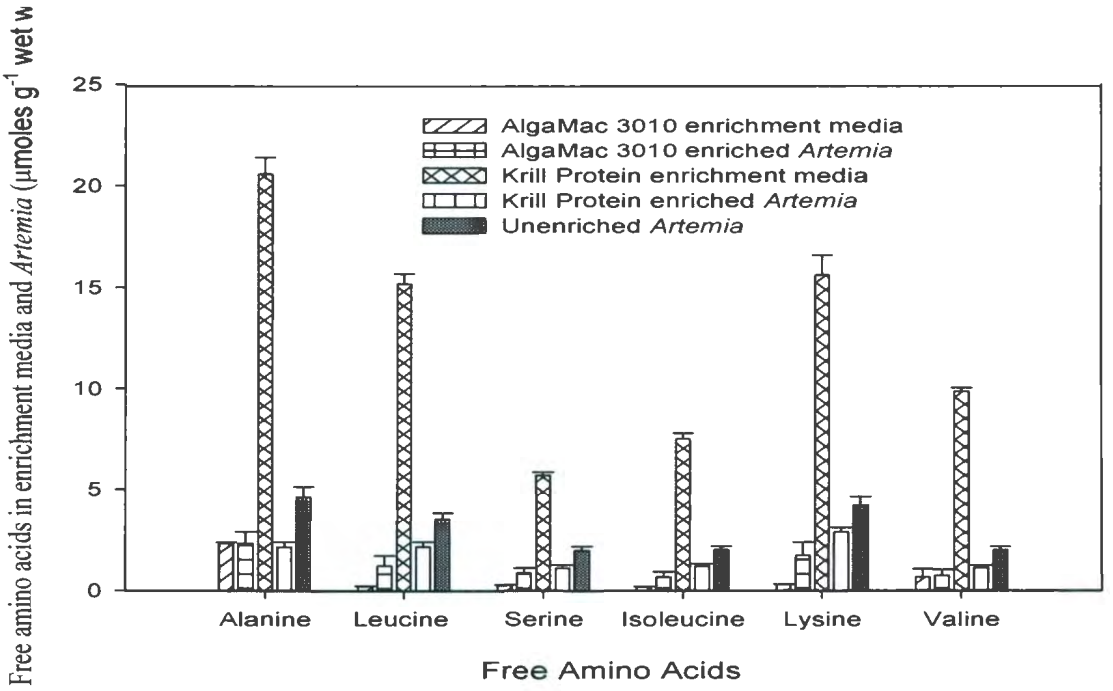

Figure 3A: Comparison of free amino acid (FAA) concentrations (µmoles g^{-1} wet wt) in AlgaMac 3010 enrichment media and a krill protein hydrolysate with FAA concentrations in *Artemia* enriched with these products. FAA concentrations in unenriched *Artemia* are shown for comparison. The enrichment media, DHA Selco, is an oil emulsion and could not be analyzed for FAA.

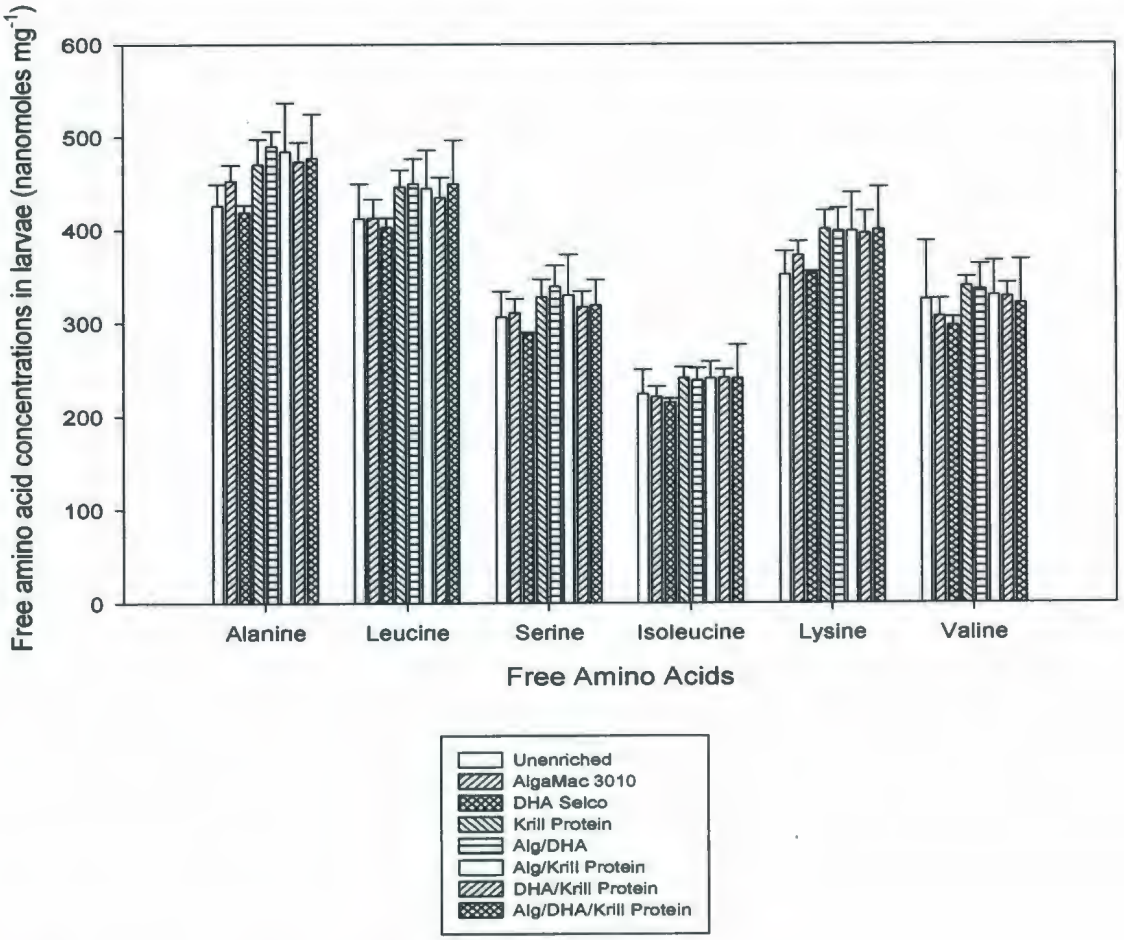

Figure 4A: Comparison of selected free amino acid concentrations (nanomoles mg^{-1}) in Atlantic cod larvae fed enriched and unenriched *Artemia* using eight different feeding regimes. Legend shows enriched *Artemia* combinations fed to each treatment. No significant differences were observed in the concentration of free amino acids in the larvae ($P<0.05$). Alg = AlgaMac 3010, DHA = DHA Selco, Krill Protein = Krill Protein Hydrolysate.