Development of foraging and digestive function in Atlantic cod (*Gadus morhua*) larvae in response to diet.

By

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Thesis Abstract

Atlantic cod (*Gadus morhua*) show great potential for mass-rearing, but much is unknown about their digestive capacity and efficiency. This thesis provides an integrated study of survival, growth, behaviour, and digestive enzyme activity in cod larvae and the variability in digestive development in response to nutritional quality of live food enrichments.

Experiments were performed investigating the effect of enrichments with live food. The first experiment used high- and low-lipid rotifers (*Brachionus plicatilis*) from hatching to 450 dd. Lipid level significantly impacted survival, behaviour and digestive enzyme activity by 100-150 dd. A second experiment examining *Artemia franciscana* enrichments from 450-650 dd resulted in larvae fed a rotation diet showed the greatest survival and growth rates, but behaviour was not impacted by enrichment type. High-protein and high-lipid diets were able to induce significantly high post-prandial digestive enzyme activities. Results indicate that diet plays a role in growth and survival, but also in the enzymatic activity of the young fish's developing digestive system.

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List of Abbreviations

3DRA:	Three day rotating *Artemia* enrichments
AA:	Arachidonic acid
ANOVA:	Analysis of Variance
ARDF:	Aquaculture Research Development Facility
BAPNA:	N-α-benzoyl-L-arginine-4-nitanilide hydrochloride
dd:	Degree-days
DHA:	Docosahexaenoic acid
dph:	Days post-hatching
EPA:	Eicosapentaenoic acid
FID:	Flame ionization detection
HLAE:	High lipid *Artemia* enrichment
HLRE:	High lipid rotifer enrichment
HPAE:	High protein *Artemia* enrichment
LLRE:	Low lipid rotifer enrichment
MS-222:	Tricaine methanesulfonate
OSC:	Ocean Sciences Centre
TAG:	Triacylglycerides
TCA:	Trichloroacetic acid
TLC:	Thin layer chromatography
UN:	Unenriched *Artemia* nauplii

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1 Development of digestive function in Atlantic cod (*Gadus morhua*) larvae.

1.1 Introduction

An important prerequisite for advancing knowledge of the trophic role of fish in the marine ecosystem is reliable information about predator-prey interactions. A key aspect of this is information on how fish feed. Acquisition, digestion, and assimilation of food are critical for fish growth and survival, especially in the larval stages when mortality is highest (Blaxter 1989, Munk 1995, Hunt von Herbing & Gallager 2000). However, the digestive systems of larval fish have structural adaptations to promote larval growth, and the changes in these adaptations with development are responses to different diets and prey concentrations (Govoni *et al.* 1986). As such, survival of larval fish depends on the successful switch to external feeding and the availability of suitable prey in sufficient quantities to promote growth.

Most fish larvae are visual, raptorial planktivores, regardless of whether their adult counterparts are indiscriminant filter feeders, pelagic carnivores, or benthic pickers (Hunter 1981). Adult Atlantic cod (*Gadus morhua*) are highly carnivorous and often feed on large prey (including other fish and large invertebrates) comprised mainly of protein and fat (Lie *et al.* 1988). However, before the fish adopts this higher predatory role, the larvae must first survive and grow throughout the energetically demanding process of metamorphosis. Fish larvae begin life by utilizing a highly lipid-rich yolk reserve during the yolk-sac

period, then begin feeding on small zooplankton, and proceed to feed on increasingly larger organisms as they grow and the digestive system matures (Andersen 2001). Cod larvae feeding solely on small prey, such as rotifers, typically do not grow very well beyond 8 mm in length as compared to cod that are fed larger prey items later in development (Pedersen & Falk-Petersen 1992).

The simple unsegmented digestive tract of newly hatched cod larvae undergoes structural and functional changes over the first month of life. The development of a simple incipient larval gut into a highly differentiated digestive system proceeds in a series of rapid periodic changes, rather than by continuous graduation (Govoni *et al.* 1986). By the time metamorphosis has ended, the digestive system is complete and the juvenile is able to assimilate nutrients in the same manner as the adult (Baglole *et al.* 1998). The organ systems responsible for food detection, digestion, and metabolism develop both structurally and functionally to ensure sufficient food intake to keep the larvae alive, support cellular growth, deliver nutrients needed for normal development, and supply energy essential for maintenance of basic physiological processes (Segner & Verreth 1995).

Of critical importance in the assimilation of prey and food into usable energy are digestive enzymes. The temporal and spatial developments of digestive enzymes in cod are just now beginning to be studied in depth (Murray *et al.* 2001; Perez-Casanova 2003), although work has been done in other species of larvae, including sea bass (*Lates calcarifer*; Walford & Lam 1993),

walleye pollock (*Theragra chalcogramma*; Oozeki & Bailey 1995), yellowtail and winter flounder (*Pleuronectes ferrunginea* and *Pleuronectes americanus*; Baglole *et al.* 1998, Parent 1998), and Atlantic halibut (*Hippogloggus hippoglossus*; Gawlicka *et al.* 2000). These studies, however, did not simultaneously examine how growth and behaviour relate to digestive development in the larvae. Comprehensive information on both behaviour and physiology in young fish is rare because of the difficulty and investment involved with culturing and experimenting on small delicate larvae.

The experiments in this thesis focus on determining the digestive capacity of Atlantic cod larvae by concurrently investigating survival, growth, foraging behaviour, and the patterns of digestive enzyme activity in response to larval diet. This will further our understanding of how these commercially important fish digest certain food types, to what capacity they can alter their digestive functions when exposed to food of differing nutrient quantity, and how growth and behaviour are affected by digestive potential during the larval stage.

This study is also important in the context of intensive aquaculture, which has great economic potential. In addition to growth, fish must also undertake other metabolic activities, such as swimming, foraging, and the ability to mount immunological defense to disease. These activities require energetic expenditure, the fuel from which is derived directly from food. It is the ability and efficiency of the fish to convert food into energy, and how this energy is metabolically partitioned, that will dictate how well the fish can perform these

activities. Therefore, insights into nutritional physiology have direct relevance to several aspects of fish biology. The results of this work have resulted in an integrated and comprehensive Master's thesis that significantly contributes to our understanding of fish physiology.

1.2 Digestive Ontogeny

Cod larvae hatch with a non-functional mouth. The digestive tract, which is located dorsally to the yolk, is a straight narrow tube divided into two sections: a short esophagus and a longer intestine (Timeyko 1987). Larvae must begin feeding within a short period after the yolk-sac nutrients have been exhausted. The time when both endogenous and exogenous foods are utilized is referred to as a "period of mixed feeding". During this period, the larvae must successfully shift from yolk sac utilization to exogenous feeding on live foods to avoid starvation. If exogenous feeding is not initiated, the larvae enter a "point of no return" or "irreversible starvation" whereby the larvae are still alive, but are too weak to feed even if food becomes available (Yin & Blaxter 1987).

At 40 degree-days of age (five days post-hatch at 8°C), cod larvae are approximately 4.5 mm, and begin to feed on external prey items, indicating their shift to exogenous feeding. By this time, the yolk sac has been 65-70% reabsorbed and the swim bladder has inflated (Kjørsvik *et al.* 1991). Significant changes in the digestive system also begin to occur. The digestive tract lumen increases in diameter and the intestinal region becomes more differentiated.

Peristaltic contractions of the intestine can be seen at this stage of development when observing the larvae under a microscope (Timeyko 1987). The liver increases in size, and the gall bladder fills with bile. The esophageal mucosa increases its folding longitudinally, while the intestinal folds are oriented transversely to increase surface area for absorption.

By 80 degree-days, the larvae are approximately 5 mm long and have fully switched to exogenous feeding. The larvae hunt vigorously, catching increasing numbers of prey organisms as they get older (Skiftesvik 1992). The yolk sac, if not already totally absorbed, may remain in the form of a thin band. The digestive tract retains its anatomical structure as before, but peristaltic movements increase, as does the folding of the intestinal mucosa (Timeyko 1987).

Metamorphosis in cod, as with similar marine finfish, is defined as the period when the larval median finfold is reabsorbed or differentiated into median fins and the axial skeletal vertebrae develop. In cod, metamorphosis from the larval to the juvenile stage occurs when the larval median finfold disappears at around 12 mm in length (Pedersen & Falk-Petersen 1992). In addition to alterations in locomotory structures, change associated with capture and digestion of food also occurs. A variation in feeding patterns is accompanied by maturation in digestive morphology and physiology with the eventual development of a distinct stomach, gastric glands, pyloric caeca, and the initiation of gastric digestion (Kjorsvik & Opstad 1989).

Several changes in digestive function are associated with the development of a functional stomach. The stomach has a food storage function, and grinds food into small particles that are passed to the intestine. In adult cod, stomach contents are acidified at the beginning of the digestive process. Proteins are denatured and converted into smaller peptides by pepsin before passage of the chyme to the intestine. The numerous pyloric caeca in the anterior part of the intestine contribute to the major post-gastric absorptive surface area, having enzyme activities similar to the rest of the intestine (Pedersen & Falk-Petersen 1992). However, until the larva reaches metamorphosis, the digestive system resembles that of other stomach-less teleosts (Morrison 1993).

Methods for determining the emergence and functionality of gastric glands in developing fish vary. Gastric glands were first observed in cod of 18-20 mm standard length by Pedersen & Falk-Petersen (1992; temperature data unavailable for conversion to degree-days), yet the point at which the glands became fully capable of secreting HCl and pepsin into the stomach is unknown from the simple contrast stain used during histological examination. A more complex *in situ* hybridization protocol for cod larvae was used by Murray *et al.* (2001) to determine pepsinogen gene expression. This method detected the presence of specific nucleic acid sequences within a cytological preparation. An RNA probe was labeled and hybridized with the sample to detect complementary sequences specific for the probe used, and was distinguished by a chromogenic reaction. Results showed the gastric glands became functional at 275-385 dd.

Although this method allowed for visual confirmation of both histological tissue development and genetic expression of pepsinogen mRNA, it is expensive, technically demanding, and time consuming especially if the probes do not already exist. Perez-Casanova (2003) used biochemical methods for determining the activity of pepsin in cod larvae and found an increase in pepsin activity associated with the onset of a functional stomach starting at 243 dd. The biochemical assay was not pepsin-specific, nor did it have any way of verifying cytological development. Nevertheless, the assay generally agreed with the results of the more detailed *in situ* technique. Such a quick and inexpensive method is a highly useful approximation of tracking pepsin activity, gastric gland and stomach function, and thus onset of metamorphosis.

1.3 Nutrients and Digestive Enzymes

Cod, like other animals, are required to use organic substances obtained from food as respiratory substrates to provide energy for metabolic processes. Once these energetic requirements have been met, the remaining resources can be utilized for growth. Carbohydrates, proteins, and lipids can all be used as energy sources by adult fish to some extent, but they are not equally suited to promote growth. A diet consisting of 60% protein, 25% fat, and 15% carbohydrate has been found to yield optimal growth in juvenile cod (Lie *et al.* 1988). However, both the overall amount and specific type of a particular nutrient are important to consider during larval development, and what is optimal

for a large fish may not be optimal for larval fish. The utilization of nutrients in fish larvae may be limited by the rate of nutrient production by these digestive enzymes, or by the capacity of nutrient transport mechanisms.

1.3.1 Proteins and proteases

Proteins can be broken down into smaller constituent amino acids. These nitrogenous components are joined together by peptide bonds to give long repeating units that ultimately make up the larger protein structure. Amino acids can be used for either energetic expenditure or protein synthesis and portioned toward the overall growth and maintenance of the fish. Some amino acids are "essential" because the fish cannot synthesize them *de novo*, and they must come from the diet. This is especially important in the larval stage, which is characterized by rapid growth rates. Young cod with less than 45% of their dietary carbon coming from protein will suffer from impaired growth and health (Lemieux *et al.* 1999).

Two proteases essential in protein digestion are pepsin and trypsin (Lauff & Hofer 1984). Pepsin is the main digestive enzyme in the stomach where it facilitates digestion by hydrolyzing and denaturing proteins. Inactive pepsinogen is secreted by the chief cells, which must then be activated in an acidic environment. Normally, HCl cleaves one peptide bond from pepsinogen, creating pepsin which can then act as a protease. Pepsin can then hydrolyze

proteins into polypeptides. These polypeptides are then further digested by trypsin in the intestine.

Trypsinogen is synthesized in the pancreas and selectively hydrolyzes and denatures proteins (Rojas-Garcia *et al.* 2001). The pancreas is diffused among the pyloric caeca in larval cod and is functional at hatch (Morrison 1993, Murray *et al.* 2001). Like pepsinogen, trypsinogen must be activated by another enzyme, in this case enterokinase, before it can properly function as trypsin in an alkaline environment. Trypsin can then in turn activate other inactive protease zymogens to further facilitate protein digestion. Trypsin functions as a protease by cleaving polypeptides into shorter chain peptides (Munilla-Moran & Saborido-Rey 1996). After cleaving, amino acids are absorbed more rapidly from these short chain polypeptides than from the equivalent amount of free amino acids (Lied & Solbakken 1984, Rønnestad *et al.* 2001).

The many branches of the pyloric caeca increase the absorptive capacity of the digestive system, and yield a more rapid flow of amino acids into the free amino acid pool. This high rate of release and apparent absorption of amino acids specific to tryptic activity shows the importance of proteases in the digestion process (Lied & Solbakken 1984, Rojas-Garcia *et al.* 2001). Perez-Casanova (2003) found cod trypsin activity at hatch, with peak activity between 80-200 dd. Trypsin has also been shown to be vitally important to the growth potential in older fish. Of several digestive enzymes studied, only trypsin could

potentially set a physiological limit on growth rate and food conversion efficiency in adult cod (Lemieux *et al.* 1999).

1.3.2 Lipids and lipases

Lipids represent a concentrated energy reserve for fish, but also function as a transport medium for lipid-soluble compounds (such as vitamins), as structural elements in cell membranes, and as precursors for a number of biologically active compounds including hormones, pigments, and growth factors (Jobling 1994). Like proteins, there are some types of lipids that cod cannot synthesize or convert from other molecules, so they must be included in the diet for proper metabolic activity and growth. Cod, like other marine fish, cannot convert 18-carbon fatty acids into longer chain fatty acids, so important substances like docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) must be obtained from the food. These are essential 20- and 22-carbon compounds during larval growth when membrane function and neural development is rapidly established. Fatty acid composition of the body tissues reflects dietary input, hence absolute quantities as well as the ratio of important fatty acids are important to proper growth (dos Santos *et al.* 1993, Rainuzzo *et al.* 1997).

To digest dietary lipids, cod larvae rely on lipases. One such important lipase is alkaline phosphatase, which is involved with the absorption and

transport of lipids as well as glucose, calcium, and inorganic phosphate (Tengjaroenkul *et al.* 2000). It is produced in the intestine by brush border cells and is very sensitive to changes in pH along the intestinal tract. Experiments with white sturgeon (*Acipenser transmontanus*) and sea bass (*Dicentrarcus labrax*) have shown that intestinal brush border enzymes like alkaline phosphatase, which are membrane-bound proteins in the intestine, have lowered enzymatic activity in response to high lipid diets (Gawlicka *et al.* 1996, Cahu *et al.* 2000). This could be due to an alteration in the fluidity of the brush border membranes in the presence of high quantities of lipids.

Research has traditionally focused on increasing the quantity of lipid in larval diets. High lipid diets have favoured larval growth, feed efficiency, protein sparing and nitrogen losses (DeSilva & Anderson 1995, Rainuzzo *et al.* 1997). It is now, however, becoming apparent that lipid levels in excess of optimal levels can have detrimental effects to fish health and growth. In addition to the digestive tract, effects of high lipids in the diet are now being seen in other body tissues. Cod store lipids in the liver, which normally retains 50-60% of dietary fats, and accounts for over 10% of the mass of the fish (Lie *et al.* 1986). Excessive amounts of dietary lipid can result in cod forming large corpulent livers, also known as "fatty liver syndrome". This diverts resources away from being used for growth, but may also hinder buoyancy regulation, liver function, and overall health, as well as flesh quality through deposition of fats into tissues (Lie *et* al. 1986, Jobling 1988, Grant *et al.* 1998). It is therefore essential that

studies attempt to determine an upper and lower threshold of lipids in larval cod diets such that maximal growth is achieved, while adverse health effects are avoided. The experiments in this thesis address both of these concerns with respect to commercially available live prey enrichments.

1.3.3 Carbohydrates and Carbohydrases

Carbohydrates constitute the smallest fraction of the diet of fish. Glucose, for example, may be of primary importance as an oxidative substrate to some cells and tissues in fish but overall, carbohydrates play a subordinate role to lipids and proteins despite variability between fish species (Hemre *et al.* 2002). The basic carbohydrase found in the digestive tract of fish is α-amylase, which is a pancreatic enzyme that is secreted into the intestine and breaks down carbohydrates into smaller polysaccharides (e.g., starch into glucose).

There is a direct correlation between amylase activity in the gut and the type of food being digested (Glass *et al.* 1987, Hidalgo *et al.* 1999). High amylase activity is characteristic of stomachless omnivorous fish species. Carnivorous species tend to have very low amylase activity but possess other carbohydrases such as chitinase (Lindsay 1987, Perez-Casanova 2003). Cod are carnivores that feed by saltatory predation, where the fish pauses while searching for prey in a small area before moving a short distance and pausing to search again (Hunt von Herbing & Gallager 2000). It stands to reason that

because cod larvae do not encounter high quantities of carbohydrates in their natural diet, that there would be little need for significant carbohydrase activity. However, this lack of carbohydrase activity has also contributed to the difficulty in mass culturing cod. For example, inexpensive carbohydrates typically added to carp feeds as binding agents and fillers have low consumption and digestibility rates in cod (Hemre *et al.* 1989, Hemre *et al.* 1990, Baskerville-Bridges & Kling 2000). This has resulted in manufactured cod feeds being a challenge to develop with both high palatability and economic practicality.

1.3.4 Trends in Enzyme Activity Studies

Studies have found that digestive enzymes are present (under controlled conditions) before larvae start to feed exogenously, and that morphological differentiation of the digestive tract corresponded to specific regionalization of the digestive enzymes. In yellowtail and winter flounder, digestive enzymes such as trypsin, esterase and alkaline phosphatase are usually present by the time the larvae start to feed exogenously at 55 dd post-hatch, and this enzymatic activity increases with age (Baglole *et al.* 1998). Relationships between digestive enzyme activities and the morphological development of digestive organs were also found by Oozeki & Bailey (1995) in larval walleye pollock. Activities of trypsin and lipase were found to be low during the transition from endogenous to

exogenous feeding. All three enzyme activities increased with age after this transition period, and then became stable.

Recent studies examining the activity of digestive enzyme activity in cod larvae have shown distinct patterns (Murray *et al.* 2001, Perez-Casanova 2003). Due to the altricial nature of the larval cod digestive system, a distinct absence of pepsin activity in early larval development was predicted and observed since there is no functional stomach at this stage. However, trypsin was observed to be active at hatching. This was predicted, since the undifferentiated intestine must be able to digest exogenous prey in an efficient manner before endogenous yolk resources are depleted. However, the assay used was not specific for trypsin and may have detected trypsin-like alkaline proteases or breakdown of zymogens like trypsinogen instead of the actual trypsin enzyme. These studies looked at the changes in enzymes over time when fed on a standard diet. However, they did not look at the adaptability of the behavioural and biochemical responses of the fish by varying the dietary levels of protein and lipids in the food.

The reasons for high larval mortality and poor growth of cod larvae on both live and prepared foods are still not clear. In fish that do not successfully initiate feeding between 25-40 dd post-hatch, there is evidence that there is a regression of the digestive tract with atrophy and muscular degeneration, and desquamation of the intestinal mucosa with the larvae dying soon after (Kjørsvik & Opstad 1989). This suggests that the determination of the digestive capacities

and limitations during the first days of life may explain why Atlantic cod larvae fail to grow. This significant reduction in feeding is one factor explaining the high mortality of larvae during mass rearing.

1.4 Growth and Foraging Behaviour

Meristics of growth in fish larvae vary. For example, the length of a fish can be expressed as standard length, fork length, or total length. The weight of a fish can be defined as wet weight, dry weight, or as percent total biomass. Examinations of larval fish growth are complicated by variables that affect how much a larva can increase its size, and potentially its survival. Factors such as temperature, light, dissolved oxygen, larval density, and prey density and quality all affect the ability of larvae to survive and grow (van der Meeren & Naess 1993; Munk 1995; Hunt von Herbing *et al.* 1996; Puvanendran & Brown 1998, 1999; Puvanendran *et al* 2002; Hunt von Herbing & Gallager 2000). By understanding the factors which determine if larval fish successfully forage, we will have a better chance of being able to predict the success of these fish early in their life history. This will also help establish a successful rearing schedule for cod to make mass production a viable commercial enterprise.

Larval and adult cod are referred to as saltatory or pause-travel predators. During saltatory predation, search for prey items only occurs when the predator is stationary and the search takes place within the entire volume of the search space (Hunt von Herbing & Gallager 2000). If the search is not successful, the

predator swims a short distance before stopping to search again. This is an intermediate predator strategy, incorporating elements of cruising predators that continuously move through the water while searching for prey, and ambush predators that remain stationary for long periods of time waiting for prey to cross the outer boundary of the search space.

For cod, first feeding occurs around 20 degree-days (Skiftesvik 1992, Perez-Casanova 2003). Successful predation on small zooplankton provides a necessary metabolic energy source, but also incurs an increased energetic expense, which the larvae must overcome for survival. Thus, one would expect not only ontogenetic changes in behaviour patterns, but also changes in response to the feeding environment. Skiftesvik (1992) found that the behaviour of cod does indeed change at the onset of exogenous feeding, where the level of activity increases but swimming speed decreases. Activity was correlated with the morphological development of the larvae: activity rose between 15-55 dd post-hatching, coinciding with the development of the fin-fold and the absorption of the yolk sac. Yin & Blaxter (1987) found a general peak in feeding rate and intensity in a variety of yolk-sac larvae within 12 hours of the yolk fully absorbing. Puvenandran *et al.* (2002) found a steady increase in swimming activity and prey capture success of cod larvae from 15 dd onwards with a 100% capture success rate by 180 dd.

Food limitation is likely a source of mortality for cod larvae in the first few weeks after hatching. Prey densities for larval cod in the wild are unknown, but

larvae reared in the laboratory at prey densities below 1000 prey/L do not survive to metamorphosis (Puvanendran & Brown 1999). However, Munk (1995) has demonstrated that cod larvae have considerable flexibility in their foraging behaviour. As prey density decreased, hungry larvae increased their swimming activity, increased their responsiveness to prey, and decreased their prey size selectivity. Hunt von Herbing and Gallager (2000) examined the foraging behaviour of cod larvae fed protozoa and copepod nauplii, and found that the percentage of successful attacks by the larvae increased with fish size. In all size classes, successful attacks had smaller attack distances and faster attack speeds than unsuccessful attacks. The slow swimming protozoa were the preferred food of first-feeding larvae, while larger larvae had higher swimming speeds and captured the larger, faster copepod nauplii. This study was unique in that it recorded both successful and unsuccessful attacks on prey, because unsuccessful attacks are an important energy expenditure and have an impact on growth and survival.

In order to optimize feeding and digestion in cod larvae, both behavioural and physiological aspects of the larvae must be considered over time in relation to different diets. This is of vital importance. By simply looking at meristics, important patterns of behaviour can be missed that have a direct impact on development, growth and survival. We want to know not only how well the larvae do in terms of growth and survival in different feeding circumstances, but we also

want to know what foraging behaviours the larvae are showing that make them fare better in specific feeding environments. Since the aim of this study is to answer practical and applied questions as they relate to overall physiological success of the fish in the wild and possibly through mass production of cod on a commercial scale, it is essential that all factors affecting growth and survival be examined. In this way, we can determine if there is an underlying correlation between digestive ontogeny, foraging behaviour, and digestive enzyme activity. This will elucidate the mechanisms by which larvae convert food into metabolic energy, how efficient this process is given a specific diet history, and how metabolism in later life is impacted by larval diet.

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2 Integrating growth, behaviour and digestive enzyme activity to assess larval Atlantic cod (*Gadus morhua*) responses to different rotifer diets.

2.1 Abstract

Atlantic cod (*Gadus morhua*) show great potential for mass-rearing, but much is unknown about their digestive capacity and efficiency. An integrated experiment was performed on cod larvae to investigate the variability in digestive development in response to the quantity of lipid in the rotifer enrichment. Survival, growth, behaviour and digestive enzyme data from hatching to metamorphosis (0-450 dd) were measured. Four treatments were used in triplicate: high lipid rotifer enrichment (HLRE), low lipid rotifer enrichment (LLRE), green water, and unfed.

Swimming activity and attacks (captures + misses) on prey were higher in the HLRE group by 100 dd and increased over time. There were no differences between digestive enzyme activity for the unfed and greened treatments, while the LLRE larvae had lower activity levels than larvae fed HLRE by 100-150 dd for all enzymes assayed. The larvae in the unfed and green water treatments did not survive past 100 dd. The LLRE cod died by 350 dd. Results suggest that a higher quantity of lipid in the rotifer enrichment will not only promote higher growth and survival in Atlantic cod larvae, but appears to provide more energy to allow larvae to capture more live prey.

2.2 Introduction

Atlantic cod (*Gadus morhua*) is being developed in several countries as a species that might lead to commercial production in the near future. Cod have good biological potential for mass culture because they are highly fecund, readily spawn in captivity, and are capable of growth rates similar to other cultured species, even at low temperatures (Howell 1984, Finn *et al.* 2002). To achieve good growth, cod require energy which is derived from the ingestion of food. Two factors influence the way food is used as a metabolic fuel by a rapidly growing larval fish: the nutritional content of the food, and the efficiency with which the larva can digest and metabolize nutrients (Jobling 1994).

Studies of larval marine fish growth have stressed the importance of successful initiation of predatory behaviours during the critical first feeding period (Hunter 1981, Kjorsvik & Opstad 1989, van der Meeren & Naess 1993, Hunt von Herbing & Gallager 2000). It is essential for larval growth that basic energetic and growth demands are met, which entails prey being captured in sufficient numbers to meet essential nutritional requirements (Puvenandran & Brown 1999). In the mass rearing of cod larvae, the rotifer (*Brachionus plicatilis*) is used as a first feeding prey but rotifers alone are not a complete diet to meet finfish larval metabolic demands (Rainuzzo *et al.* 1989).

For Atlantic cod, optimizing live feed enrichments first requires understanding the digestibilities and retentions for three major nutrient classes: carbohydrates, proteins and lipids. Carbohydrates are not a major constituent of

the larval cod diet, and as such cod larvae lack digestive carbohydrase activity in the first two months of life (Perez-Casanova 2003). Thus, most research is now focused on the importance of proteins and lipids.

During the last twenty years, studies on live food enrichments for larval teleosts have focused on the quantity and quality of lipids in their formulas. It has been widely reported that high quantities of lipids in the first feeding diet will improve larval development (Rainuzzo *et al.* 1997, 1998). Lipids are important as they form the basis of cellular membranes and are vital for proper neural development, but they also act as transporters of lipid-soluble compounds such as vitamins, and are structural precursors for hormones (Jobling 1994). Increasing the overall energy of the diet is further considered beneficial for a high feeding efficiency, protein sparing and for reducing phosphorous and nitrogen losses (Kaushik and Olivia-Teles 1985, Cahu *et al.* 2000). These enrichments focus on providing larvae with essential lipids for proper tissue development (Rainuzzo *et al.* 1997, Cahu *et al.* 2000), while at the same time not providing excessive amounts of lipids that may become deposited in the liver and drive up production costs (Lie *at al.* 1986, Grant *et al.* 1998, Morias *et al.* 2001).

Recent studies have shown trends in the development of digestive enzyme activities over time in sea bass, red drum (*Sciaenops ocellatus*), and Atlantic cod and haddock (*Melanogrammus aeglefinus*) (Infante & Cahu 1994, Lazo *et al.* 1998, Perez-Casanova 2003, Murray *et al.* 2001). However, the ability of the larvae to adapt and vary their digestive development in response to

differences in diet has thus far not been investigated, nor has enzymatic activity been studied and analyzed concurrently with growth and behavioural data. This experiment addresses the following question: Does the level of lipid in rotifer enrichment affect the survival, growth, behavioural and biochemical responses of Atlantic cod larvae?

2.3 Materials & Methods

2.3.1 Larviculture and diets

Atlantic cod eggs were obtained from OSC broodstock, disinfected, and incubated in 500 l tanks at 6°C until ~70% had hatched (~80 dd), which was taken as day 0 of the experiment. Cod were acclimated and transferred to 3000 l experimental tanks, stocked with 50 larvae/l with constant aeration at 8 °C. An initial water flow of 2 l/min and low light intensity were gradually increased to 8 l/min and 2000 lux over the first two weeks of the experiment. Temperature ranged from 8-10°C over the course of the experiment in the 3000 l tanks.

Rotifers (*Brachionus plicatilis*) were used as the live prey for the duration of this experiment. Rotifers were reared in 3000 l tanks, starting with 450 l freshwater and 450 l seawater at 23-28 °C, with constant gentle aeration. Initial stocking density was less than 700/ml. Rotifers were cultured on baker's yeast *Saccharomyces cerevisiae* and culture Selco. Each day, a population and egg count were performed on a number per volume basis, then 420 l of water (315 l

filtered seawater and 105 l freshwater) were added to the tank. This was repeated daily for six days, after which the rotifers were removed from the tank, washed, concentrated, and placed in a 300 l conical tank for enrichment.

Two diets were used in triplicate: a high lipid rotifer enrichment (HLRE) with 21% dw TAG (triacylglycerol), and a low lipid rotifer enrichment (LLRE) with 5% dw TAG. Single tanks with larvae were "greened" with *Isochrysis* algae only, or were unfed, without replicates for ethical reasons. Enrichments were added to the rotifer tanks twice in a twelve hour period at 9:00 pm and 3:00 am. The enriched rotifers were harvested at 9:00 am, rinsed, and put into 10 l seawater in 20 l buckets and kept in the same room as the experimental tanks. Air stones were added to the buckets to provide constant aeration. Ten liters of seawater were added to the buckets to further dilute the rotifers and reduce their temperature. This helped prevent rotifers from settling out of the tank before larvae had an opportunity to feed. Aliquots of 10 ml were taken at several locations throughout the experimental tanks with larvae three times daily and counted to determine prey density (data not presented). Rotifers were added to the tanks as necessary to sustain an optimal prey density of 4000/l (Puvenandran & Brown 1999).

2.3.2 Enrichment analyses

During the first week of the experiment, triplicate samples of rotifers (unenriched rotifers to serve as a baseline for comparison, and the LLRE and

HLRE diets) were taken for dry weight and lipid analyses. Rotifer 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 (TLC/FID) with an Iatroscan (Parrish 1987). Extracts were spotted on silica gel coated Chromarods and a three stage development system was used to separate lipid classes.

2.3.3 Growth and behavioural data

Growth data for standard length and myotome height were taken every 50 dd (~7 days) with a stereomicroscope and a calibrated eyepiece micrometer. Condition factor and length specific growth rate were calculated as per Jobling (1994):

Condition Factor = Myotome height (mm) / Standard Length (mm);

Length Specific Growth Rate = $[\text{Ln}(SL_{t2}) - \text{Ln}(SL_{t1}) / t_2 - t_1]$, where

SL_{t2} is the Standard Length (mm) at time interval 2,

SL_{t1} is the Standard Length (mm) at time interval 1, and

$t_2 - t_1$ is the difference (days) between time intervals 2 and 1.

Behavioural data were taken every 50 dd (7 days) using a two minute focal animal technique, where an individual larva is observed for two minutes.

"Swimming activity" was defined as the percent time a larva moved through the water column by movements of the caudal body area, and "attacks" were defined as the frequency (number per time) of captures (bites and ingests) and misses (failed captures) of prey (Puvenandran & Brown 2002) A Psion computer with observation software was used to record and compile data. Ten larvae were observed per tank per treatment.

2.3.4 Digestive enzyme activity data

Triplicate pooled samples of larvae were taken for biochemical determination of digestive enzyme activity at 0, 20, 50, 100, 150, 200, 250, 300, 350, 400, and 450 dd post-hatching. Three hundred larvae were collected at hatch to obtain the minimum 0.5 ml tissue necessary for each assay, and the number of fish collected over time decreased as the fish grew. After repeated visual verification, larvae were collected between 9:30 and 10:00 am to ensure empty guts and reduce the impact of prey enzymes. A small aquarium net was used to remove larvae from the tanks, and fish were transferred to a 1 l cup with 0.07 mg/l of MS-222 in seawater. A plastic 1 ml pipette was used to remove larvae from the cup by gently sucking the larvae up into the transparent neck of the pipette and larvae were then counted. Larvae were then released onto a framed mesh of 500 µm netting. Salt water and MS-222 were allowed to drain off the fish, which were then rinsed with fresh water and also drained. The larvae

were finally placed in 1.5 ml Eppendorf tubes and stored at -80°C until they were processed and analyzed.

Processing the tissues for analysis required defrosting the samples on ice (~4°C), diluting with 4 parts (w/v) 150 mM NaCl, then using an automated tissue grinder to create whole body homogenates. These homogenates were transferred to 1.5 ml Eppendorf tubes and centrifuged for 10 minutes at 12,000 *g* at 4°C. The supernatants were aliquoted in 0.5 ml Eppendorf tubes and stored at -80°C until assayed for total protein concentration, general proteases, trypsin-like alkaline proteases, pepsin-like acidic proteases, general lipases, and alkaline phosphatase. Assays were conducted in triplicate for each replicate sample, and were performed at room temperature by diluting with two volumes of the respective assay buffer.

Total protein concentration of the homogenate was assayed with the BioRad Protein Assay (Bradford 1976). Bovine gamma globulin was used to produce a standard curve from which concentrations were derived. These total protein concentrations were then used to standardize the subsequent enzyme activity results to larval size as the fish grew over the course of the experiment.

General protease activity was measured using the azocasein hydrolysis assay (Ross *et al.* 2000). Samples were incubated in 2.5 volumes of 5.0 mg/ml azocasein dissolved in 100 mM ammonium bicarbonate (pH 7.8). Samples were constantly shaken for 20 hours at 25°C, after which the reaction was stopped by

adding 0.3 volumes of 20% TCA (trichloroacetic acid). These mixtures were centrifuged for 10 minutes at 17,000 *g*, and 100 µl of supernatant was added to 100 µl 0.5 M NaOH in a microplate. Optical density was measured at 450 nm. Enzyme specific activities are reported as units of activity per mg total protein (one unit represents the amount of enzyme that will increase the absorbance of the sample 0.001 Optical Density units per 20 hours).

Trypsin-like alkaline protease activity was assayed based on the methods in Gawlicka *et al.* (2000). The substrate was prepared by diluting 4.4 mg BAPNA in 50 µl DMSO, then adding 5 ml of a 100 mM ammonium bicarbonate buffer (pH 7.8) to yield a 2 mM BAPNA solution. Cod samples (25 µl) were incubated with 50 µl of the 2 mM BAPNA substrate and 25 µl buffer for 5 minutes at room temperature (~25°C) in a microplate. The blank contained 50 µl buffer and 50 µl substrate. Optical density was then measured at 450 nm in 30 second intervals over 30 minutes to get an initial rate of reaction, which was then used to calculate the enzymatic activity of the sample. Enzyme activities are reported as units of activity per mg protein (one unit represents 1 µmol *p*-nitroaniline liberated during one minute of hydrolysis).

Pepsin-like acidic protease activity was assayed according to methods in Anson (1938). The substrate was prepared by diluting 0.2 g of 2% hemoglobin stock solution in 10 ml distilled water, mixing vigorously and filtering through a glass wool filter. Eight milliliters of this solution then had ~2 ml HCl added to adjust the pH to 2.0. Cod samples (50 µl) were incubated for 10 minutes at room

temperature (~22°C) with 250 µl of the final 1.6% hemoglobin substrate in a microplate. The reaction was stopped by adding 500 µl of 5% (w/v) TCA, and then centrifuged at 3600 *g* for 6 minutes. The blank contained 250 µl substrate and 500 µl 5% TCA. Optical density of the supernatant was measured at 280 nm using quartz cuvettes. Enzyme activities are reported as units of activity per mg total protein (one unit represents 1 µmol tyrosine liberated during one minute of hydrolysis).

General lipase activity was assayed according to methods in Gawlicka *et al.* (2000). A detergent solution was prepared by adding 1.0 ml of 10 % Triton X-100 to 9.0 ml 100 mM ammonium bicarbonate buffer (pH 7.8). The substrate was a 10 mM solution of *p*-nitrophenyl myristate in 100% ethanol. A microplate containing wells with 25 µl buffer, 25 µl substrate and 50 µl detergent solution was incubated at room temperature (~22°C) for 15 minutes, then 4 µl of room temperature cod homogenates were added. Blanks contained 50 µl buffer, 50 µl detergent solution, and 4 µl substrate. Optical density was measured at 405 nm in 30 second intervals over 30 minutes to get an initial rate of reaction, which was then used to calculate the enzymatic activity of the sample. Enzyme activities are reported as units of activity per mg protein (one unit represents 1 µmol *p*-nitrophenol liberated during one minute of hydrolysis).

Alkaline phosphatase activity was assayed according to methods in Gawlicka *et al.* (2000). A 100 mM ammonium carbonate-magnesium chloride buffer was made by adding 0.79 g ammonium bicarbonate to 0.020g magnesium

bicarbonate and diluting in 100 ml distilled water. The substrate was a 20 mM solution of *p*-nitrophenyl phosphate disodium prepared by diluting 74.22 mg *p*-nitrophenyl phosphate disodium in 10 ml of the buffer solution. A microplate containing wells with 55 µl buffer and 25 µl sample were incubated at room temperature (~22°C) for 5 minutes, then 20 µl of room temperature substrate was added. Blanks contained 80 µl buffer and 20 µl substrate. Optical density was measured at 405 nm in 30 second intervals over 30 minutes to get an initial rate of reaction, which was then used to calculate the enzymatic activity of the sample. Enzyme activities are reported as units of activity per mg protein (one unit represents 1 µmol *p*-nitrophenol liberated during one minute of hydrolysis).

2.3.5 Statistical analyses

Prior to analysis, survival, growth, behaviour, and enzyme data were tested for assumptions of normality and homogeneity of variance before an ANOVA (analysis of variance) was employed. Distribution of the data, plots of residuals, and predicted values were examined. There was no significant tank effect ($F_{2,8}<4.07$, $p>0.05$ for all treatments), and data from treatments were pooled. Statistical analyses were performed with Minitab statistical software (version 13). One-way ANOVAs were performed to detect differences between the means of treatments using a significance level of $\alpha=0.05$. The data were normal and homogeneous, thus transformation or randomization was not required before statistical analyses were employed.

2.4 Results

2.4.1 Enrichment analysis

The HLRE contained the highest quantity of overall lipids when standardized to dry weight of the sample, and the LLRE rotifers were not significantly different from the unenriched rotifers (Table 2.1). The proportion of individual lipid classes was higher in the HLRE for triacylglycerols and free fatty acids, while the LLRE had proportionately more acetone mobile polar lipids. The relative amounts of ethyl esters, sterols and phospholipids were similar for rotifers in both enrichments.

Three fatty acids were evaluated: arachidonic acid (AA, 20:4ω-6), eicosapentaenoic acid (EPA, 20:5ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), as well as the sum of the ω3 and ω6 fatty acids. The LLRE did not differ from the unenriched baseline rotifers, but the HLRE were significantly higher than both the LLRE and unenriched rotifers (Table 2.1). The DHA:EPA ratio for the LLRE was 0.3, while the HLRE ratio was 3.7. The ω3:ω6 ratio for the LLRE was 0.9, while the HLRE ratio was 2.4; however, AA concentrations were the same in both unenriched and HLRE rotifers.

Table 2.1. Lipid class and fatty acid class composition of unenriched rotifers (as a baseline), LLRE (low lipid rotifer enrichment) and HLRE (high lipid rotifer enrichment) diets. Values are means of triplicates ± standard error. Values in the same row with the same superscript are not significantly different ($F_{2,8}$< 4.46, p>0.05).

	Rotifer enrichments:		
	Unenriched	LLRE	HLRE
Total lipid (µg/mg dw)	32.0±9.4 [a]	44.4±4.8 [a]	78.3±8.6 [b]
Total lipid (%):	3.2 ± 0.5 [a]	4.4± 0.1 [a]	7.8 ± 0.7 [b]
Lipid class (% total lipid):			
Ethyl esters	13.3±1.5 [a]	14.7±2.0 [a]	12.7±1.4 [a]
Triacylglycerols	15.5±1.7 [a]	5.1±1.2 [b]	20.6±1.7 [a]
Free fatty acids	4.3±0.9 [a]	2.7±1.0 [a]	20.3±1.6 [b]
Sterols	6.7±0.7 [a]	4.2±0.6 [b]	3.0±0.1 [b]
Acetone mobile polar lipids	3.1±0.1 [a]	42.7±3.4 [b]	19.2±1.1 [c]
Phospholipids	55.2±2.7 [a]	21.4±2.3 [b]	21.6±2.8 [b]
Fatty acids (µg/mg dry weight):			
AA	0.4±0.1 [a]	0.5±0.1 [b]	0.4±0.3 [a]
EPA	0.4±0.1 [a]	0.5± 0.2 [a]	1.2±0.5 [b]
DHA	0.1±0.1 [a]	0.2±0.1 [a]	4.5±2.2 [b]
Σω3	1.0±0.4 [a]	1.4±0.3 [a]	6.3±0.9 [b]
Σω6	1.1±0.4 [a]	1.8±0.3 [a]	2.6±1.1 [b]
Fatty acid proportions and ratios:			
%AA	1.5±0.1 [a]	1.4±0.11 [a]	1.1±0.3 [b]
%EPA	1.7±0.2 [a]	1.6±0.13 [a]	3.4±1.9 [b]
%DHA	0.5±0.1 [a]	0.5±0.07 [a]	12.4±4.5 [b]
DHA:EPA	0.3±0.3 [a]	0.3±0.1 [a]	3.7±0.2 [b]
Σω3: Σω6	0.9±0.2 [a]	0.8±0.1 [a]	2.4±0.1 [b]

2.4.2 Survival and growth

Larvae in the green water and unfed treatments died 100 dd after hatching. All three tanks containing cod larvae fed the LLRE died by 300 dd. A total of 4613 ± 1358 (mean ± standard error) larvae survived in the HLRE treatment (~3.1% survival).

Both myotome and standard length for the unfed and green water treatments decreased compared to their size at hatch. Myotome height was significantly higher for larvae fed HLRE by 150 dd ($F_{1,59}$=28.06 , $p<0.001$), and standard length was significantly greater by 200 dd ($F_{1,59}$=14.54, $p<0.001$) compared to larvae fed LLRE. Myotome height and standard length for LLRE larvae plateaued from 100-300 dd. HLRE larvae, however, continued to grow over the course of the experiment (Figure 2.1 A&B).

Condition factor showed a similar trend to that of length. HLRE fed larvae had significantly higher condition factor than LLRE larvae by 200 dd ($F_{1,59}$=39.68, $p<0.001$). The HLRE larvae continually increased condition factor over the course of the experiment, while the LLRE larvae displayed a slow overall decline in condition factor, and both green water and unfed treatments decreased condition until death at 100 dd (Figure 2.2 A).

Length specific growth rates were negative after the initial sampling date for greened and unfed larvae, and both LLRE and HLRE larvae slowed their growth rates over the course of the experiment (Figure 2.2 B). However, the rate

of growth slowed significantly faster in the LLRE larvae by 200 dd ($F_{1,59}$=20.80, $p<0.001$) compared to the HLRE larvae (Figure 2.2 B).

2.4.3 Behaviour

The proportion of time larvae spent swimming was not significantly different between the greened and unfed treatments, where both treatments declined to 100 dd. Swimming activity for larvae fed HLRE was fairly constant. HLRE larvae spent 38.2% of their time swimming by the end the experiment, while larvae fed LLRE significantly reduced swimming activity after 100 dd ($F_{1,5}$=12.49, p=0.024) and declined until death (Figure 2.3 A).

With the exception of 150 dd, attacks on rotifers were observed to be significantly lower in LLRE-fed larvae after 100 dd ($F_{1,5}$=11.96, p=0.026), while HLRE larvae increased their attacks up to 200 dd, after which the predation rate stabilized (Figure 2.3 B). Attacks on prey were not measured in the green water and unfed tanks, since no rotifers were introduced to the tanks.

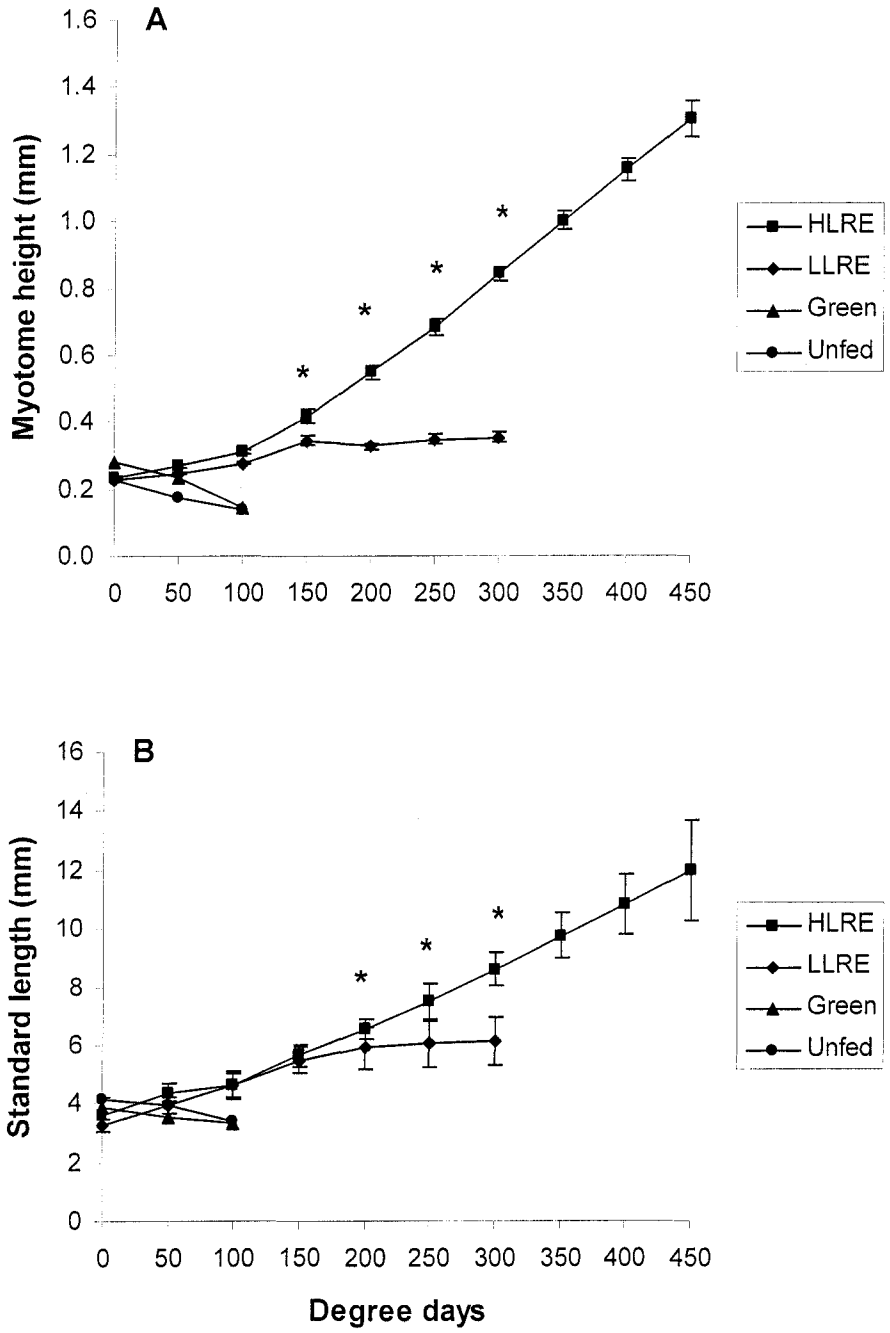

Figure 2.1. (A) Myotome height (mm) and (B) Standard length (mm) of cod larvae over time among four feeding treatments.
HLRE (high lipid rotifer treatment, n=30), LLRE (low lipid rotifer enrichment, n=30), Greened (n=10) and Unfed (n=10). Bars represent one standard error of the mean.
* denotes significant difference between the HLRE and LLRE treatments ($p>0.05$).

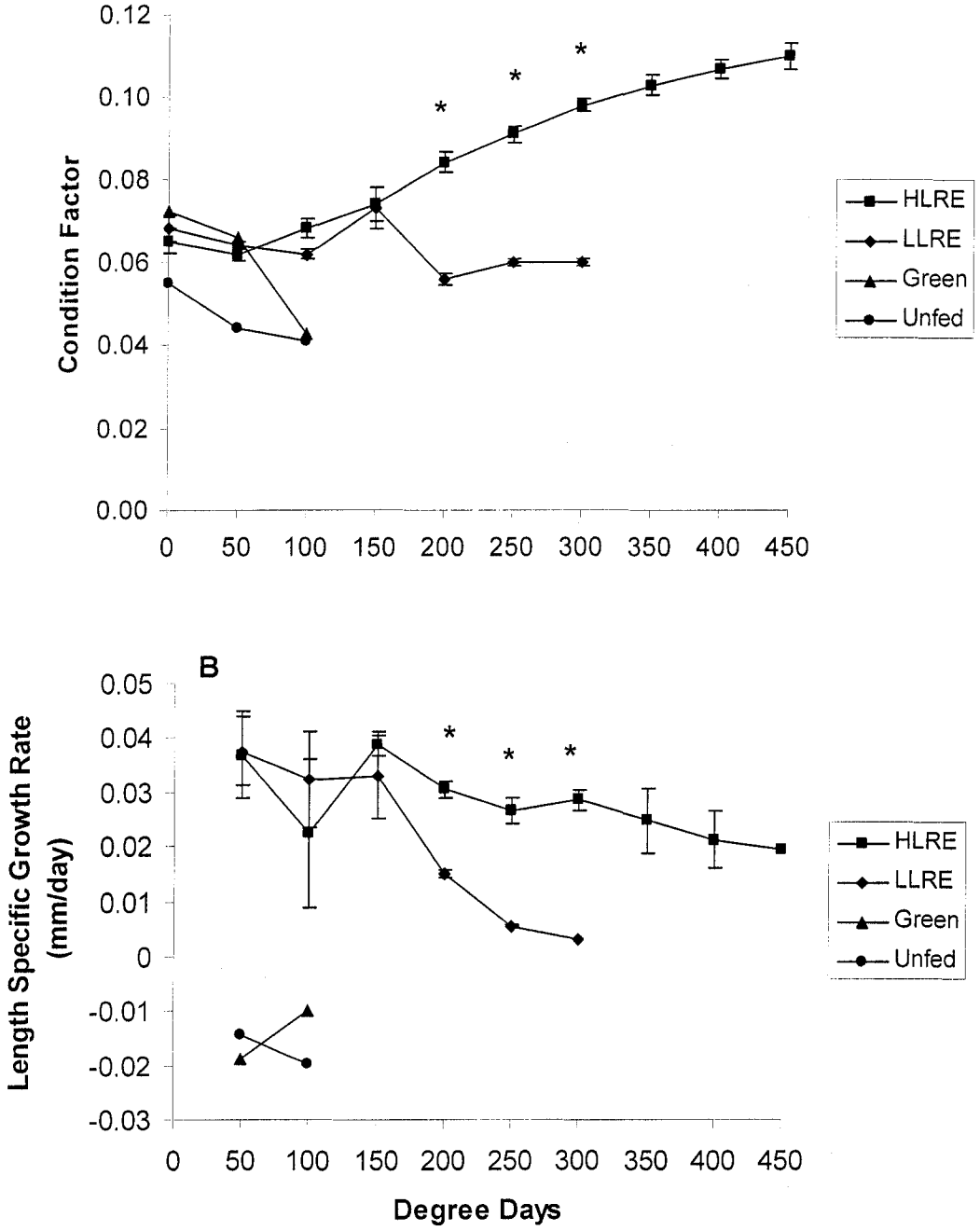

Figure 2.2. (A) Condition factor and (B) Length specific growth rate (mm/day) of cod larvae over time among four feeding treatments.
HLRE (high lipid rotifer treatment, n=30), LLRE (low lipid rotifer enrichment, n=30), Greened (n=10) and Unfed (n=10). Bars represent one standard error of the mean.
* denotes significant difference between the HLRE and LLRE treatments ($p>0.05$).

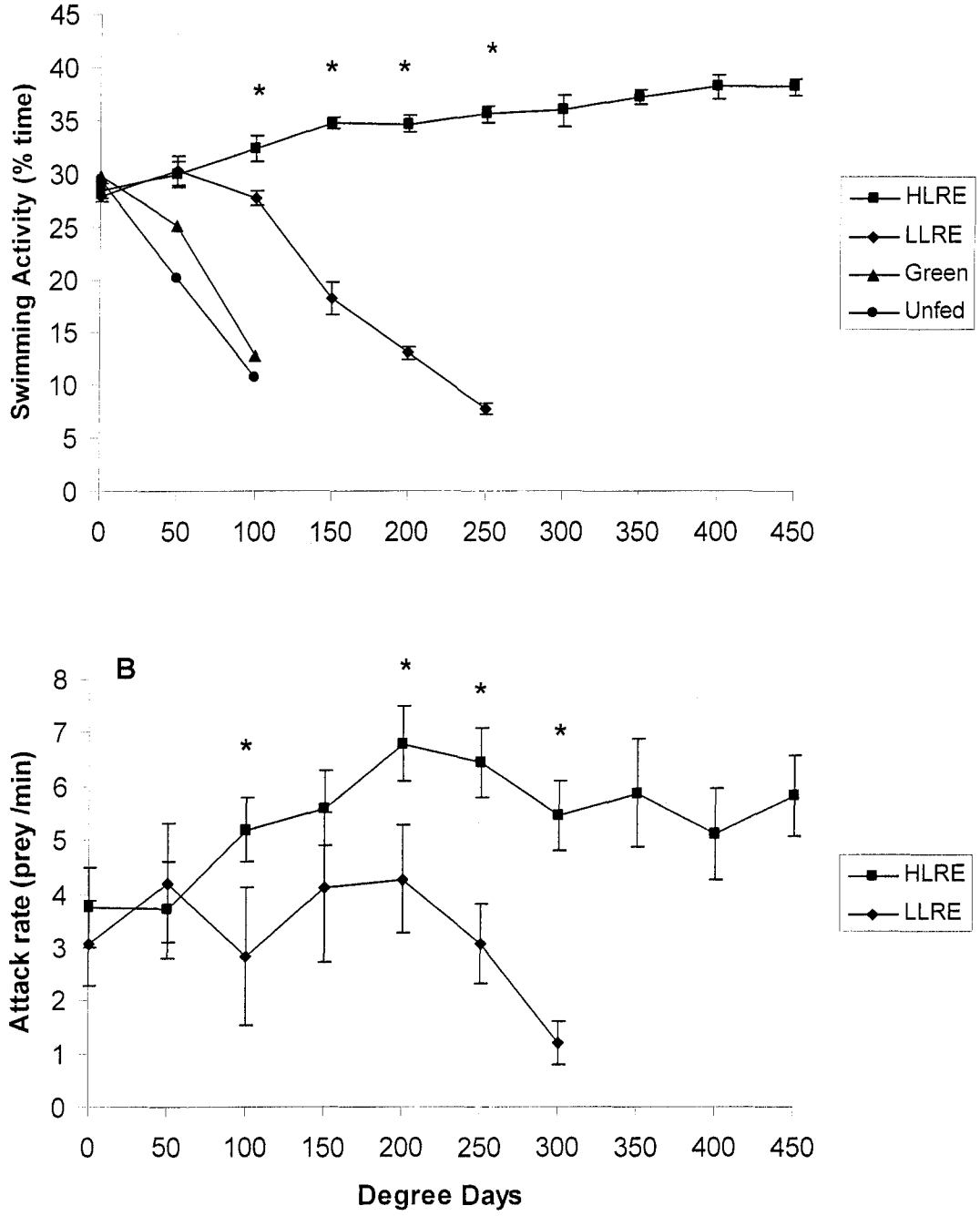

Figure 2.3. (A) Swimming activity (% time) and (B) Attack rate (captures+misses; prey/min) of cod larvae over time among four feeding treatments.
HLRE (high lipid rotifer treatment, n=30), LLRE (low lipid rotifer enrichment, n=30), Greened (n=10) and Unfed (n=10). Bars represent one standard error of the mean.
* denotes significant difference between the HLRE and LLRE treatments ($p>0.05$).

2.4.4 Digestive enzyme activity

The activity of digestive enzymes differed between treatments. The unfed and greened treatments had their highest enzyme activity levels at hatching, followed by a consistent decline in activity for all enzymes measured (proteases, trypsin, pepsin, lipases, and alkaline phosphatase), as well as overall protein concentration of the larvae (Figures 2.4-2.9). Comparing the activity of digestive enzymes between cod fed rotifers enriched with either HLRE or LLRE, a similar pattern was observed. Since the larvae fed LLRE did not survive past 250 dd, there are no data for that treatment beyond that time.

What is interesting however, is the point at which the enzyme activity levels start to significantly differ. The HLRE larvae had significantly higher values for general proteases ($F_{1,8}$=36.39, $p<0.001$; Figure 2.5) and alkaline phosphatase ($F_{1,8}$=27.18, $p<0.001$; Figure 2.9) by 100 dd, and for overall protein content ($F_{1,8}$= 30.58, $p<0.001$; Figure 2.4), trypsin ($F_{1,8}$= 44.75, $p<0.001$; Figure 2.6), pepsin ($F_{1,8}$= 66.12, $p<0.001$; Figure 2.7), and lipase activity ($F_{1,8}$=102.58 ,$p<0.001$; Figure 2.8) by 150 dd. By 250 dd of age, larvae fed LLRE had reached their lowest activity levels of the experiment, akin to levels found in green water and unfed treatments before their death at 100 dd.

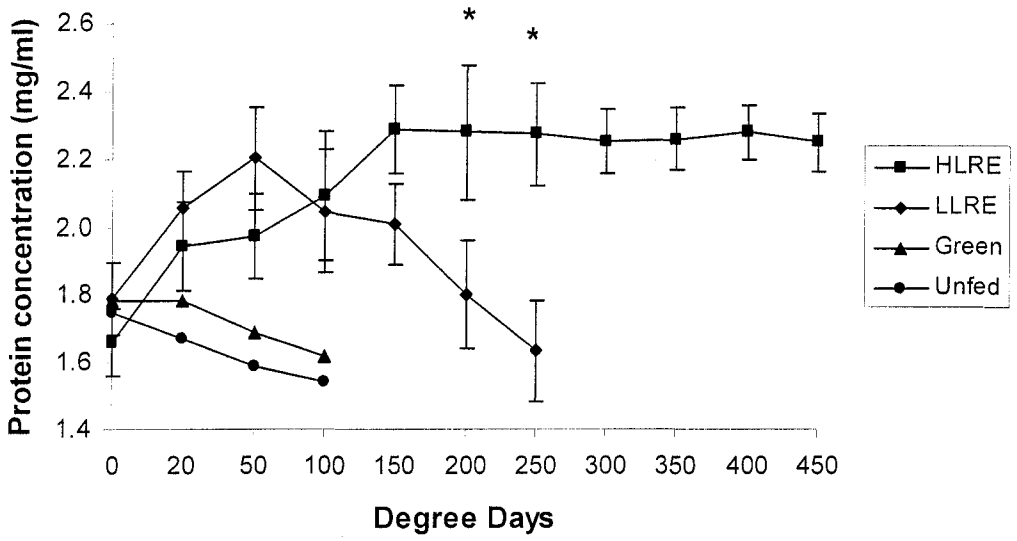

Figure 2.4. Total protein concentration (mg/ml) of 0.5 ml cod larvae over time among four feeding treatments. Points represent means of triplicates for HLRE (high lipid rotifer enrichment) and LLRE (low lipid rotifer enrichment), and the single tank means for the Greened and Unfed treatments. Bars represent one standard error of the mean.
* denotes significant difference between HLRE and LLRE treatments (p>0.05).

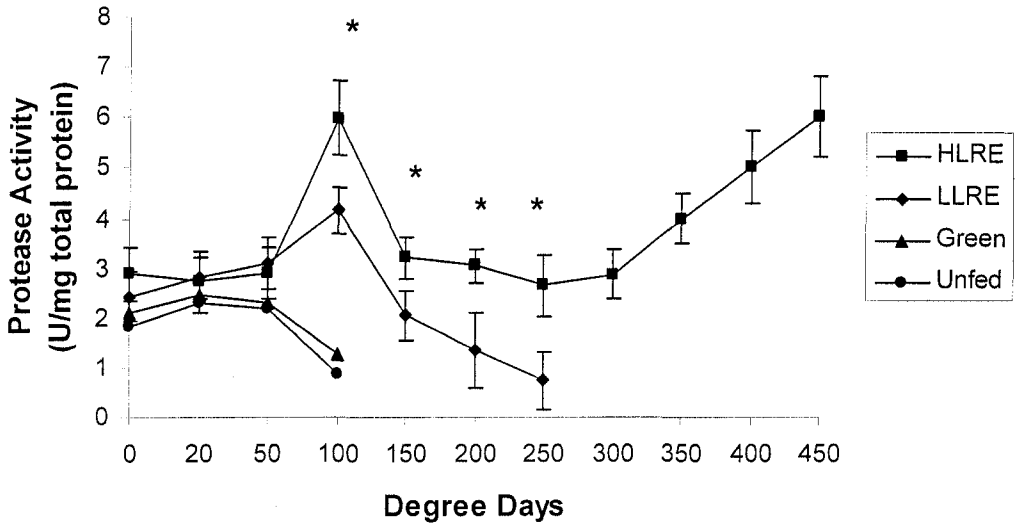

Figure 2.5. General protease activity (U/mg protein) on azocasein substrate in 0.5 ml cod larvae over time among four feeding treatments. Points represent means of triplicates for HLRE (high lipid rotifer enrichment) and LLRE (low lipid rotifer enrichment), and the single tank means for the Greened and Unfed treatments. Bars represent one standard error of the mean. * denotes significant difference between HLRE and LLRE treatments (p>0.05).

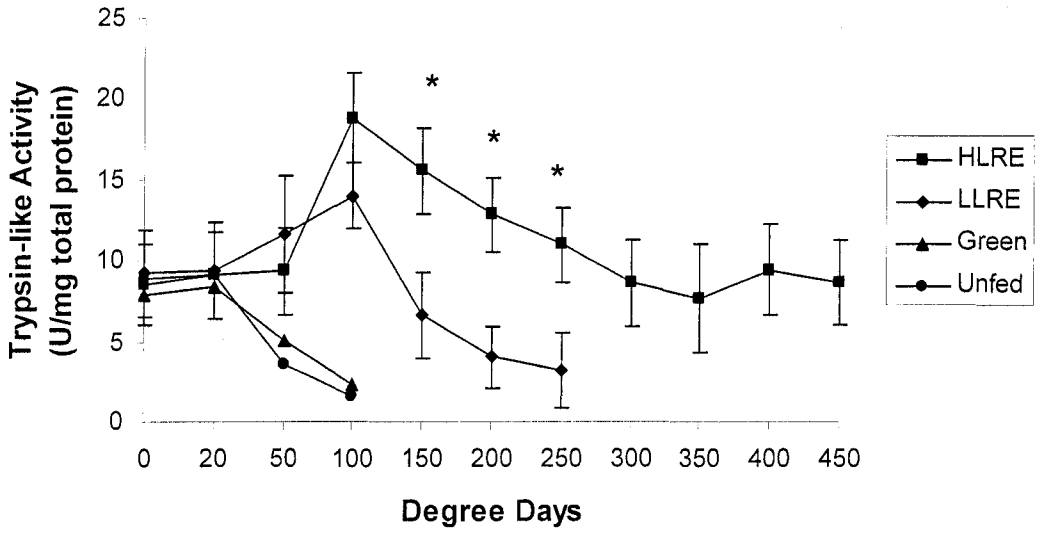

Figure 2.6. Trypsin-like activity (U/mg protein) on BAPNA substrate in 0.5 ml cod larvae over time among four feeding treatments. Points represent means of triplicates for HLRE (high lipid rotifer enrichment) and LLRE (low lipid rotifer enrichment), and the single tank means for the Greened and Unfed treatments. Bars represent one standard error of the mean. * denotes significant difference between HLRE and LLRE treatments ($p>0.05$).

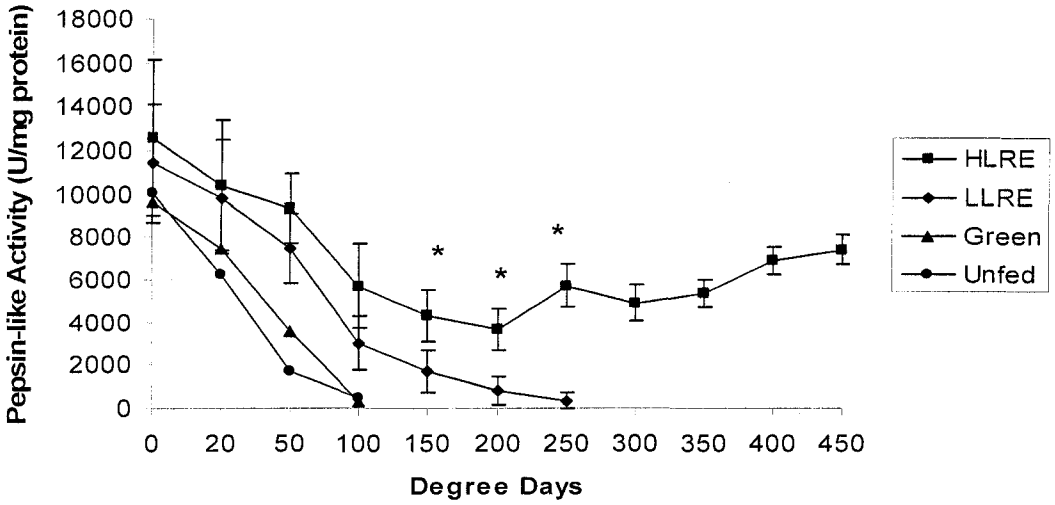

Figure 2.7. Pepsin-like activity (U/mg protein) on hemoglobin substrate in 0.5 ml cod larvae over time among four feeding treatments. Points represent means of triplicates for HLRE (high lipid rotifer enrichment) and LLRE (low lipid rotifer enrichment), and the single tank means for the Greened and Unfed treatments. Bars represent one standard error of the mean. * denotes significant difference between HLRE and LLRE treatments ($p>0.05$).

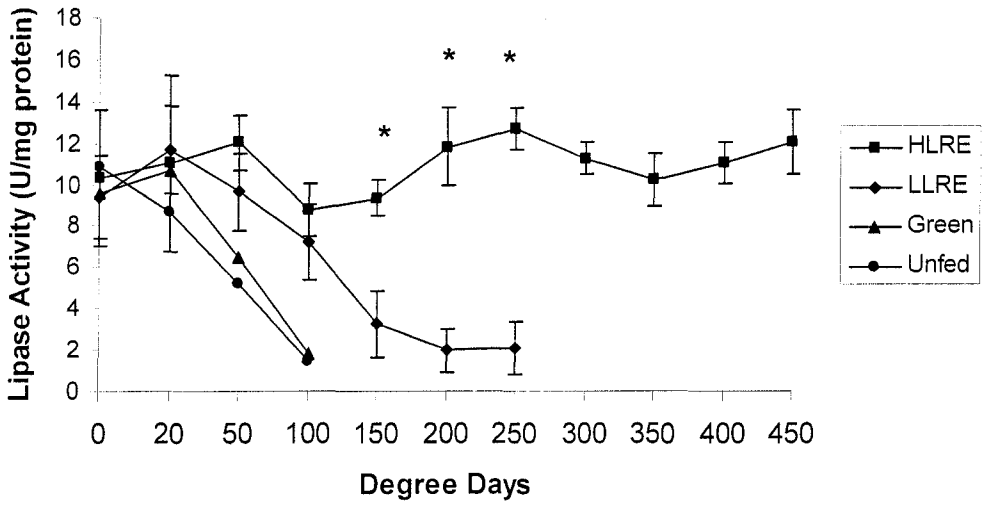

Figure 2.8. General lipase activity (U/mg protein) on *p*-nitrophenyl myristate substrate in 0.5 ml cod larvae over time among four feeding treatments. Points represent means of triplicates for HLRE (high lipid rotifer enrichment) and LLRE (low lipid rotifer enrichment), and the single tank means for the Greened and Unfed treatments. Bars represent one standard error of the mean. * denotes significant difference between HLRE and LLRE treatments ($p>0.05$).

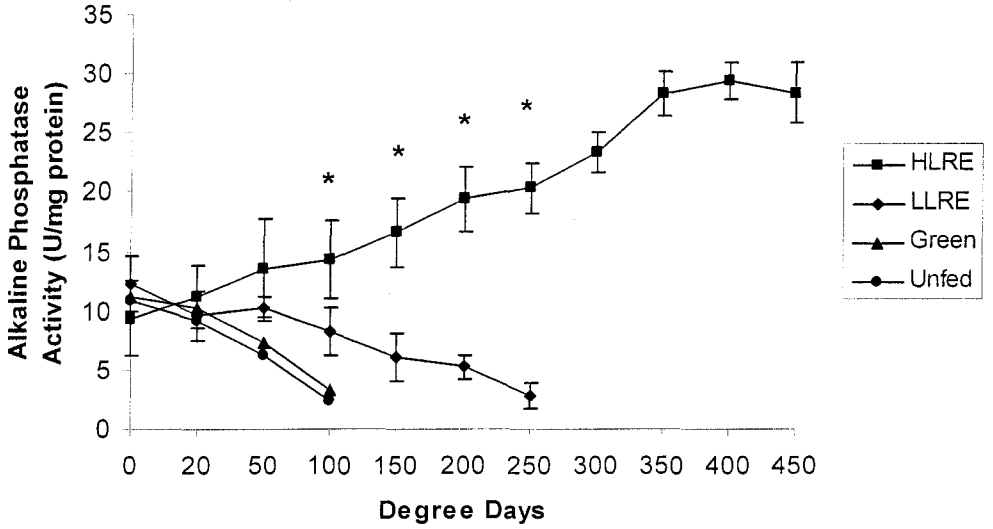

Figure 2.9. Alkaline phosphatase activity (U/mg protein) on *p*-nitrophenyl phosphate substrate in 0.5 ml cod larvae over time among four feeding treatments. Points represent means of triplicates for HLRE (high lipid rotifer enrichment) and LLRE (low lipid rotifer enrichment), and the single tank means for the Greened and Unfed treatments. Bars represent one standard error of the mean. * denotes significant difference between HLRE and LLRE treatments ($p>0.05$).

2.5 Discussion

The cod in the HLRE treatment showed the best survival, whereas the LLRE fish only survived until 250 dd and the unfed and greened treatments did not survive past 100 dd. The final survival rate of 3.1% in the HLRE tanks may seem low until considering the large numbers of larvae needed for the biochemical assays. At least 0.5 ml of wet tissue was required for each assay replicate. At hatch, this resulted in 300-350 larvae collected in triplicate for each tank. The numbers of fish necessary to make up the minimum volume of tissue decreased as individual larval mass increased. As such, tank populations were not only affected by diet, but also the disturbance of removing individuals for sampling. However, equal disturbances were performed in all tanks on a given sample day, so the effect of sampling was similar across the treatments.

Performance data indicate that HLRE is a better live prey diet than LLRE prey, since all replicates of LLRE-fed cod died by 250-300 dd. However, mortality in the low lipid treatment was preceded by a predicable pattern of decreased growth, condition, and foraging activity. Growth and condition of the larvae were significantly higher in the high lipid treatment by 150-200 dd. Larval swimming activity and prey ingestion rates were also significantly higher in the HLRE treatment by 100 dd.

Activity data are a good first indication of poor performance (Skiftesvik 1992), since fish that are not receiving energy to meet metabolic demands have less energy to expend for activities like swimming or prey capture, even when the

prey swim slowly and in predictable patterns, and are easily caught by saltatory predators like cod larvae (Buskey *et al.* 1993, Hunt von Herbing & Gallager 2000). The HLRE larvae grew and consistently increased their overall growth as well as swimming activity and attack rate. Cod larvae in the LLRE treatment, however, showed characteristics similar to those of starved larvae, such as decreased foraging activity and increased buoyancy (Laurence 1978, Kjorsvik *et al.* 1991, Skiftesvik 1992). Tissue degradation was found in other species of marine fish larvae under similar conditions of starvation (Govoni *et al.* 1986, Yin & Blaxter 1987). This would suggest that even though ample prey were available, the larvae in this experiment did not possess the energy required to ingest prey given that the energy derived from previous foraging activity was not sufficient to support metabolic demands of growth.

Ellertsen *et al.* (1980) reported that cod larvae, at the onset of exogenous feeding, had a successful predation rate of 32-62%, which increased to over 90% by the end of yolk-sac absorption. As such, even though a high activity level would increase the odds of ingesting live prey, low activity levels in cod larvae may be a strategy to conserve energy and cope with starvation, or at least delay the onset of irreversible starvation.

In addition to decreased foraging activity, the digestive efficiency of cod can be inefficient under poor feeding conditions. If sufficient nutrition is not available to a larva, impaired development of the fins results, as well as the inability of the fish to increase its overall size (and maneuverability), and rapid

degradation of the digestive tissues (Ellertsen *et al.* 1980, Yin & Blaxter 1987, Kjorsvik *et al.* 1991). With tissue degradation comes a reduction in the ability of the gut to process food for energy. With respect to this experiment, even if larvae were still capable of prey capture in the early stages of starvation, reduced activity and incomplete digestion, possibly due to gut tissue degradation, likely contributed to the onset of starvation. The time to irreversible starvation of cod has been reported as 70 dd when no food was available (Laurence 1978). In this experiment, when nutritionally deficient food was available significant differences in survival, growth and behaviour was apparent at 100 dd. Given that the live feed was the same in both treatments, the data suggest that the quantity or quality of lipid in the enrichment, and not the live prey itself, led to decreased survival, growth, swimming activity and ingestion rates. Qualitatively, the high lipid diet was similar to one or both of the other rotifer treatments in terms of ethyl esters, triacylglycerols, sterols, phospholipids, and AA proportions or concentrations.

A positive correlation has been found for DHA:EPA ratios and larval growth in yellowtail flounder and gilthead seabream (Copeman *et al.* 2002, Rodriguez *et al.* 1997). Mirroring the lipid composition of marine fish eggs has been suggested as a starting point for determining nutritional requirements of newly hatched larvae. A typical DHA:EPA ratio of 2:1 has been found in several marine larval species and suggested as adequate for larval feeding (Sargent *et al.* 1999). In the current experiment, the LLRE had a DHA:EPA ratio of 0.3:1,

equivalent to that of unenriched rotifers. The HLRE, however, had a significantly higher ratio of 3.7:1. In light of the growth, lipid, and behavioural data in this experiment, the LLRE diet was deficient in vital lipids. In herring, diets deficient in DHA have been shown to change the fatty acid composition of neural tissues and decrease foraging efficiency (Bell *et al.* 1995). Atlantic cod have been shown to possess high levels of DHA in both eye and brain tissues (Bell & Dick 1991). Since cod are visual feeders, inadequate amounts of DHA may inhibit their ability to successfully forage. This deficiency of DHA in the eye tissue of cod larvae leading to visual impairment could be a major factor explaining the marked decline in larval cod foraging activity by 100 dd. This decreased foraging activity was followed by lower growth measures and digestive enzyme activities as compared to the HLRE larvae by 150 dd and death by 250 dd.

Digestive enzyme activity reflected the ability of the larvae to digest available food items for metabolic energy, and were similar to general patterns found in other larval species, such as Senegal sole (Ribiero *et al.* 1999). Furthermore, the results of the current experiment concur with results from experiments on rats, milkfish (*Chanos chanos*) and seabass where a high lipid diet elicited higher enzyme activities in the animals as compared to those fed a lower lipid diet (Borlongan 1990, Spannagel *et al.* 1996, Zambonino-Infante & Cahu 1999). It is difficult, however, to make meaningful comparisons between studies that use vastly different rearing temperatures, diets, sampling methods, and assays to determine digestive enzyme activity. The biochemical assays of

the current experiment used similar methods to, and results concur with, those of Perez-Casanova (2003). In both cases, trypsin- and pepsin-like enzymes were all present and active at hatching. The previous study, however, examined activity with one feeding regime. The results of the current experiment show similar overall trends in enzyme activity for both types of proteases, but the activity of lipase and alkaline phosphatase were higher in cod larvae fed HLRE in this experiment than cod larvae in the Perez-Casanova (2003) study. The overall patterns of digestive enzyme activity in the two studies are similar, but differences in enrichments elicited distinct biochemical responses. Additionally, Perez-Casanova quantified the contribution of rotifer digestive enzymes to that of the whole body homogenates of Atlantic cod larvae. Rotifer enzymes contributed 16.4% to the total general lipase activity, 10.8% to trypsin-like enzyme activity, and 3.5% to general protease activity, and ~0% to pepsin-like activity. As such, the amount of digestive enzymes in the rotifer live food was minimal and did not significantly impact the results of the enzyme assays.

Enzyme activity levels obtained from the trypsin and pepsin assays are not specific for these enzymes, as they detect trypsin-like alkaline proteases and pepsin-like acid proteases. Furthermore, the assays were performed on whole body homogenates, which further complicates interpretation as interference may result from proteases and protease inhibitors in tissues other than from the digestive system. For example, one would not expect to see pepsin activity in early larval development since the larvae do not have a functional stomach until

metamorphosis. The resultant activity of pepsin-like enzymes in this experiment is most likely due to other acidic proteases like aspartic proteases in the pepsin family of digestive enzymes.

The activities of general lipases and alkaline phosphatase in the HLRE treatment were higher than in the study by Perez-Casanova (2003) for larvae beyond 250 dd. There are two possibilities to explain this increase in activity: (1) the increase in activity is due to a decrease in feeding activity as the larvae approach metamorphosis, initiating an increase in the need to hydrolyze stored lipids for energy (Martinez *et al.* 1999), or (2) feeding cod larvae a diet with high lipids for energy induces the digestive system to increase its lipid digesting capabilities. Data from the behavioural portion of this experiment do not support the first option, but instead support the theory that increases in enzyme activity are in fact due to the influence of food. Since alkaline phosphatase has been generally accepted as a marker of intensity of nutritional absorption in the intestine of larvae teleosts (Segner *et al.* 1989), the results of this experiment will have implications in assessing the digestive capacity of fish if this increase in enzyme activity is due to diet and not to feeding activity.

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3 Meristic, behavioural, and enzymatic responses of peri-metamorphic Atlantic cod (*Gadus morhua*) larvae to enriched *Artemia* diets.

3.1 Abstract

The period around metamorphosis is one of the critical periods in the commercial production of Atlantic cod (*Gadus morhua*). Metamorphosis is a period of drastic physiological change, but the digestive capacity of young fish undergoing metamorphosis while confronted with a change in diet remains largely unknown. To examine the metamorphic capability of larval cod, larvae were fed *Artemia franciscana* from 450-650 dd that were unenriched (UN), high lipid enriched (HLAE), high protein enriched (HPAE), or on a three day rotation of these diets (3DRA).

Larvae fed the three day rotating *Artemia* diet showed the greatest growth rates and survival. The time the fish spent swimming and attacks on live prey were not significantly different between treatments. Digestive enzyme activity assayed before and after feeding showed the HLAE and HPAE induced higher activity in lipid and protein digesting enzymes, respectively. DHA:EPA and $\Sigma\omega3$:$\Sigma\omega6$ for the larval tissues at the end of the experiment showed HLAE and 3DRA treatments had ratios closest to values for cod eggs. Evidence suggests that dietary quality not only plays a role in growth and survival of cod larvae as they approach metamorphosis, but also in the enzymatic capability of the digestive tract to respond to live prey of differing nutritional content.

3.2 INTRODUCTION

The digestive system of cod during early first feeding is largely undifferentiated, although morphological differences do exist in the epithelium of the foregut, midgut and hindgut. As larvae age, mortality decreases and the capacity to store energy and avoid starvation increases (Jordaan & Brown 2003). Metamorphosis begins when larvae reach 12-15 mm in length and is evident by the disappearance of the median fin-fold and the emergence of median fins and the complete development of the axial skeleton (Pedersen & Falk-Petersen 1992). Concomitant with alterations in locomotory structures in cod are changes in feeding behaviour, and digestive physiology which matures with the eventual development of a distinct stomach, gastric glands, pyloric caeca, and the initiation of gastric digestion with a marked increase of pepsin activity (Timeyko 1987, Hjelmeland *et al.* 1995).

Successful mass-rearing of Atlantic cod (*Gadus morhua*) depends on larvae receiving enough energy to support metabolic demands of growth and survival. Cod larvae begin life using a highly lipid-rich yolk reserve, then switch to predation on live food. As larvae grow and the digestive system matures to metamorphosis, they spend more time actively seeking out prey while sustaining faster swimming speeds (Hunt von Herbing & Gallager 2000). These developments in foraging enable the larvae to select for increasing numbers of larger prey with a high overall nutritional content.

Culturing of cod larvae normally involves three diets: *Brachionus plicatilis* (rotifers), *Artemia franciscana (Artemia)*, and final weaning onto formulated food. Standard protocols feed rotifers to larvae for the first 250-350 dd post-hatch and, after a brief co-feeding period, *Artemia* until the fish reach 25 mm in length when weaning is initiated (Brown *et al.* 2003). Rearing efforts are now attempting to shift the diets such that formulated feed can be introduced to the fish at an earlier age, thus saving time, money, and resources (Callan *et al.* 2003). However, there has yet to be developed a formulated food for cod that yields growth rates comparable to live feeds while minimizing cannibalism (Lazo *et al.* 2000, Brown *et al.* 2003).

Shields *et al.* (2003) observed that cod larvae are able to ingest *Artemia* from as early as 50 dd, but these larvae experienced high incidences of swim bladder over-inflation and high mortality during metamorphosis. Successful weaning of cod larvae has been achieved by as early as 150 dph and 8.5 mm standard length but survival was low (Kling 1998, Baskerville-Bridges & Kling 2000). Survival and growth were improved with a brief co-feeding period with *Artemia* while the larvae were being weaned onto dry feed. Callan *et al.* (2003) have outlined two specific influences of *Artemia* on the ability of larval cod to accept a formulated diet. First, *Artemia* provide a visual and chemical cue to help increase the ability of the larvae to capture particles in the water column. Second, the biochemical composition of *Artemia* aids larval digestion and assimilation of a formulated diet through contribution of the prey's own digestive

and autolyzing enzymes. The contribution of digestive enzymes from *Artemia* has been reported to be less than 10% of overall enzyme activity for striped bass (*Morone saxitilus*), sea bass (*Dicentrarchus labrax*) and Atlantic halibut (*Hippoglossus hippoglossus)* (Ozkizilcik *et al.* 1996; Cahu & Zambonino Infante 1995, 1997; Gawlicka *et al.* 2000). As such, the exogenous enzymes within *Artemia* do not significantly contribute to the live prey's own digestion when assayed in larval whole body homogenates.

Patterns of digestive enzyme activity in cod have been described over larval development (Perez-Casanova 2003). However, that study did not compare enzyme activities of cod larvae with more than one diet. In order to have a more complete picture of the digestive demands and capacity of cod around metamorphosis, the behavioural and biochemical responses of the fish to varying amounts of protein and lipid in the food must be examined.

Although *Artemia* are a larger prey and preferred by cod as they grow, they are nutritionally deficient in highly and polyunsaturated fatty acids (HUFAs and PUFAs), and as such require some sort of enrichment before being fed to larvae (Bell *et al.* 2003). I conducted a study to examine the performance of cod larvae on different *Artemia* enrichments to determine if particular enrichments promote different digestive enzyme activities in cod and improve the growth and survival of larvae. This experiment incorporates growth, behaviour, and digestive enzyme activity measurements during *Artemia* feeding. The goal of this work was to determine how diets affect performance and digestive ability of cod

larvae, and then use this information to assist in the development of better enrichment formulations.

3.3. MATERIALS & METHODS

3.3.1 Larviculture and diets

General rearing protocols were outlined in Chapter 2 for cod larvae from hatching to 450 dd. Once the surviving larvae from the high-lipid rotifer enrichment treatment had reached the end of that experiment, they were randomly assigned to one of eight 3000 l tanks at ~10°C in equal stocking densities with 24 hour light and a filtered seawater flow of 8 l/min. Four *Artemia franciscana* enrichment diets were used in duplicate: high lipid (HLAE), high protein (HPAE), unenriched *Artemia* nauplii (UN), and a daily rotation of all three enrichments (3DRA). According to manufacturer's crude analysis, the HPAE had 65% protein and 10% lipid by weight, whereas the HLAE was 20.8% protein and 56% lipid by weight.

Table 3.1. Lipid class and fatty acid composition of unenriched *Artemia franciscana* nauplii (UN), *Artemia* enriched with high protein (HPAE), and the *Artemia* enriched with high lipids (HLAE). Values are means of triplicates ± standard error. Values in the same row with the same superscript and not significantly different ($p>0.05$). Data from Nicole Rowsell, MSc thesis in progress.

(**ug/mg dw**):	UN	HPAE	HLAE
Total lipid	24.2±2.0 [a]	27.1±1.8 [a]	33.7±2.0 [b]
Triacylglycerols	9.4±0.6 [a]	14.0±1.2 [b]	18.5±0.1 [c]
Phospholipids	2.2±0.3 [a]	5.6±1.0 [b]	4.2±0.2 [c]
18:1ω9	3.2±0.2 [a]	5.4±0.5 [b]	3.9±0.1 [c]
18:3ω3	2.8±0.2 [a]	2.7±0.2 [a]	2.5±0.4 [a]
20:4ω6 (AA)	0.2±0.02 [a]	0.2±0.02 [a]	0.3±0.01 [b]
20:5ω3 (EPA)	0.4±0.05 [a]	0.7±0.05 [b]	0.6±0.02 [a]
22:6ω3 (DHA)	0.04±0.01 [a]	0.1±0.008 [b]	1.3±0.04 [c]
Σω3	4.1±0.3 [a]	4.2±0.3 [a]	4.3±0.8 [a]
Σω6	1.2±0.1 [a]	2.1±0.1 [b]	1.7±0.02 [c]
DHA:EPA	0.08 [a]	0.14 [b]	2.3 [c]
EPA:AA	2.0 [a]	3.5 [b]	2.0 [a]
Σω3:ω6	3.5 [a]	2.1 [b]	2.5 [c]

Artemia cysts were hydrated and decapsulated according to manufacturer instructions. In 18-24 hours, the nauplii hatched out in 300 l tanks with 30°C seawater. Nauplii needed for the unenriched treatment were removed, while the remaining *Artemia* were enriched overnight (at 9:00 pm and 3:00 am). The enriched *Artemia* were harvested at 9:00 am, thoroughly washed with 20°C water, and put into 6 l seawater in 10 l buckets and kept in the same room as the experimental tanks. Air stones were added to the buckets to provide constant aeration, and 4 l of seawater was added to the buckets to further dilute the *Artemia* and acclimate the prey to the tank temperature. This prevented prey from settling out of the tank before larvae had an opportunity to feed. *Artemia* were added to the tanks three times daily as necessary to sustain a mass culture protocol prey density of 1000/l (Lori Thorne-ARDF staff, pers. comm.)

3.3.2 Growth and behavioural data

Growth data for standard length and myotome height were taken every 50 dd (~7 days) with a stereomicroscope and a calibrated eyepiece micrometer. Condition factor and length specific growth rate were calculated as per Jobling (1994):

Condition Factor = Myotome height (mm) / Standard Length (mm);

Length Specific Growth Rate = $[\text{Ln}(SL_{t2}) - \text{Ln}(SL_{t1}) / t_2 - t_1]$, where

SL_{t2} is the Standard Length (mm) at time interval 2,

SL_{t1} is the Standard Length (mm) at time interval 1, and

$t_2 - t_1$ is the difference (days) between time intervals 2 and 1.

Behavioural data were taken every 50 dd (7 days) using a two minute focal animal technique, where an individual larva is observed for two minutes. "Swimming activity" was defined as the time in which a larva moved through the water column by movements of the caudal body area, and "attacks" were defined as the frequency (number per time) of captures (bites and ingests) and misses (failed captures) of prey (Puvenandran & Brown 2002). Observations were made approximately five minutes after the live food was added to the tank for the morning feeding. A Psion computer with observation software was used to record and compile data. Ten larvae were observed per tank per treatment.

3.3.3 Digestive enzyme activity data

Triplicate pooled samples of larvae were taken for biochemical determination of digestive enzyme activity at 500, 550, 600, and 650 dd. Larvae were collected for the pre-prandial (before feeding) samples between 9:30 and 10:00 am, and one hour later for the post-prandial (after feeding) samples. After repeated visual verification, larvae were collected between 9:30 and 10:00 am to ensure empty guts and reduce the impact of prey enzymes in the pre-prandial larval samples. A small aquarium net was used to remove larvae from the tanks, and fish were transferred to a 1 l specimen cup with 0.07 mg/l of MS-222 in

seawater. Anaesthetized larvae were removed from the container with small forceps. Salt water and MS-222 were allowed to drain off the fish, which were then rinsed with fresh water. The larvae were finally placed in 1.5 ml Eppendorf tubes and stored at -80°C until processed and analyzed.

The procedures for processing the tissues and performing assays were similar to those previously outlined in Chapter 2. Briefly, the whole body homogenates that had been stored at -80 °C were defrosted on ice (~4°C), diluting with 4 parts (w/v) 150 mM NaCl, then using an automated tissue grinder to create whole body homogenates. These homogenates were centrifuged for 10 minutes at 12,000 *g* at 4°C. Supernatants were then aliquoted in 0.5 ml Eppendorf tubes and stored at -80°C until assayed for digestive enzyme activity. Assays were conducted in triplicate for each replicate sample, and were performed at room temperature by diluting with two volumes of the respective assay buffer.

Total protein concentration of the homogenate was assayed with the BioRad Protein Assay (Bradford 1976), and then used to standardize the subsequent enzyme activity results to larval size as the fish grew over the course of the experiment. General protease activity was measured using the azocasein hydrolysis assay (Ross *et al.* 2000). Trypsin-like alkaline protease activity was assayed based on the methods in Gawlicka *et al.* (2000) using a BAPNA substrate. Pepsin-like acidic protease activity was assayed with a hemoglobin substrate according to methods in Anson (1938). General lipase activity was

assayed according to methods in Gawlicka *et al.* (2000) where *p*-nitrophenyl myristate was the substrate for the reaction. Finally, alkaline phosphatase activity was also assayed according to methods in Gawlicka *et al.* (2000), but used a substrate of *p*-nitrophenyl phosphate disodium.

3.3.4 Larval tissue lipid analyses

Triplicate samples of pooled cod larvae were taken for lipid analyses from all four feeding treatments at 450 dd (before *Artemia* had been introduced as a prey item) and again at 650 dd, the end of the experimental period. 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 (TLC/FID) with an Iatroscan (Parrish 1987). Extracts were spotted on silica gel coated Chromarods and a three stage development system was used to separate lipid classes.

3.3.5 Statistical analyses

Prior to analysis, survival, growth, behaviour, and enzyme data were tested for assumptions of normality and homogeneity of variance before an ANOVA (analysis of variance) was employed. Distribution of the data, plots of residuals, and predicted values were examined. There was no significant tank

effect ($F_{1,5}$ < 5.4, p>0.05 for all treatments), and data from treatments were pooled. Statistical analyses were performed with Minitab statistical software (version 13). One-way ANOVAs were performed to detect differences between the means of treatments using a significance level of α=0.05. The data were normal and homogeneous, thus transformation or randomization was not required before statistical analyses were employed.

3.4 RESULTS

3.4.1 Survival and growth

Survival was highest in the HLAE and 3DRA treatments ($F_{3,7}$= 9.75, p<0.01 and $F_{3,7}$= 5.04, p<0.05). Survival in these treatments were 87.6% and 79.0%, while the HPAE and unenriched survival rates were 17.9% and 21.4%, respectively.

Growth data (Table 3.2) showed that cod fed the HPAE and HLAE diets had greater myotome depth ($F_{3, 29}$= 12.54 and 12.96, p<0.001), while larvae fed 3DRA were significantly longer at the end of the experiment as compared to the other treatments ($F_{3, 29}$= 8.21, p<0.001). However, examining the percentage increase in body dimensions showed that the 3DRA had a 99.4% increase in myotome height while the HPAE and HLAE only increased ~61%, while the UN diet cod only increased myotome height by 21.2% (Figure 3.1). Similar findings were evident from the standard length data where the UN, HPAE and HLAE

length measures were not significantly different (19-28%), but the 3DRA fish were 92.6% longer by 650 dd. Because of the absolute values of the myotome and length measures in mm, the length specific growth rate was significantly higher in the 3DRA treatment ($F_{3,29}$= 24.01, $p<0.001$). The treatment with the second highest length specific growth rate, HLAE, was almost one-third the value of the 3DRA value at 650 dd (0.012 vs. 0.033).

Values for condition factor were highest for larvae in the HLAE and HPAE treatments (Figure 3.2; $F_{3,29}$= 11.47 and 12.83, $p<0.001$), but like the other meristic data, the highest percentage increase in condition factor over the course of the experiment was found in the 3DRA treatment larvae. Larval wet weight, interestingly, was highest in the UN group by 650 dd at 60.53 mg ($F_{3, 29}$= 10.24, $p<0.001$), followed closely by the HPAE and HLAE treatments (although they were not significantly different 57.3-60.5 mg), but ultimately followed the trend whereby the 3RDA showed the greatest overall percentage increase of 139% by 650 dd.

Table 3.2. Mean growth data for peri-metamorphic cod larvae fed one of four *Artemia franciscana* enriched treatments over 200 degree-days (UN = Unenriched nauplii, HPAE = high protein *Artemia* enrichment, HLAE = high lipid *Artemia* enrichment 3DRA = three day rotation of *Artemia* enrichments) n=20, mean ± standard error. Values in rows with the same superscripts are not significantly different (p>0.05).

	UN	HPAE	HLAE	3DRA
Myotome (mm):				
450 dd	1.5 ± 0.05 [a]	1.5 ± 0.1 [a]	1.5 ± 0.1 [a]	1.1 ± 0.02 [b]
650 dd	1.8 ± 0.09 [a]	2.5 ±0 .1 [b]	2.5 ± 0.1 [b]	2.2 ±0 .01 [c]
Length (mm):				
450 dd	13.5 ± 0.2 [a]	13.3 ± 0.2 [a]	14.1 ± 0.2 [a]	10.5 ± 0.3 [b]
650 dd	16.1 ± 0.5 [a]	16.5 ±0.5 [a]	18.0 ±0 .6 [ab]	20.2 ± 0.2 [b]
Length Specific Growth Rate (µm/day):	8.9 ± 0.8 [a]	10.8 ± 1.0 [a]	12.4 ± 1.3 [a]	32.75± 2.5 [b]
Condition Factor:				
450 dd	0.11 ± 0.005 [a]	0.12 ± 0.005 [a]	0.11 ±0.006 [a]	0.11 ±0 .004 [a]
650 dd	0.11 ± 0.004 [a]	0.15 ± 0.005 [b]	0.14 ±0.005 [b]	0.11 ± 0.002 [a]
Larval wet weight (mg):				
450 dd	29.5 ± 3.7 [a]	26.5 ± 3.8 [a]	27.5 ± 3.2 [a]	17.6 ± 2.6 [b]
650 dd	60.5 ± 7.2 [a]	57.3 ± 6.4 [a]	58.2 ± 7.4 [a]	42.1 ± 5.4 [b]

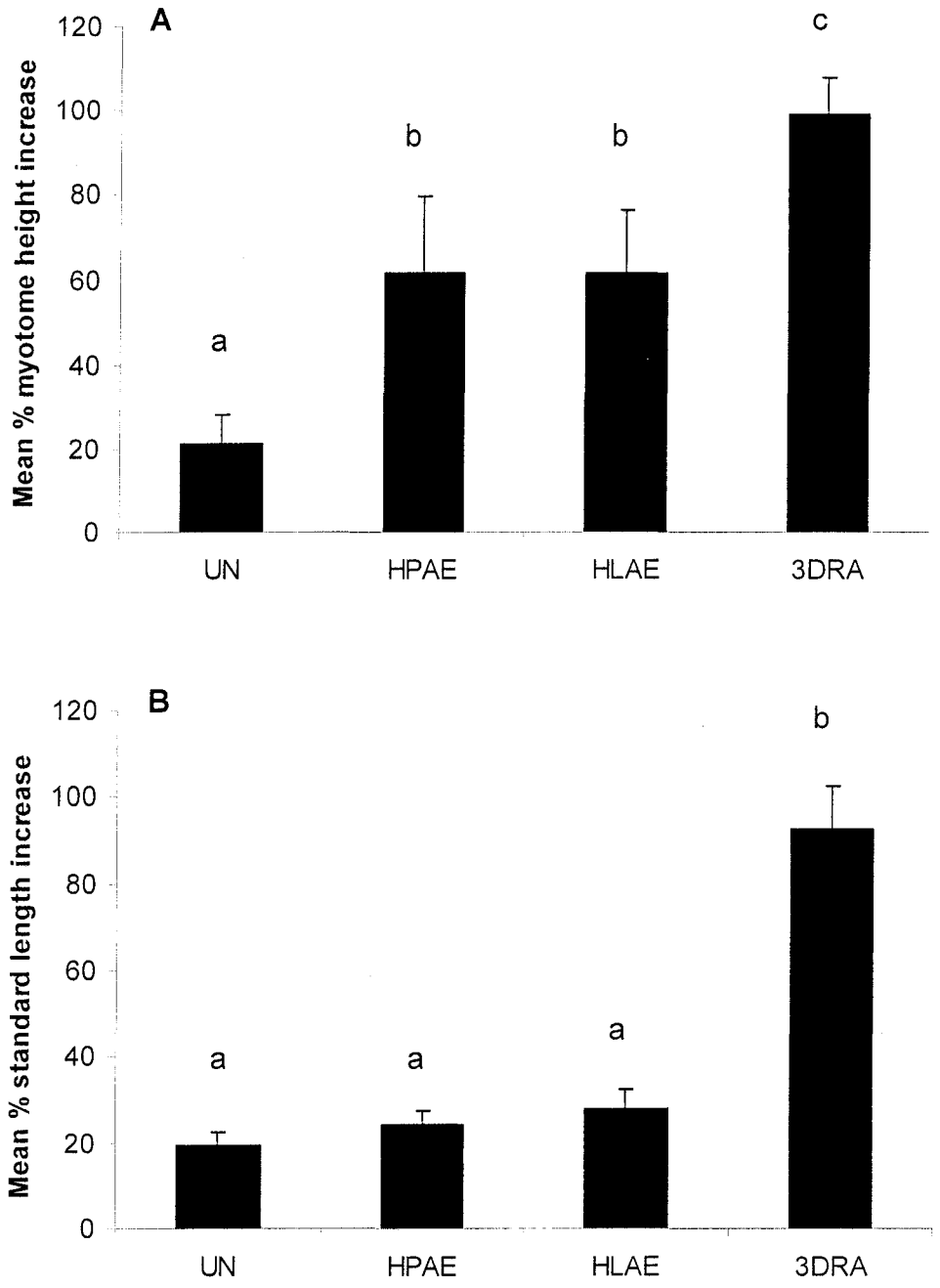

Figure 3.1. Mean percent increase in myotome height (A) and standard length (B) for peri-metamorphic cod larvae from 450-650 dd over four feeding treatments.
UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean, n=20. Bars with the same superscript are not significantly different ($p>0.05$).

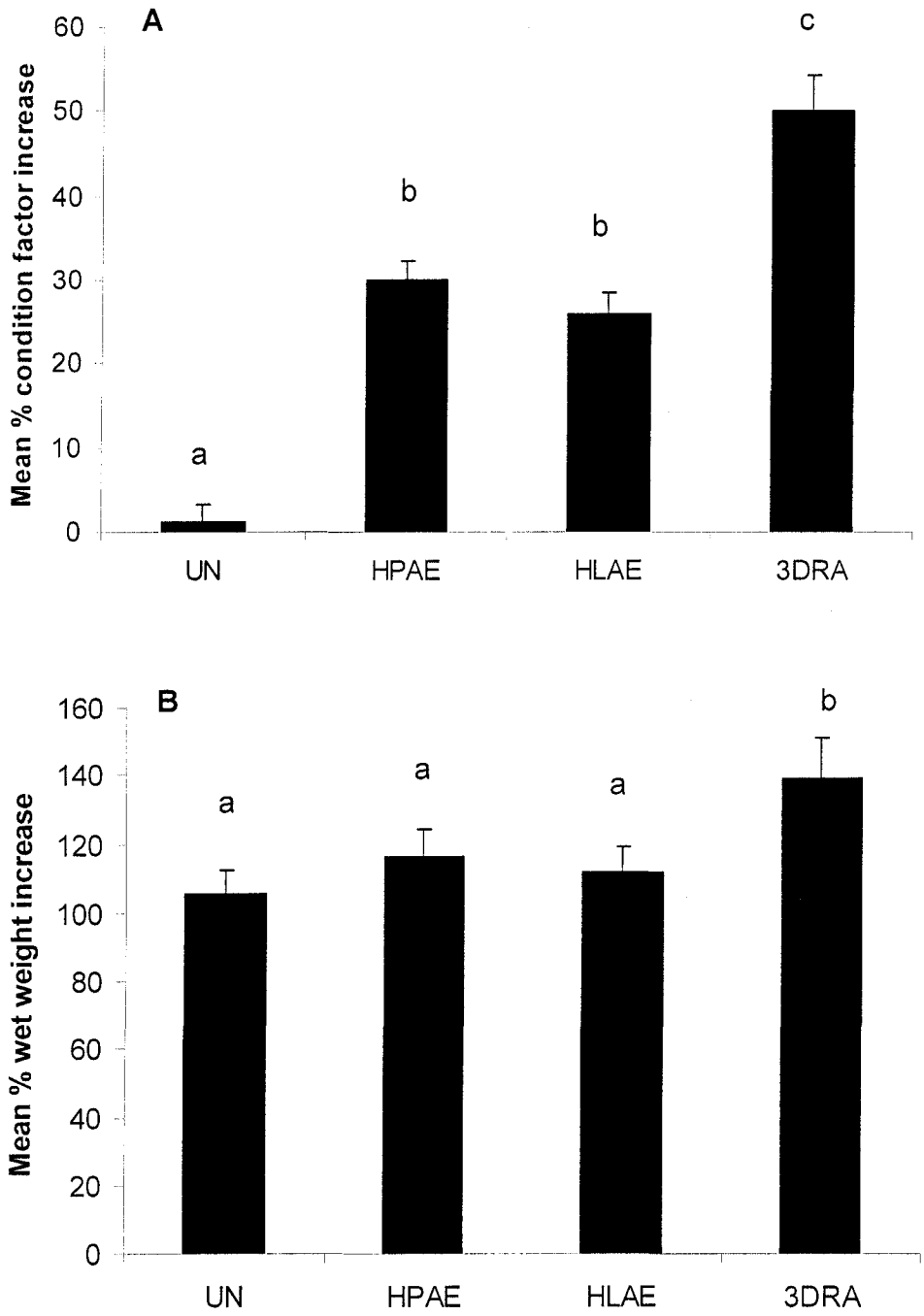

Figure 3.2. Mean percent increase in condition factor (A) and wet weight (B) for peri-metamorphic cod larvae from 450-650 dd over four feeding treatments.
UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean, n=20. Bars with the same superscript are not significantly different ($p>0.05$).

3.4.2 Behaviour

Behavioural data showed no significant differences between or among treatments at any point during the experiment for either proportion of time the larvae spent actively swimming (Figure 3.3), or attack rate on live prey (Figure 3.4; $F_{3, 29}<2.92$, $p>0.05$). Larvae were observed to spend, on average, 36-39% of their time swimming, and caught 7-8.5 *Artemia* per minute while foraging.

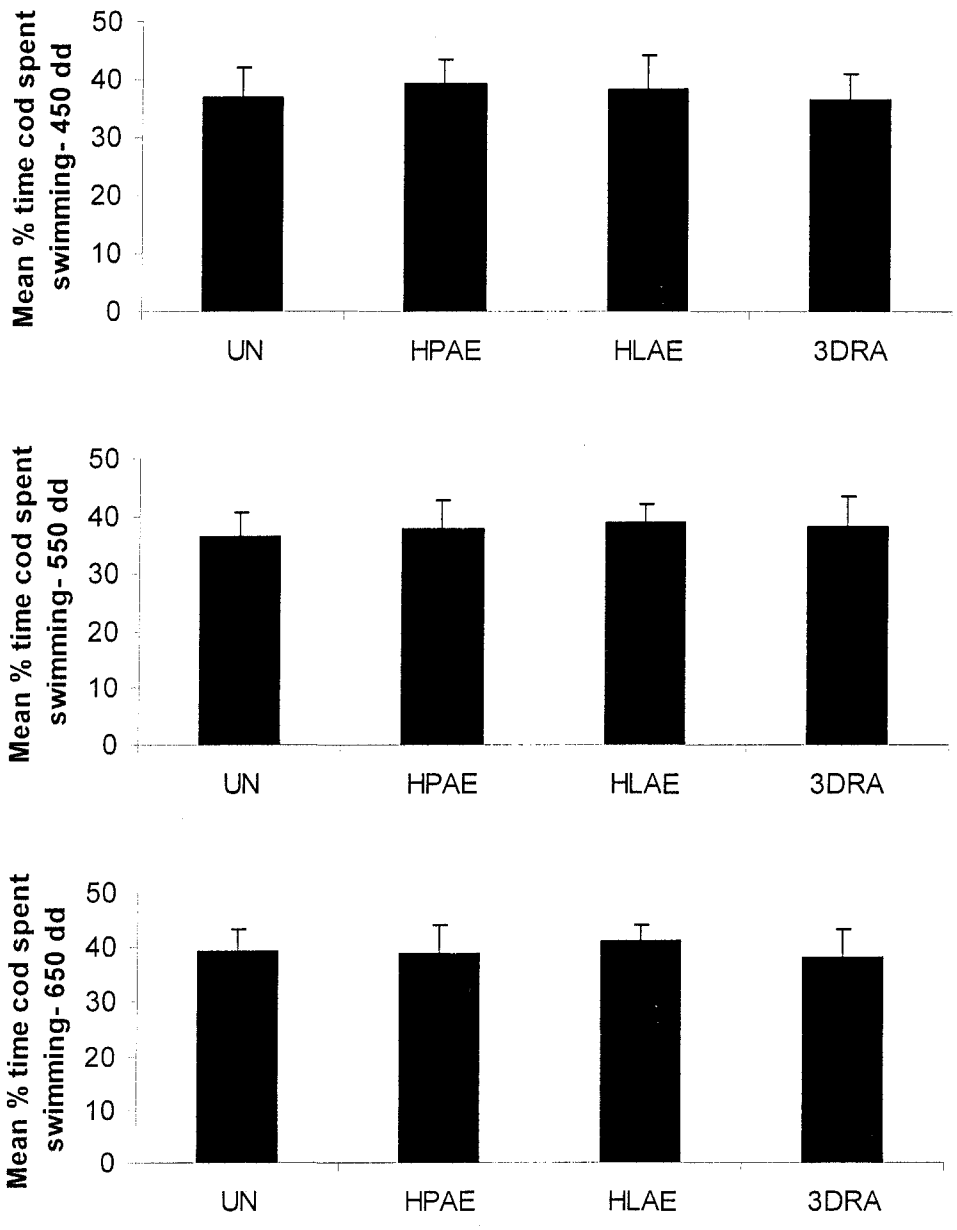

Figure 3.3. Mean percent time that peri-metamorphic cod larvae spent swimming from 450-650 dd over four feeding treatments.
UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean, n=20. There were no significant differences between treatments at 450, 550, or 650 dd ($p>0.05$).

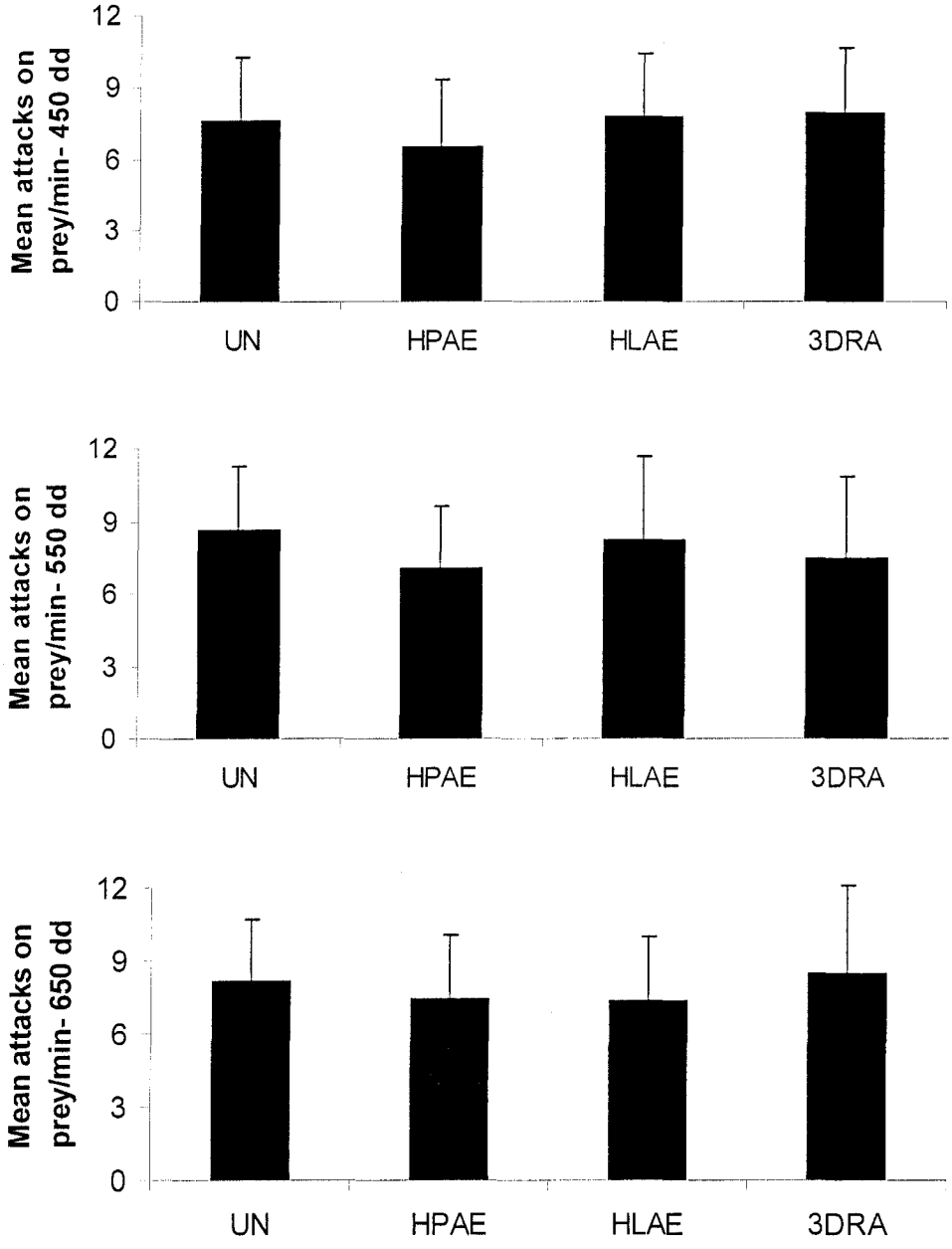

Figure 3.4. Mean attacks on prey per minute of peri-metamorphic cod larvae from 450-650 dd over four feeding treatments.
UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean, n=20. There were no significant differences between treatments at 450, 550, or 650 dd ($p>0.05$).

3.4.3 Digestive enzyme activity

Measures for post-prandial enzyme activity were higher than pre-prandial measures for all samples taken. Digestive enzyme activity data showed significant elevations in post-prandial activity for enzymes one would expect to act on the *Artemia* enrichments: lipases were higher after eating prey with a high lipid enrichment, proteases were higher after eating prey with a high protein enrichment. Trypsin, pepsin, and general proteases had increased enzyme activity after eating the HPEA diet on 500, 550, and 600 dd ($F_{3,8} > 4.07$, $p<0.05$; Figures 3.5-3.7). General lipases and alkaline phosphatase were elevated in cod feeding on HLAE for 500, 550, and 600, but interestingly, also for cod fed on the rotating diet at 600 dd only (Figures 3.8 & 3.9).

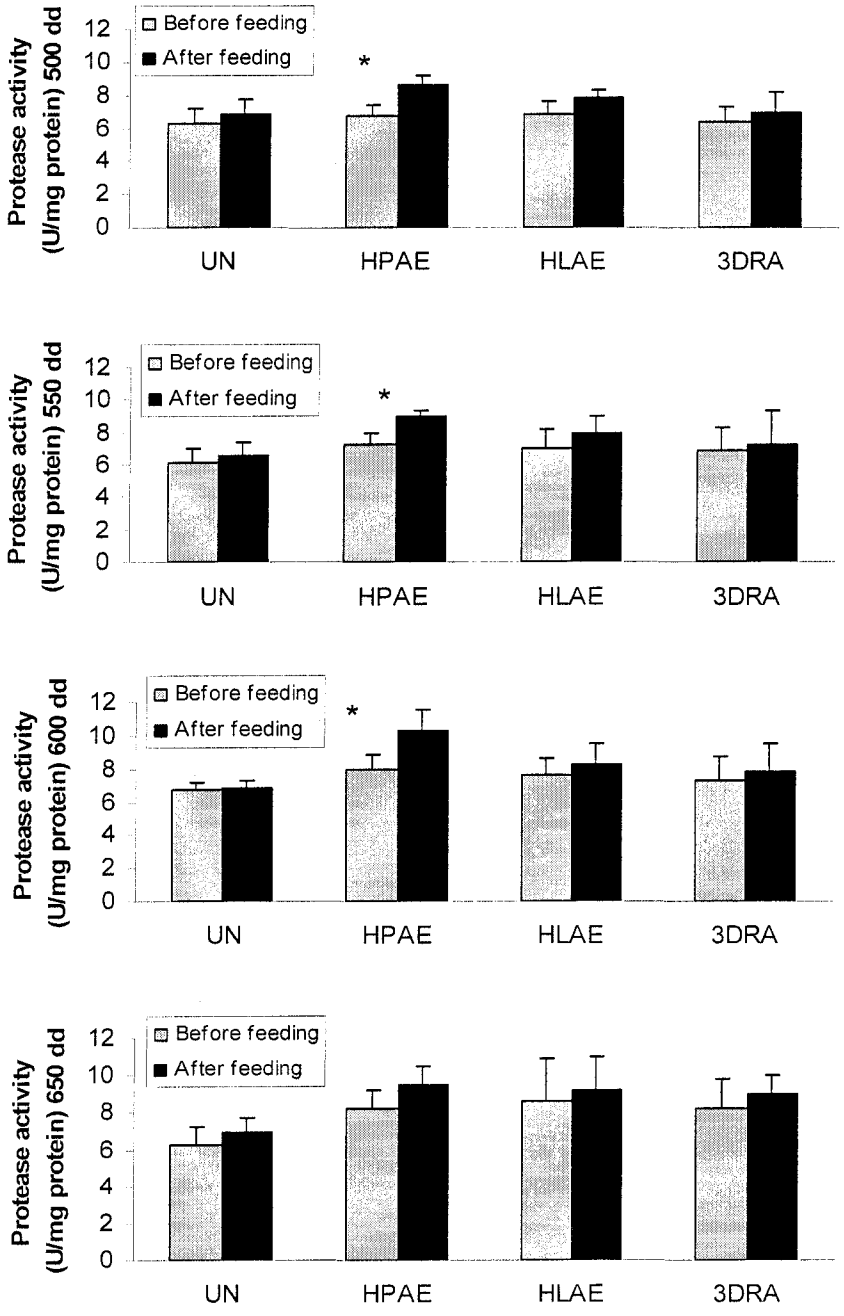

Figure 3.5. Activity of general protease (U/mg protein) on azocasein substrate in 0.5 ml peri-metamorphic cod larvae before and after eating one of four feeding treatments from 500-650 dd. UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean. * denotes significantly higher cod enzyme activity after eating the *Artemia* as compared to before eating ($p>0.05$).

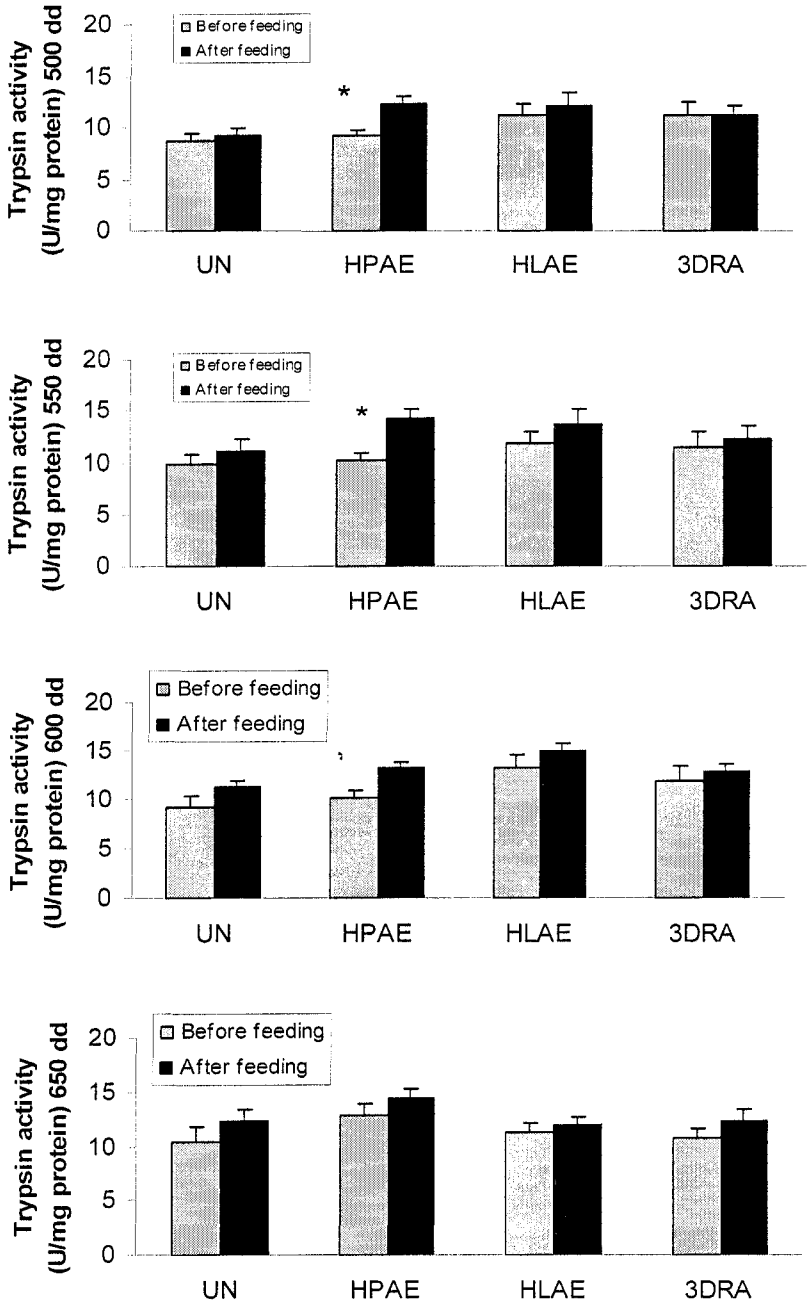

Figure 3.6. Activity of trypsin-like enzymes (U/mg protein) on BAPNA substrate in 0.5 ml peri-metamorphic cod larvae before and after eating one of four feeding treatments from 500-650 dd. UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean. * denotes significantly higher cod enzyme activity after eating the *Artemia* as compared to before eating ($p>0.05$).

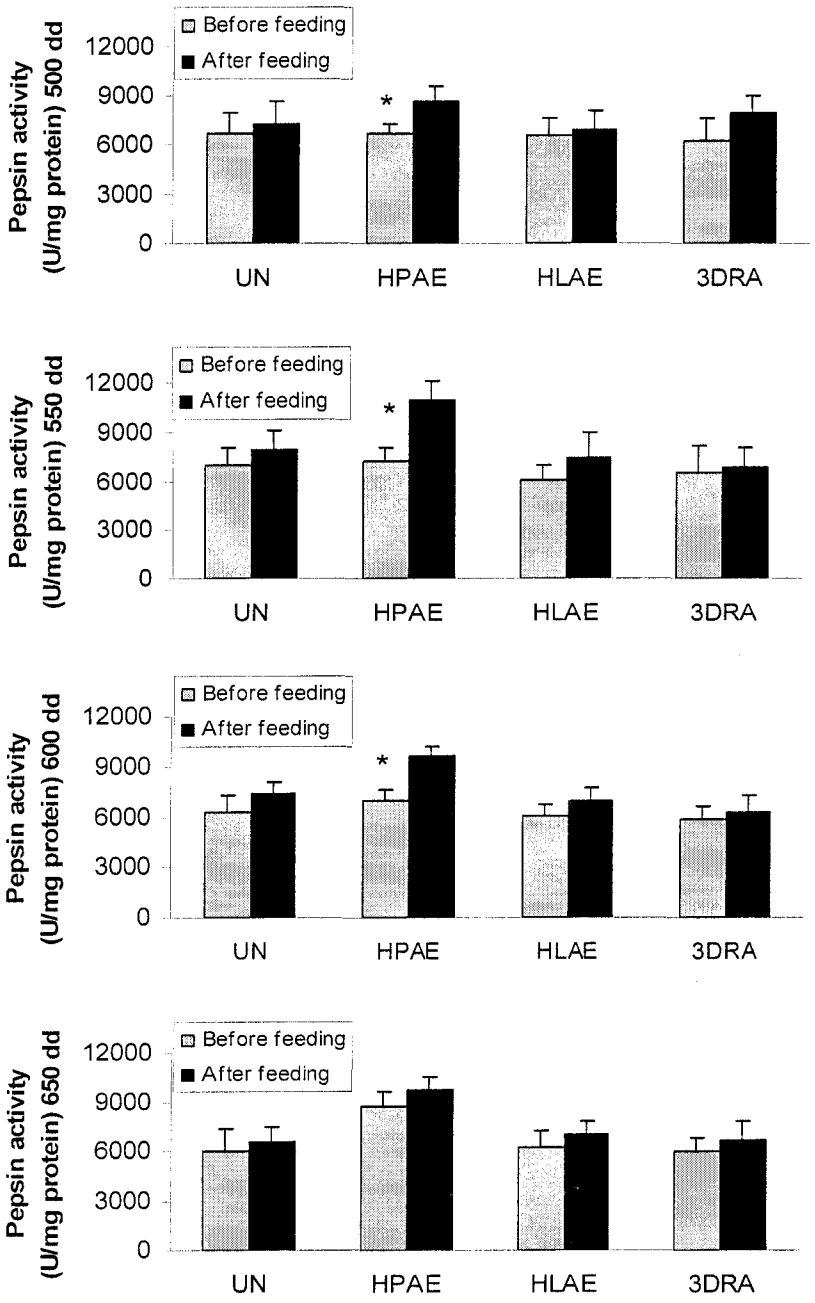

Figure 3.7. Activity of pepsin-like enzymes (U/mg protein) on hemoglobin substrate in 0.5 ml peri-metamorphic cod larvae before and after eating one of four feeding treatments from 500-650 dd. UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean. * denotes significantly higher cod enzyme activity after eating the *Artemia* as compared to before eating ($p>0.05$).

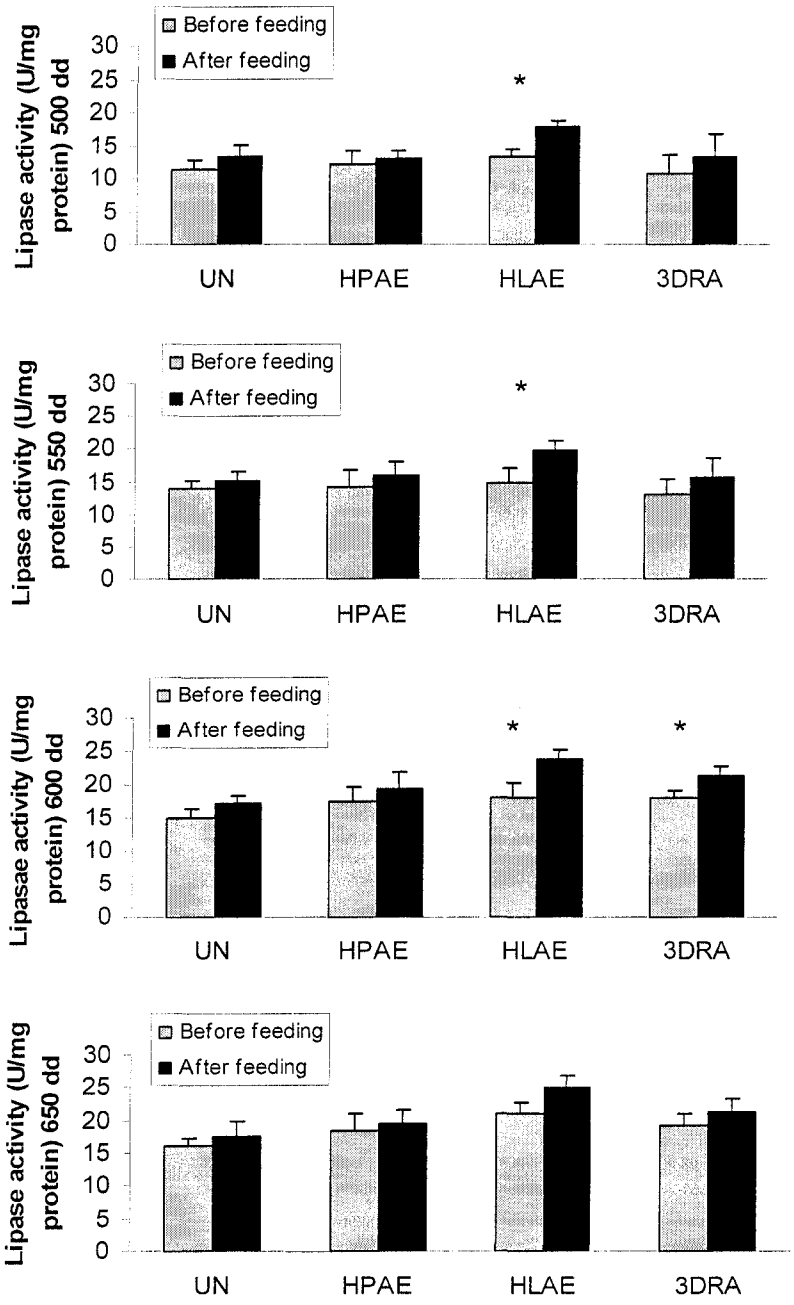

Figure 3.8. Activity of general lipases (U/mg protein) on *p*-nitrophenyl myristate substrate in 0.5 ml peri-metamorphic cod larvae before and after eating one of four feeding treatments from 500-650 dd. UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean. * denotes significantly higher cod enzyme activity after eating the *Artemia* as compared to before eating ($p>0.05$).

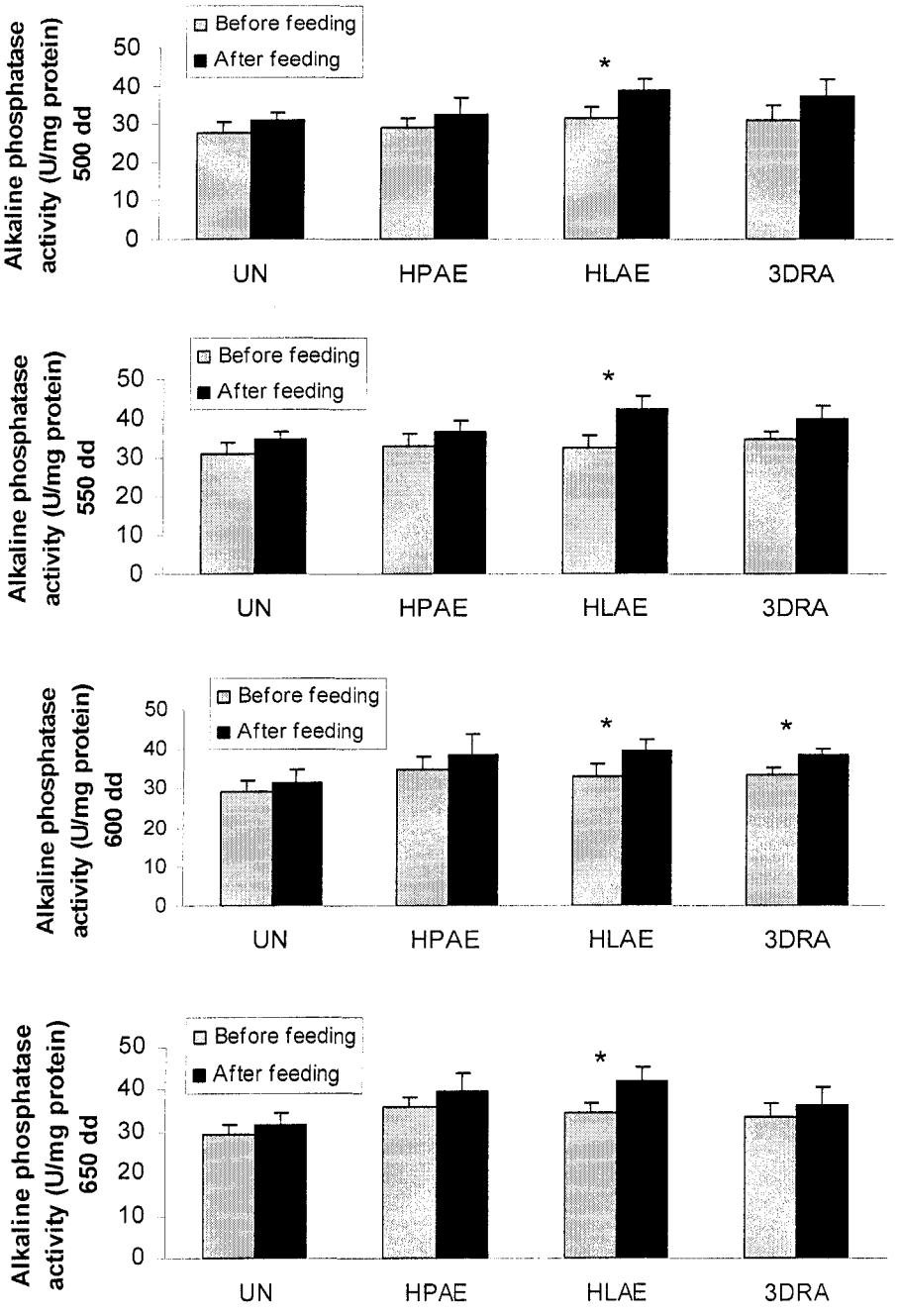

Figure 3.9. Activity of alkaline phosphatase (U/mg protein) on *p*-nitrophenyl phosphate substrate in 0.5 ml peri-metamorphic cod larvae before and after eating one of four feeding treatments from 500-650 dd. UN (unenriched nauplii), HPAE (high protein Artemia enrichment), HLAE (high lipid Artemia enrichment), 3DRA (three day rotation of Artemia). Bars represent one standard error of the mean. * denotes significantly higher cod enzyme activity after eating the *Artemia* as compared to before eating ($p>0.05$).

3.4.4 Larval tissue lipid analysis

The lipid composition of the live food enrichments (Table 3.1) shows the treatments differed for total lipid, triacylglycerols, phospholipids, 18:1ω9, 22:6ω3 (DHA), and Σω6. However, the amounts of 18:3ω3, 20:4ω6 (AA) and Σω6 were similar for all three enrichments. The highest DHA:EPA was found in the HLAE enrichment (2.3:1), the highest EPA:AA was seen in the HPAE (3.5:1) and the highest ω3:ω6 was determined to be in the UN (3.5:1).

Lipid composition of the larval tissues showed that 450 dd old cod at the beginning of the experiment had the lowest total lipid content at 46.86 µg/mg, while 650 dd old cod fed unenriched *Artemia* nauplii had the highest overall lipid content with 134.41 µg/mg, but this was not significantly different from the HPAE or HLAE treatments ($p>0.05$, Table 3.2).

Lipid class data showed an overall similarity for the 450 dd cod tissues before *Artemia* feeding began and the 650 dd cod tissues for the 3DRA treatment, with similar values for proportions of ethyl esters, triacylglycerols, acetone mobile polar lipids, and phospholipids ($p>0.05$; Table 3.3). The HPAE, HLAE and UN treatments showed the highest proportions of acetone mobile polar lipids. The UN and HPAE treatments had high values for ethyl esters, triacylglycerols, and phospholipids. Triacylglycerol and phospholipids percentages were highest in the UN and HPAE treatments. The 3RDA fish had the highest proportion of free fatty acids as compared to the other groups.

Table 3.3. Lipid class composition of peri-metamorphic cod larvae fed one of four *Artemia franciscana* enrichments from 450 to 650 dd. Unenriched *Artemia* nauplii (UN), high protein *Artemia* enrichment (HPAE) , high lipid *Artemia* enrichment (HLAE), and a three day rotation of *Artemia* enrichments (3DRA). Values are means of triplicates ± standard error. Values in the same row with the same superscripts are not significantly different (p>0.05).

	450 dd cod	650 dd cod:			
	Start *Artemia*	UN	HPAE	HLAE	3DRA
Total lipid (µg/mg wet weight)	46.9±26.3 [a]	134.4±46.4 [b]	117.6±53.5 [b]	95.1±8.0 [b]	55.6±30.3 [a]
Lipid class (% total lipid):					
Ethyl esters	0.10±0.02 [a]	6.6±1.4 [b]	4.16±1.19 [b]	2.6±0.4 [c]	0.7±0.3 [a]
Triacylglycerols	7.0±2.5 [a]	24.6±3.7 [b]	22.85±2.31 [b]	11.3±7.9 [a]	8.6±5.4 [a]
Free fatty acids	6.5±3.1 [a]	10.7±5.6 [a]	8.00±2.13 [a]	9.8±1.4 [a]	13.8±2.44[b]
Sterols	16.1±2.9 [a]	11.1±2.3 [b]	10.55±1.20 [b]	11.9±0.8 [b]	12.8±1.1 [b]
Acetone mobile polar lipids	6.6±3.8 [a]	0.9±1.7 [b]	2.48±1.51 [b]	0.6±0.3 [b]	5.8±3.2 [a]
Phospholipids	61.4±1.3 [a]	41.0±6.0 [b]	48.85±4.31 [b]	62.3±7.5 [a]	57.7±8.2 [a]

The absolute amounts of lipid classes (Table 3.4) generally agree with the proportions of lipid classes (Table 3.3). The UN and HPAE had the highest triacylglycerols in both proportion and absolute amounts (μg/mg weight). Phospholipids were proportionately similar in the HLAE, 3DRA and 450 dd start cod tissues, while absolute amounts of phospholipids were highest in the UN, HPAE, HLAE and 3DRA when compared to larvae at the start of the experiment, before *Artemia* had been fed.

An interesting result is the ratio of the important fatty acids to each other. The Σω3:Σω6 ratios were lowest at the beginning of the experiment (2.4:1), higher in the UN (2.9:1) HPAE and HLAE (3.0:1) treatments, and higher still in the 3DRA-fed cod (3.6:1). The DHA:EPA ratio was the highest in the cod at the beginning of the experiment (7.8:1), and ranged from 0.8-2.0:1 for cod tissues after consuming the four enrichments over the course of the experiment. The EPA:AA ratio, conversely, was lowest in the 450 dd old cod (0.7:1), highest in the HPAE cod (3.3), with intermediate values from 1.6-2.2:1 for the remaining treatments.

Table 3.4. Lipid class and fatty acid total amounts (standardized to wet weight of sample) of cod larvae fed one of four *Artemia* enrichments from 450 to 650 dd post-hatching (UN = Unenriched nauplii, HPAE = high protein *Artemia* enrichment, HLAE = high lipid *Artemia* enrichment, 3DRA = three day rotation of *Artemia* enrichments). Values are means of triplicates ± standard error. Values in the same row with the same superscript are not significantly different ($F_{1,5}$<6.61, p>0.05).

(µg/mg wet weight)	450 dd	650 dd			
	Start *Artemia*	UN	HPAE	HLAE	3DRA
Total lipid	46.9±26.3[a]	134.4±46.4[b]	117.6±53.5[b]	95.1±8.0[c]	55.6±30.3[ac]
Triacylglycerols	4.3±2.3 [a]	34.1±15.2 [b]	26.1± 9.0 [b]	10.7± 4.6 [a]	6.0±3.3 [a]
Phospholipids	28.4±17.6 [a]	56.9± 27.3 [b]	58.7±31.5 [b]	59.4±10.2 [b]	33.3±14.3[ab]
18:1ω9	3.0±1.4 [a]	25.9±8.7 [b]	24.0±9.2 [b]	13.1±6.3 [c]	8.8±2.7 [c]
18:3ω3	0.40±0.18 [a]	13.3±7.2 [b]	10.6±4.3 [b]	5.9±2.4 [bc]	4.0±2.2 [c]
20:4ω6 (AA)	1.3± 0.2 [a]	2.7±1.0 [b]	1.60±0.5 [ab]	2.6± 0.9 [b]	1.3±0.5 [a]
20:5ω3 (EPA)	0.9± 0.3 [a]	5.9±2.9 [b]	5.3±1.8 [b]	4.4± 1.1 [b]	2.1±0.8 [a]
22:6ω3 (DHA)	7.2± 2.0 [a]	4.5±1.6 [a]	4.9± 2.3 [a]	8.6±2.9 [a]	4.1±1.5 [a]
Σ ω3	8.9±3.3 [a]	27.4±16.4 [b]	23.6±12.0 [b]	20.8±9.4 [b]	11.4±4.6 [a]
Σ ω6	3.7±2.0 [a]	9.3± 2.7 [b]	7.85±3.2 [b]	6.9±1.1 [b]	3.2±0.7 [a]
Σω3:Σω6	2.4 [a]	2.9 [b]	3.0 [b]	3.0 [b]	3.6 [c]
DHA:EPA	7.8 [a]	0.8 [b]	0.9 [b]	2.0 [c]	1.9 [c]
EPA:AA	0.7 [a]	2.2 [b]	3.3 [c]	1.7 [b]	1.6 [b]

3.5 DISCUSSION

Survival data clearly illustrate the importance of lipids in the larval cod diet, as the survival of the 3DRA and HLAE treatments was four times higher than the HPAE and UN treatments. Growth data indicate that a diet of *Artemia* containing a variety of enrichments, like that in the 3DRA treatment, is better than feeding a constant diet of a single enrichment. Lipids are an important nutrient in the early stages of larval development but once the fish reach a certain size, additional types of nutrients, particularly proteins, can be utilized. For example, by 250 dd, the cod digestive system matures to a point where the stomach becomes developed and functional such that gastric proteases can be produced in significant quantities (Timeyko 1986, Perez-Casanova 2003, Murray *et al.* 2001). The inclusion of proteins in the diet is important during rapid growth of larval fish because component amino acids are necessary for energetic requirements and building new proteins within the tissues (DeSilva & Anderson 1995).

The unenriched *Artemia* nauplii used in this experiment yielded cod tissues with the highest lipid content of the four treatments at 650 dd (Table 3.3). This is somewhat counter-intuitive, until one considers the age of the nauplii, as opposed to the older enriched *Artemia*. The unenriched nauplii were removed from the rearing cones no later than 24 hours after hatching and fed to the cod for that treatment. The other three enrichment treatments were at least 48 hours old when fed to the larvae due to the enrichment process. *Artemia* have been shown to significantly decrease their lipid content from levels at hatching as the

Artemia metabolize the lipids for energy (Coutteau & Mourente 1997). Evjemo (*et al.* 2003) determined newly hatched nauplii to have 20.3% lipid content on a dry weight basis, which drops to 18.2% by 48 hours after hatching, and 17.6% by 72 hours. If the unenriched nauplii treatment had used 48 hour-old unenriched *Artemia*, results would have shown a result more reflective of unenriched *Artemia* at the same stage of lipid loss as the other three diet treatments. However, mass culture currently uses 24 hour old unenriched nauplii as a live prey item, and as such, the results presented here still have practical implications for currently used protocols in larval cod rearing.

Zheng *et al.* (1996) determined the optimum DHA *Artemia* enrichment for larval Pacific cod (*Gadus macrocephalus*) to be 1.6-2.1% dry weight. They also reported larvae fed *Artemia* enriched with EPA or AA showed no significant improvements in larval survival, growth or vitality. On its own, DHA is a superior enrichment for *Artemia* being fed to cod. However, research is now being done to examine the interactions of HUFAs on larval development. This is especially important for marine fish, since they cannot significantly desaturate or elongate 18-carbon fatty acids and require supplemental HUFAs in the diet (Wu *et al.* 2002). *Artemia* are difficult to enrich with high levels of DHA, and the HUFAs tend to be partitioned into triacylglycerols used for energy storage, instead of phospholipids. Phospholipids are especially important in the larval stages of fish when development of membranes and neural tissue are being established (Bell *et al.* 2003).

Ratios of important fatty acids provide a more meaningful measure than individual amounts of HUFAs when examining how well cod perform on a specific diet, since length and condition factor are not comprehensive indicators of fish performance. One standard reference has been to compare the fish tissues to the fatty acids found in their eggs, or when possible, from wild larvae. Cod eggs have DHA:EPA and EPA:AA ratios of 2.0:1 and 8.7:1, respectively, while older wild-caught larvae have DHA:EPA ratios of 1.8-2.0:1 and EPA:AA ratios of 8-13:1 (Klungsoyr *et al.* 1989). Bell *et al.* (2003) suggest feeding marine flatfish larvae enriched live prey with a minimum DHA:EPA of 2:1 and EPA:AA of 5:1. These values are greater than those found in unenriched *Artemia* with ratios for DHA:EPA of 0.08:1, and an EPA:AA of 2.0:1 (Table 3.1), which reemphasizes the need for enrichments (Estevez *et al.* 1999). The HLAE had the most optimal ratios of the diets used, with DHA:EPA of 2.3:1 and EPA:AA of 2.0:1 (Table 3.1).

In the current experiment, the HLAE and 3DRA diets yielded cod tissues with DHA:EPA ratios of 2.0:1 and 1.9:1, indicating that the high lipid enrichment and the three day rotation of enrichments supplied the cod with fatty acids such that proper cell membrane function is retained during rapid growth and metabolism to a balance similar to that found in eggs and wild larvae (Sargent *et al.* 1999). Deficiencies in these important fatty acids, and therefore imbalances in the ratios of HUFAs, result in reduced growth, dermal abnormalities, elevated muscle edema, decreased capacity for immunological defense, increased permeability of cellular membranes, underdeveloped swim bladders, reduced

reproductive performance and increased mortality (Halver 1989, Lovell 1993, Rainuzzo *et al.* 1997).

By assaying the cod before and after eating, the digestive enzymes were shown to increase the activity of lipases and proteases when high levels of lipid and protein are present in the enrichment. Experiments in rats, as well as rainbow trout, carp and seabass, have shown that diet composition impacts digestive enzyme secretion, especially pertaining to dietary fats (Kawai & Ikeda 1972, 1973; Spannagel *et al.* 1996; Zambonino-Infante & Cahu 1999). Additionally, there may be a threshold of dietary lipid content below which little adaptation of the digestive system is possible and above which significant adaptation occurs (Brannon 1990). Pre- and post-prandial digestive activities did not significantly differ among treatments beyond 600 dd, except for alkaline phosphatase in HLAE-fed cod. At this stage in development, cod were between 16-20 mm standard length, depending on treatment. Metamorphosis is well underway at this point, as was illustrated by the external emergence of median fins, but also the internal activity of gastric proteases like pepsin in this experiment.

The question now becomes whether the absence of significant elevations in the post-prandial enzyme activities beyond 600 dd were the result of changes to composition of the live food enrichments, or as a consequence of growth and development of new organs and tissues, and perhaps gut evacuation rates. For the enzymes where no elevation was seen, it is likely a result of the larvae's

increasing tissue deposition, which alters the enzyme activity results since the values are standardized to the amount of protein in the whole body homogenate. However, in the case of alkaline phosphatase, metamorphosis brings a marked maturation of the development of the brush border membrane of enterocytes (Martinez *et al.* 1999). With this brush border maturation comes a significant increase in the activity of alkaline phosphatase to the degree that significant elevations are seen even when the protein content of the homogenate has been taken into account, especially when fish are fed on a high lipid diet. Similar results have been seen in Senegal sole (*Solea senegalensis*) and sea bass (*Dicentrarchus labrax*) fed high lipids (Ribiero *et al.* 1999, Cahu *et al.* 2000). Pepsin, however, began to significantly increase activity earlier in metamorphosis (well before 600 dd, as evidenced by enzyme activity data from Chapter 2), and no such trend was found during the current experiment for cod in the HPAE treatment.

Although the behaviour and foraging activity of cod in the four diet treatments were not affected by the enrichment, growth and digestive enzyme activities were significantly affected by diet. The most dramatic changes were seen in the 3DRA-fed cod. A feeding protocol of *Artemia* with a variety of commercial enrichments, and thus a diverse source of nutritional components improves growth and survival. This enables mass culture to yield fish that become larger faster, and allows metamorphosis and weaning to be completed earlier in the culturing process.

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4 Summary

The experiments in this thesis focused on determining the foraging capacity of Atlantic cod larvae by examining growth, behaviour, and patterns of digestive enzyme activity in response to larval diet. Fish must be able to obtain energy from ingested food to undertake necessary metabolic activities, such as swimming, foraging, and the ability to mount immunological defenses to disease. It is the ability of the fish to convert food into energy and how this energy is partitioned that will determine how a fish's growth and performance are affected. Therefore, insights provided by studies of foraging ability and physiology, such as this one, have direct relevance to aspects of fish biology as well as economic ramifications with respect to the mass rearing of Atlantic cod.

In intensive culture, larval fish are initially reared on live food and at some later stage, weaned onto a formulated diet. However, the quality of the live food varies greatly and places significant constraints on larval rearing. Much of the research now being done with species that have been identified as having commercial potential is focused on developing formulated diets capable of sustaining optimal growth from first-feeding onwards (Brown *et al.* 2003). A consequence of the difficulty in rearing larvae on formulated diets is that nutritional requirements of larvae cannot be directly or accurately determined (Fraser *et al.* 1988). However, by studying the changes in biochemical composition that occur during early larval development, nutritional requirements of first-feeding larvae can be indirectly determined.

Chapter 2 illustrated the importance of lipid in the rotifer-feeding phase of cod development. Live prey is essential to cod culture, but supplemental nutrients must be added. The low lipid algae enrichment was not able to support larval survival beyond 250 dd. The high lipid enrichment, however, resulted in significantly increased larval growth and survival, as well as increased swimming activity and prey capture over the same period. Digestive enzyme activity levels in the current experiments were higher for lipases and alkaline phosphatase than previously reported for cod over the same period by Perez-Casanova (2003). Thus, there is evidence that high quantities of lipid in the diet may elicit higher activity levels for lipid-digesting enzymes. The pre- and post-prandial digestive enzyme activities of older larval cod in Chapter 3 suggest that diet influences the growth, lipid composition of the tissues, and digestive enzyme activity in peri-metamorphic cod larvae, but not the behaviour or activity of these animals. The digestive system of peri-metamorphic cod can be induced to elicit biochemical responses to the diet, at least for a brief period of time.

Two points must be kept in mind when interpreting the results from Chapters 2 and 3 and adapting mass-culture protocols. First, these cod remained on a live food diet longer than is accepted practice in commercial cod culture. As such, there may be aspects of larval growth that are not being taking into account when weaning is initiated at an early age and metamorphic development is not complete. If larvae are growing at a rate that an enriched *Artemia* diet cannot support, digestive enzyme activity may be affected, inducing

elevations or depressions that would not have occurred if the fish had been weaned onto dry pellets by the normal 350-500 degree days of age. However, Shields *et al.* (2003) contradict current cod-rearing protocols and recommend that *Artemia*, and subsequent formulated food, should be withheld until the completion of metamorphosis. Furthermore, they saw no advantage to offering freshly-hatched nauplii during the rotifer-*Artemia* transition and advise feeding the cod enriched *Artemia* directly. Additional experiments on the impact of both rotifer and *Artemia* enrichments on digestive enzyme activity when cod are weaned at progressively earlier ages should be performed to determine an optimal rotifer to *Artemia* transition regime.

Second, the enzyme assays are not specific for the individual enzymes analyzed. The pepsin assay detects pepsin as well as pepsin-like acid proteases, so it cannot be stated with absolute certainty that the data give a precise profile of what is occurring in the gut. However, the data do provide a basis for comparative studies of enzyme activity. By comparing the relative activities of the digestive enzymes and how they are affected by diet, we can use this information to better understand how these larvae successfully digest food, which is then incorporated into metabolic energy. It is this energy that will be used in every aspect of the fish's activity and ultimately impact overall health and growth, which is essential to any financially viable aquaculture operation.

In conclusion, the digestive ontogeny of larval cod is affected by the diet. Lipids are important during the rotifer start-feeding phase of development, while a

combination of *Artemia* enrichment types provide the best growth in peri-metamorphic cod. The results of this thesis can be applied to future experiments examining earlier weaning, possible reduction of *Artemia* feeding, overall effectiveness of different live food enrichments on cod growth, as well as improving broodstock nutrition and avoiding fatty liver syndrome. It is expected that the information from studies of this nature can eventually lead to more economically efficient hatchery protocols, with the end result of larger and healthier cod being sent to market.

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Appendix 1: Information on live food and enrichments

Chapter 2:

Rotifers used: *Brachionus plicatilis*
Cultured at the Ocean Sciences Centre, Memorial University of Newfoundland.

LLRE: Low lipid rotifer enrichment = *Isochrysis* sp.
Cultured at the Ocean Sciences Centre, Memorial University of Newfoundland.

HLRE: High lipid rotifer enrichment = AlgaMac® 2000
Aquafauna Bio-Marine, Inc.
P. O. Box 5, Hawthorne, California. 90250 USA

Chapter 3:

Artemia used: *Artemia franciscana* cysts
INVE Aquaculture Inc.
P. O. Box 3105, Ogden, Utah. USA. 84409

UN: unenriched 24 hour-old nauplii

HLAE: High lipid *Artemia* enrichment = AlgaMac® 3050
Aquafauna Bio-Marine, Inc.
P. O. Box 5, Hawthorne, California. USA. 90250

HPAE: High protein *Artemia* enrichment = Spray-dried krill hydrolysate (SD-KH2)
Specialty Marine Products Ltd.
4160 Marine Drive, West Vancouver, BC. Canada. V7V 1N6