ONTOGENY OF THE CORTICOSTEROID STRESS RESPONSE AND EFFECT OF DIFFERENTIALLY ENRICHED LIVE FEED ON GROWTH, LIPID COMPOSITION AND ACUTE STRESS TOLERANCE OF LARVAL ATLANTIC COD, GADUS MORHUA

SARAH KATE MATHESON WESTELMAJER

ONTOGENY OF THE CORTICOSTEROID STRESS RESPONSE AND EFFECT OF DIFFERENTIALLY ENRICHED LIVE FEED ON GROWTH, LIPID COMPOSITION AND ACUTE STRESS TOLERANCE OF LARVAL ATLANTIC COD, *Gadus morhua*.

BY

© SARAH KATE MATHESON WESTELMAJER

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Aquaculture

Memorial University of Newfoundland

February 2008

St. John's Newfoundland

Abstract

The ontogeny of the corticosteroid stress response was examined in hatchery-reared Atlantic cod (*Gadus morhua*). Pooled whole-body samples of larvae at rest and following a 30 second (s) air exposure were taken at multiple intervals throughout development from 2 days pre-hatch up to metamorphosis at 59 days post-hatch (dph). Extracted samples were analysed using a commercially available enzyme-linked immunoassay kit, which was subsequently validated for use with whole-body samples. A significant increase in whole-body immunoreative corticosteroid (IRC) concentration in response to the air-exposure stress was observed in larvae at hatch (0 dph), which likely indicates the functioning of the hypothalamus-pituitary-interrenal axis (HPI) and thus the endogenous production of cortisol by the larvae at this time. Two stress hyporesponsive periods (SHRP) were detected, between 0-30 dph and at 50 dph, and likely correspond with two critical periods of neurological and immunological development, during which the production and release of cortisol is restricted. Potentially stressful aquaculture procedures should be avoided during these SHRPs.

The effect of differentially enriched live-food organisms, rotifers (*Brachionus plicatilis*), and brine shrimp (*Artemia* sp.), on the lipid content, growth and stress tolerance of larval Atlantic cod was also examined. The lipid and fatty acid content of larval tissue was directly related to the lipid and fatty acid content of enriched live feed. Larvae fed rotifers and *Artemia* enriched with elevated proportions of the highly unsaturated fatty acids,

DHA (docosahexaenoic acid; 22:6ω3), AA (arachidonic acid; 20:4ω6), and ω6DPA (docosapentaenoic acid; 22:5ω6) showed best overall growth and survival following an acute 15 s air-exposure and transfer stress. Differences in post-stress survival between treatments were most likely attributable to variations in larval EPA (eicosapentaenoic acid; 20:5ω3)/AA and DHA/DPA content. Corticosteroid levels remained elevated in larvae with maximal post-stress survivorship, and may indicate the requirement of larval cod for a prolonged rise in cortisol following a stress event. Suggested DHA/EPA/AA ratios for rotifer and *Artemia* enrichments that yielded best growth and stress tolerance in Atlantic cod larvae were 7/2/1 and 5/2/1 respectively.

Acknowledgements

First and foremost, I would like to thank my husband Todd, without whom I never would have chanced moving 7,000 kms away from all friends and family to pursue the best opportunity in aquaculture research. He kept me sane (and fed) during months of non-stop hatchery work, lab work and writing. Above all, he never gave up on me and he never let me give up on myself.

I am also very grateful to my incredible parents who have stood by me through thick and thin. Thank you so much for always being there for me, trying very hard to understand just what it was that I was researching, and of course, helping plan a wedding in the middle of everything!

To my supervisor, Dr. Joe Brown, thank you so much for taking a chance on a environmental biology major from BC who had wild dreams of convincing all Canadians of the benefits of aquaculture. You inspired me every day and made sure I progressed both as a student and as a person. You are sorely missed. I hope I've made you proud.

A world of thanks to my co-supervisors Drs. Chris Parrish and Stewart Johnson who voluntarily took in an orphaned Masters student in the middle of her program. I would not have been able to finish this degree without your unwavering support, guidance and words of encouragement.

Many thanks to Dr. V. Puvandendran (Puvy) whose door was always open and who is the main reason why I was able to successfully raise two batches of cod from egg to juvenile. He was also instrumental in collecting many ontogenetic samples from the late stages of development that were used in chapter 2.

Jeanette Wells was generously helpful with time and advice concerning lipid and fatty acid analysis.

Thanks to Drs. David Berlinsky and William King for inviting me into your lab and sharing your knowledge on cortisol assays.

Thanks to everybody at the ARDF/JBARB and the OSC. Danny Boyce, Lori Thorne, Francine Fortune, Jennifer Monk, Rodney Healy, Dwight Drover, Connie Short, Jennifer Hall, Jim Devereaux and the workshop staff, Ken Langdon, all the custodians, all my fellow grad students.

Funding for this research was provided by AquaNet (AP-34). I would sincerely like to thank everyone who was involved with the AquaNet project. This dedicated group of people made this degree possible for me.

Table of Contents

Table of Contents cont.

Table of Contents cont.

List of Tables

List of Figures

List of Abbreviations

AA	arachidonic acid (20:4ω6)
ACTH	adrenocorticotropic hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
C_{20}	20-carbon
C_{22}	22-carbon
CF	condition factor
COX	cyclooxygenase
CV	coefficient of variation
dd	degree days
DHA	docosahexaenoic acid (22:6ω3)
DPA	docosapentaenoic acid (22:5ω6)
dph	days post-hatch
dw	dry weight
EFA	essential fatty acid
ELISA	enzyme-linked immunoassay
EPA	eicosapentaenoic acid (20:5ω3)
FA	fatty acid
GR	glucocorticoid receptor
h	hours
HPA	hypothalamus-pituitary-adrenal
HPI	hypothalamus-pituitary-interrenal
HUFA	highly unsaturated fatty acid
IRC	immunoreactive corticosteroid
LA	linoleic acid (18:2ω6)
LNA	linolenic acid (18:3ω3)
LSGR	length-specific growth rate
min	minutes
MUFA	monounsaturated fatty acid
MyH	myotome height
PG	prostaglandin
PGE2	arachidonic acid-derived prostaglandin
PGE3	eicosapentaenoic acid-derived prostaglandin
PUFA	polyunsaturated fatty acid
RIA	radioimmunoassay
s	seconds
SE	standard error
SFA	saturated fatty acid
SHRP	stress hyporesponsive period
SL	standard length

ww	wet weight

1.1 General Introduction

The culture of Atlantic cod (*Gadus morhua*) on a viable commercial scale shows great potential and is currently in operation in Norway and being developed by countries such as the United Kingdom, Canada and the United States. In comparison to the culture of salmonid species, existing operations for the culture of cod are generally small and experimental in nature or are a means of supplementary income for people in rural areas (Svåsand *et al.* 2004). Further expansion of the industry has been hindered by the inability to reliably produce large quantities of high quality juveniles at several intervals throughout the year. This bottleneck in production stems predominantly from the high mortality that occurs during the larval phase (1 – 50 days post egg hatching) and metamorphosis. Cod eggs hatch at a very early stage of development, and are thus especially vulnerable to environmental fluctuations and starvation (for review see Brown *et al.* 2003). Consequently, the consideration of all aspects that affect larval diet and feeding, from light intensity and photoperiod to prey densities and nutritional profiles, is critical for ensuring successful rearing.

The nutritional requirements of larval teleosts appear to be species-specific, but some commonalities exist for the three major classes of nutrients, the carbohydrates, proteins and lipids. Larval fish, including Atlantic cod, typically have greater protein and lipid requirements than adult fish because of their high growth rates, but require little to no carbohydrate (DeSilva and Anderson 1995, Pérez-Casanova *et al.* 2006). Lipids, and specifically fatty acids, have been of recent interest in research, because they are

inexpensive compared to proteins, a good energy source for developing larvae, and they also play major roles in neurological development, pigmentation and various hormone systems. Larval cod are unable to endogenously produce significant amounts of some highly unsaturated fatty acids (HUFAs), and therefore they must be provided in the diet. These essential fatty acids (EFAs) include those of the ω3 and ω6 varieties and more specifically docosahexaenoic acid (DHA; 22:6ω3), eicosapentaenoic acid (EPA; 20:5ω3) and arachidonic acid (AA; 20:4ω6).

In a hatchery setting, cod larvae are fed predominantly two types of small zooplankton, *Brachionus plicatilis* (rotifers) and the brine shrimp *Artemia sp.* Unfortunately, both rotifers and *Artemia* are nutritionally deficient when compared to wild copepods, which are the natural diet of cod larvae (Rainuzzo *et al.* 1989, Dehert 1999, Harel and Place 1999, Hamre *et al.* 2002). As a result, these live-food organisms are enriched with an emulsion high in protein, lipids and HUFAs before being fed to the larvae. There are many commercial enrichments currently available to aquaculturists, and each differs in its nutritional profile. An optimal cod-specific diet has yet to be determined, mostly because the exact nutritional requirements of larvae at this stage are still unknown. As a consequence of this knowledge gap, the focus of recent larviculture research has been on the relationship between DHA, EPA and AA in the diet and how changes in the ratio of these three EFAs affect growth, survival, feeding behaviour, and the development of digestive capacity (Shields *et al.* 2003, Cutts *et al.* 2006, O'Brien-MacDonald *et al.* 2006, Park *et al.* 2006, Garcia *et al.* 2007a,b).

The physiological response of fish to stress is adaptive such that it allows a fish to better cope with an immediate stressful situation. Yet, stress is a big problem for aquaculture because repeated or constant exposure to stress, particularly during certain necessary husbandry practices such as net confinement, air exposure, tank transfer and salinity changes, can result in decreased growth, decreased immunocompetance, and ultimately mortality (Barton and Iwama 1991, Barton 1997). Changing the ratio of DHA:EPA:AA in live-food diets is known to have a significant effect on larval survival following the application of such husbandry practices (Kanazawa 1997, Harel *et al.* 2001, Koven *et al.* 2001, 2003, Van Anholt *et al.* 2004). These results can be at least partially explained by the roles DHA, EPA and AA play in the regulation of the release of the stress hormone cortisol via the hypothalamus-pituitary-interrenal (HPI) axis. Most notably, an overabundance of AA in the diet, in relation to the amount of EPA, has been shown to increase basal cortisol levels in unstressed and chronically stressed larvae (Harel *et al.* 2001, Koven *et al.* 2003). In contrast, larvae fed a mid-level of AA show decreased cortisol levels and better post-stress survival, compared with larvae fed no to low amounts of AA (Harel *et al.* 2001, Koven *et al.* 2003, VanAnholt *et al.* 2004). Determining the balance between DHA, EPA and AA that will confer the best ability to cope with a stress is, therefore, critical when considering the nutritional profile of larval diets.

What is known about the ontogeny of the cortisol stress response in larval teleosts is primarily based on salmonid and tropical marine fish research. High levels of maternally derived cortisol in newly fertilized eggs have been found in many species including Chinook (*Oncorhynchus tshawytscha*: Feist and Schreck 2002) and Chum salmon (*Oncorhynchus keta*: De Jesus *et al.* 1992), yellow perch (*Perca flavescens*: Jentoft *et al.* 2002), tilapia (*Oreochromis mossambicus*: Hwang *et al.* 1992), and Japanese flounder (*Paralichthys olivaceus*: De Jesus *et al.* 1991). During development, cortisol levels decrease as the embryo approaches hatch. After hatching, basal cortisol levels rise steadily; the timing of this increase appears to be species-specific and starts anywhere from a few days to several weeks post-hatch (De Jesus *et al.* 1991, Hwang *et al.* 1992, Szisch *et al.* 2005). Basal cortisol levels also increase significantly during the early stages of metamorphosis in marine fish larvae, (De Jesus *et al.* 1991, Tanaka *et al.* 1995, Szisch *et al.* 2005), while for salmonids, a similar peak occurs when the yolk is completely absorbed and the fry emerge from gravel beds (De Jesus *et al.* 1992). In cod culture, metamorphosis is a period of mass mortality, which is assumedly caused by increased cannibalism and high stress levels associated with the rapid physiological and morphological changes that the larvae undergo. To date, however, there have been no studies to verify that stress levels are indeed elevated during this period of rapid development.

The stage at which cortisol is first produced endogenously in response to a stressor varies between groups, such that some show a hypo-responsive period of several weeks post-

hatch (Pottinger and Mosuwe 1994, Barry *et al.* 1995) while others show a rapid development of the cortisol response (Pepels and Balm 2004). Cortisol in cold-water marine larvae was not quantified until King and Berlinsky (2006) were able to measure corticosteroids in pooled samples of cod larvae as early as the day of hatch. A significant cortisol response to an acute air-exposure stress was first observed in larvae at 8 dph; however, a small, but statistically insignificant, increase in whole-body corticosteroids was also detected in stressed larvae at hatch. It is therefore possible, that the cortisol stress response develops earlier than 8 dph, and possibly as early as 3 dph when first-feeding is initiated, since a similar pattern was reported for gilthead seabream (*Sparus aurata*: Szisch *et al.* 2005).

Consequently, the objectives of the first experiment in this study were: (1) to determine whether the HPI axis and the subsequent release of corticosteroids in response to an acute stress are active earlier than the previously reported 8 dph, (2) to investigate if changes in basal cortisol levels correspond to times at which changes are made in the feeding protocol, and (3) to find out if basal stress levels do indeed peak at metamorphosis by examining the corticosteroid levels prior to, during, and post metamorphosis.

The effect of commercially available enrichment products on the lipid, and more specifically the fatty acid, profiles of rotifers, *Artemia* and the cod larvae fed these enriched live-food organisms was the focus of the second experiment in this study. The objectives were: (1) to determine if cod larvae fed differentially enriched live food

exhibit differences in growth throughout the rearing period, (2) to determine if cod larvae fed differentially enriched live food respond differently to an acute stress, (3) to relate variations in dietary HUFA ratios with differences observed in growth, post-stress survival and post-stress corticosteroid release.

1.2 Literature Review

1.2.1 *History of Cod Culture*

The seeking, capturing, processing, and selling of Atlantic cod, *Gadus morhua*, have always been intimately tied with the lives and culture of the people of Eastern Canada and especially Newfoundland. Unfortunately, the progressive decline in wild stocks forced the closure of the Canadian commercial cod fishery in 1992. This moratorium along with a worldwide drop in catches of most fish species, has markedly increased the value of fresh fish products, and has placed renewed interest in the culture of cod in Newfoundland.

The culture of cod is not a novel idea. The successful production of large quantities of larval Atlantic cod for stock enhancement began in Norway over 100 years ago (Svåsand and Moksness 2004). In the mid-1980's, hatcheries in Norway and the United Kingdom were raising small batches of cod from egg to adult. Unfortunately, extremely high mortality rates during larval and juvenile stages, in addition to a brief resurgence in the Norwegian cod fishery forced the abandonment of these trials. At this time in Newfoundland, considerable drops in the size of cod and cod catches were being noticed and wild cod "ranching", young cod caught by fishermen and grown in net pens for 5 – 6 months, was being attempted (Brown *et al.* 2003). Regrettably, the start of the moratorium hindered further development of this industry. The last fifteen years have generated a significant amount of intensive research into larviculture practices, which in turn has lead to the establishment of basic hatchery rearing protocols and the formation of

a few pilot-scale on-growing sea farms off the southern shore of Newfoundland. Although these trials have been successful, juvenile production is still too low to support a major industry and more focus needs to be put into minimizing the bottleneck that occurs at the larval stage (Svåsand *et al.* 2004). In order for this industry to continue to expand, large numbers of inexpensive high quality juveniles need to be readily available throughout the year. This requires an optimization of current hatchery procedures in order to decrease production costs, mostly associated with the production of live food, and decrease mortality while simultaneously maintaining or even increasing growth. Currently, the only large-scale intensive cod hatcheries are located in Norway; however, pilot-scale hatcheries in both Scotland and Canada are increasing production on a yearly basis with the expectation of supporting a viable industry in the future.

1.2.2 *Specifics of Cod Culturing*

The culture of all cold-water marine finfish, with few exceptions, is appreciably more challenging than salmonid-culture due to the presence of a prolonged larval phase. Larvae of Atlantic cod, and other potential aquaculture species such as haddock (*Melanogrammus aeglefinus*) and Atlantic halibut (*Hippoglossus hippoglossus*), hatch at a very early stage of development and thus are much smaller, more delicate and more vulnerable to mortality than their salmonid counterparts.

The beginning of the larval phase in finfish is marked by the transition from an endogenous food source, the yolk, to an exogenous, environmentally derived food source

(De Silva and Anderson 1995). Unlike most salmonids, early weaning of marine larvae, including cod, onto an inert particulate diet has had only limited success, and co-feeding with live-food diets is generally still required to ensure sufficient growth and survival (Kanazawa *et al.* 1989, Holt 1993, Kolkovski *et al.* 1997, Baskerville-Bridges and Kling 2000a). This is mostly a result of small body size, and thus extremely small gape, a rudimentary digestive system, the need for prey movement to induce a feeding response, as well as the poor acceptability of many microparticulate diets due to high nutrient leaching, short residence times in tanks, unpalatability and lower digestibility (for a general review see Cahu and Infante 2001, for cod see Baskerville-Bridges and Kling 2000b, Hamre 2006). Consequently, a large part of raising cod is the concurrent culture of high quality live-food organisms, typically the small zooplankton *Brachionus plicatilis* (rotifers) and the brine shrimp *Artemia sp.* Unfortunately, both rotifers and *Artemia* are nutritionally deficient when compared to the wild zooplankton species on which cod larvae would naturally be feeding. This adds an additional step to the culturing process whereby the live-food organisms are enriched with nutrients, predominantly lipids and protein, before being fed to the larvae.

Another very important aspect of raising cod is the condition of abiotic factors in the culture environment. Fluctuations in water quality parameters such as temperature, salinity and dissolved gas concentrations, which are often very costly for a hatchery to control, cause high levels of acute and chronic stress that have a major impact on larval growth, development and survival. There are also numerous critical periods throughout

the larval phase where specific cues must be present and felt by the larvae in order to stimulate successful morphological, physiological and behavioural development. Before larvae become juveniles, they must undergo a series of rapid morphological and physiological changes, typically referred to as metamorphosis, where "temporary larval systems" are replaced by fully differentiated tissues, organs and systems. The quality of resulting juveniles will be determined by the timing and accuracy of changes throughout the critical periods and metamorphosis. If environmental conditions are substandard or if high stress levels mask physiological cues, larval mortality will be high and any surviving juveniles will be of very poor quality.

To facilitate and promote the expansion of cod aquaculture in Newfoundland and other Atlantic regions, larval rearing must be streamlined to benefit from the rapid growth potential of cod under ideal conditions. More research into basic dietary requirements and the balance between specific nutrient classes is still needed to produce a cod-specific diet, especially during the larval phase. Also, a better understanding of when a response to external stressors is initially expressed and how this response changes throughout larval development is essential in creating and modifying daily husbandry practices to maximize growth, survival and juvenile quality.

1.2.3 *Lipid Nutrition*

The specific nutritional requirements of larval teleosts vary between species, but some common trends have been elucidated. Carbohydrates, proteins and lipids are the three

major nutrient classes that have been the focus of most larval nutrition research. Larvae typically have greater protein and lipid requirements than adult fish (for review see De Silva and Anderson 1995). Carbohydrates, on the other hand, are not a major constituent of larval diets, especially for cod, since larval forms of this species do not show significant digestive carbohydrase activity before two months of age (Pérez-Casanova *et al.* 2006). Some lipids can be used as inexpensive energy sources, and therefore typically constitute a major part of both live-food enrichments and formulated feeds. Fish-based oils, however, are costly lipid sources, but are indispensable for larval diets in that they contain essential fatty acids, which play many vital roles in growth and development. In recent years, lipids and specifically fatty acids have been the primary focus of larval nutrition research owing to their properties as highly efficient energy storage molecules as well as playing major roles in membrane structure and function, neurological development, pigmentation and various hormone systems.

The five major classes of lipids are fatty acids, triacylglycerols, phospholipids, sterols and sphingolipids (DeSilva and Anderson 1995). Fatty acids (FA) are carboxylic acids with long hydrocarbon side chains. Free fatty acids are rare in nature and can be more commonly found esterified into larger molecules such as triacylglycerols, wax esters and phospholipids. Wax esters and triacylglycerols are neutral lipids and are the most common molecular forms for storing metabolic energy in plants and animals. Polar lipids, such as phospholipids and sphingolipids, along with sterols (especially cholesterol) form the major constituents of all biological membranes. Certain fatty acids can be synthesized

de novo by finfish, including those with no double bonds (saturated) and one double bond (monounsaturated), and are considered to be non-essential. Conversely, several long chain highly unsaturated fatty acids (HUFAs) especially those with a 20 – 22 carbon chain length and with the first double bond occurring closer than the 9th carbon (ω9) from the methyl end, cannot be synthesized by most marine finfish and therefore must be provided in the diet (Olsen *et al.* 2004). These essential fatty acids (EFAs) include the most commonly studied ω3 and ω6 varieties, docosahexaenoic acid (DHA; 22:6ω3), eicosapentaenoic acid (EPA; 20:5ω3) and arachidonic acid (AA; 20:4ω6). In most marine food webs, ω3 fatty acids dominate over the ω6's by a factor of 5-20; however, the importance of AA has also recently been emphasized (Bell and Sargent 2003).

A similar pattern is found in embryonic and larval marine fish tissue, such that typically, the most abundant HUFA is of an ω3 type (Sargent *et al.*, 1989). In most species, levels of DHA increase during embryonic development and after the onset of exogenous feeding indicating a preferential sequestering of this ω3 fatty acid (for a general review see Izquierdo 1996, Izquierdo *et al.* 2000, for cod see Fraser *et al.* 1988). Moreover, during periods of starvation DHA, the ω6 HUFA AA, and in some species EPA are retained at the expense of other fatty acids in order to maintain essential cellular structure and biochemical functions (Izquierdo 1996, Rainuzzo *et al.* 1997, Izquierdo *et al.* 2000). On the other hand, when starved, some species such as the turbot (*Scophthalmus maximus*: Rainuzzo *et al.* 1994), the common dentex (*Dentex dentex*: Mourente *et al.* 1999), and the Senegal sole (*Solea senegalensis*: Mourente and Vásquez 1995) conserve

large amounts of AA at the expense of EPA, the ω3 HUFA of the same 20-carbon length. This highlights the ubiquitous importance of DHA, AA and to a certain extent EPA in the development of marine finfish larvae.

The successful rearing of cod larvae, as well as the larvae of other marine teleost species, requires these essential lipids to be provided in adequate amounts in the diet (Sargent *et al.* 1995, 2002). As previously stated, rotifers and brine shrimp (*Artemia*), the most commonly cultured live-food organisms for larval cod, are, unfortunately very poor in these HUFAs as well as other essential nutrients (Rainuzzo *et al.* 1989, Harel and Place 1999). However, relatively simple methods that take advantage of the filter-feeding behaviour of these organisms have been developed in order to incorporate specifically formulated particles into the rotifers and *Artemia* nauplii before feeding to larval fish (for a review of techniques see Leger *et al.* 1986). This process is known as live-food enrichment.

Enrichment of live feed with DHA results in increased growth, survival, and resistance to stress as well as a decreased incidence of deformities, albinism and improper eye migration in flatfish (Rainuzzo *et al.* 1997, Sargent *et al.* 1999, Izquierdo *et al.* 2000, Gapasin and Duray 2001). To date, no negative effects of dietary DHA enrichment have been reported in finfish larvae. In fact, the total amount of DHA that can be supplied to growing fish larvae is almost entirely constrained by our technical ability to enrich live-food organisms. *Artemia* and to a certain extent, rotifers, quickly metabolize DHA once

ingested and it is therefore difficult to deliver extremely high levels of dietary DHA to fish larvae (Rainuzzo *et al.* 1997). The supplementation of EPA in the diet of larval finfish is also known to improve growth and survival (for review see Izquierdo *et al.* 2000). Unlike DHA, however, there is a limit to the positive effects of EPA supplementation. The balance between DHA:EPA in the diet and tissues appears to be the most important factor since these two fatty acids compete for the same enzymes that catalyze the formation of membrane phospholipids. If there is too much EPA in the larval tissues, DHA is displaced from the very important sn-2 position of phospholipids, which causes a significant decrease in larval growth (Izquierdo *et al.* 2000) and survival (Takeuchi *et al.* 1992, Zheng *et al.* 1996). Recently, the importance of adequate amounts of AA in larval diets has been emphasized (Bell and Sargent 2003). Again, the ratio of AA, especially with regard to EPA, has to be considered since these two fatty acids also compete for enzymes and the subsequent production of eicosanoids. This concept is discussed in detail in the section 1.2.5 *Effect of Lipid Nutrition on Stress Response* below.

Many different commercial enrichment products are currently available and each varies in its total HUFA content as well as the ratio of certain fatty acids to others. Moreover, variations in enrichment protocols, including stocking density of live feed, water quality, and enrichment duration and concentration, can significantly impact the nutritional quality of the live feed, especially in the case of *Artemia* (Furuita *et al.* 1996, Navarro *et al.* 1999). Some research has been done on the characterization of specific HUFA ratios in live-feed enrichments that optimize larval growth and survival, however, the optimal

DHA:EPA:AA ratio appears to be species-specific, and the best clues have predominantly come from examining the ratio found in embryos and natural prey organisms of a given species (Sargent *et al.* 1999, Izquierdo *et al.* 2000, Bell and Sargent 2003, Robin and Peron 2004). The optimal ratio for sea bass has been found to be 2:1:1, while the optimal ratio for flatfish such as turbot and halibut is closer to 2:1:0.1 (Sargent *et al.* 1999). While the exact ratio has yet to be determined for larval Atlantic cod, the ratio found in cod eggs as well as the ratio in many wild copepods on which larval cod feed, are known. Cod eggs from the resident broodstock at the Ocean Sciences Centre's (Memorial University of Newfoundland) Aquaculture Research and Development (ARDF) facility were analyzed and the DHA:EPA:AA ratio was found to be approximately 2:1:0.1 (unpublished data). Similar DHA:EPA ratios have been found in various wild zooplankton species present in the North Atlantic Ocean; however, AA content tends to be considerably lower (Dehert 1999, Hamre *et al.* 2002). The relative importance of these three fatty acids in larval cod nutrition has yet to be completely described and more research is ongoing to determine the ratio that will maximize larval growth and survival.

1.2.4 *Stress*

Since the commercialization of cultured seafood products and the intensive rearing of large quantities of aquatic and marine animals, the concept of stress and its effects on product quality and quantity have been the focus of much research. Stress is an inevitable occurrence in both wild and hatchery/grow-out environments, and can be triggered by a

wide range of stimuli, from being chased by a predator to poor water quality. Daily culture practices including handling, grading, and transport as well as culture conditions such as crowding, accumulation of waste products, low oxygen and temperature fluctuations are all known to induce a stress response in fish (for review see Barton and Iwama 1991, Schreck and Li 1991, Barton 2002). The successful rearing of finfish thus requires the development of husbandry techniques that aim to minimize stress, especially during critical periods when the fish are most vulnerable to changes in homeostasis. The entire larval stage and especially metamorphosis are examples of such periods where high mortality is observed, potentially as a consequence of high stress levels.

Stress is a difficult concept to study since it is very difficult to define both accurately and specifically. Over the years there have been numerous definitions put forth, but none has been successful in covering all biochemical, physiological, behavioural and psychological aspects of a typical stress response as well as addressing the concept of an adaptive value (for review see Barton 1997). The most general and typically used definition in fish biology research is that stress is 'a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced' (Brett 1958). This definition, however, does not consider the importance of the immediate response to a stressor, which allows an animal to either cope with or escape from the stressful situation by diverting energy and resources away from nonessential processes to areas of the body that enable a "fight

or flight" reaction. Thus, the initial response to a short term or acute stressor is truly an adaptive response that gives an animal the best chances of survival (Barton 1997). Conversely, if the animal cannot escape and is subjected to the stress for a chronic period of time, suppression of growth, reproduction and the immune system occurs and permanent damage and/or mortality results. This aspect of the stress response is considered to be maladaptive (Barton and Iwama 1991).

Once a stressor is perceived, the stress response is triggered, and can be composed of several behavioural, cellular and physiological changes. Behaviours that are affected by stress include thermoregulation, chemoreception, orientation, swim performance, foraging, feeding, and predator avoidance (Schreck *et al.* 1997). Cellular changes can include changes in heat shock protein synthesis (Iwama *et al.* 1999) as well as decreases in cell and nucleus size (Barton and Iwama 1991). The physiological stress response is divided into a nervous pathway and a hormonal pathway given the mode of stimulation. In finfish, the nervous pathway involves the direct stimulation of chromaffin tissue in the head kidney by the hypothalamus and results in the release of catecholamines such as epinephrine. The catecholamines, in turn, stimulate the release of glucose from storage molecules and also act directly on the heart and gills/opercula increasing heart and ventilation rate. These effects occur within seconds of perceiving the stressor, in contrast to the hormonal branch (discussed further below), which involves signaling between a number of tissues and takes several minutes to appear. The various stress responses have also been classified in terms of the level of organization such that the primary response

involves chemical/hormonal changes, the secondary response involves changes at the cellular level, and the tertiary response includes "whole animal" effects (Barton and Iwama 1991). The bulk of research on stress has predominantly focused on measuring easily quantifiable physiological parameters within the primary and secondary responses. Recently, however, there has been an increase in the number of studies measuring responses at the "whole animal" level including metabolic rate, disease resistance and swimming capacity (e.g. Sloman *et al.* 2000, Iguchi *et al.* 2003, Herbert and Steffensen 2005, Lankford *et al.* 2005).

The most commonly quantified physiological indicators of the stress response in finfish have been the corticosteroid hormones, of which cortisol is the most predominant. An increase in plasma or whole body corticosteroid concentration following the application of a number of different stressors has been well documented (for review on plasma levels see Barton and Iwama 1991, for whole body concentrations: Barry *et al.* 1995, Stephens *et al.* 1997a,b, Sakakura *et al.* 1998). The release of corticosteroids is a primary hormonal response, which occurs following the immediate release of catecholamines via the nervous pathway. In the hormonal pathway, once the fish perceives the stressor, the hypothalamus secretes corticotrophin-releasing hormone, which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which, in turn, is released into the circulatory system. When the ACTH reaches the kidney, corticosteroids are released from interrenal cells. This pathway is more commonly known as the hypothalamus-pituitary-interrenal (HPI) axis.

The effect of circulating corticosteroids is mediated by the presence of cytosolic glucocorticoid receptors (GR) in target tissue cells. Receptor activation occurs when GR binds to its ligand cortisol. These ligand-receptor complexes can then move into the nucleus and regulate the expression of several glucocorticoid-responsive genes (Charmandari *et al.* 2005, Mommsen *et al.* 1999). This process can initiate numerous tertiary stress responses, which go on to affect growth, behaviour, immune system function, and reproduction (Mommsen *et al.* 1999, Barton and Iwama 1991).

1.2.5 *Effect of Lipid Nutrition on Stress Response*

In general, fatty acids play vital roles in membrane structure and function, the regulation of metabolism and the growth of new tissue, especially the eye, brain and nervous system. Certain EFAs, including DHA, EPA and AA, are also thought to be directly involved in the stress response of fish. EPA and AA are precursors for paracrine hormones such as prostaglandins (PG), leukotrienes, thromboxanes, hydroxyeicosatetraenoic acids and epoxytetraenoic acids, which are collectively known as eicosanoids (Lands 1991). The AA-derived eicosanoid, prostaglandin (PGE_2) is thought to directly stimulate the release of cortisol, through the activation of the HPI axis (Gupta *et al.* 1985). The less biologically active EPA-derived prostaglandin (PGE_3) is thought to regulate the production of PGE_2 by the active competition between EPA and AA for the cyclooxygenase (COX) enzymes that convert these HUFAs into PGs (Lands 1991). As a result, the ratio of EPA:AA in tissue is most likely involved in the proper regulation of cortisol at rest and during a stress response. AA-derived leukotrienes are

also known to stimulate the production of ACTH, which is the pituitary hormone directly responsible for the release of cortisol via the HPI axis (Hirai *et al.* 1985, Barton and Iwama 1991). Although DHA and docosanoids are not known to act directly on the HPI axis, they are still thought to play a role in the modulation of the kinetics of the stress response in fish (Harel *et al.* 2001).

This is where nutrition plays a critical role, since, unlike amino acids and proteins, the fatty acid composition of membrane phospholipids is not significantly controlled by enzymes (genetically determined), and therefore the fatty acid ratios in membrane and storage lipids will directly reflect the fatty acid profile of the diet (DeSilva and Anderson 1995, Olsen *et al.* 2004). Consequently, the ratio of fatty acids, and especially EFAs, is just as important as the specific amounts put into a diet. An overabundance or a paucity of one fatty acid could have a considerable impact on a variety of vital processes, one of which being the stress response.

Early studies on the effect of lipid nutrition on the post-stress survival of larval fish focused on DHA and EPA supplementation of artificial feed. High DHA levels increased the overall survival of larval red sea bream (*Pagrus major*) as well as promoted post-stress survival after an acute 30 s air exposure or decreased dissolved oxygen levels (Kanazawa 1997). For Japanese flounder (*Paralichthys olivaceus*) larvae, Tago *et al.* (1999) also found that high levels of both DHA and EPA significantly improved survival

after exposure to various chronic stressors such as increasing water temperature and decreasing dissolved oxygen concentration.

In terms of current studies on the effect of live-feed enrichment on the stress response of larval fish, the focus has switched to overall DHA:EPA:AA ratios and the measurement of whole-body cortisol levels. High dietary DHA was found to increase post-hypersalinity stress survival better than high dietary AA in larval striped bass (*Morone saxatilis*), but maximal survivorship was observed in the dietary treatment containing high levels of DHA and midlevels of AA (Harel *et al.* 2001). A similar result was seen in gilthead seabream larvae, such that those fed a diet high in both DHA and AA had better survival following an acute air exposure compared with larvae fed a diet high in DHA only (Koven *et al.* 2001). If the supplementation of AA in the diet occurs after the stress, however, differences in survival are not seen (Koven *et al.* 2001). These results clearly indicate that the presence of AA in larval diets is essential for dealing with acute stressors, but that there is an apparent threshold over which the effects of AA supplementation become negative. In contrast, with respect to a chronic stress, such as a change in salinity, increased dietary AA was not found to increase survival in gilthead seabream larvae (Koven *et al.* 2003).

In terms of cortisol regulation, dietary supplementation of AA prior to an acute air exposure stress significantly reduces post-stress cortisol levels in gilthead seabream larvae (Van Anholt *et al.* 2004). Progressive increases in levels of AA in the diet,

however, cause increasing basal cortisol levels in both unstressed and chronically stressed larvae of the same species (Harel *et al.* 2001, Koven *et al.* 2003). This suggests that a specific amount of AA is required in the diet and thus also in cellular membranes in order for the HPI axis to function appropriately and to respond rapidly to a stressor. An increase in cortisol post-stress is not prevented, but the return to homeostasis appears to be more rapid and thus the negative and mal-adaptive effects of long-term cortisol exposure are mitigated. The ratio of AA to EPA in the diet and cellular makeup is still the most critical aspect, such that the presence of large quantities of AA causes the activation of the HPI axis and the release of cortisol during periods of no stress. As a result, EPA must be present in the diet and the tissues at a specific concentration in order to regulate the production of AA–derived eicosanoids. Simple increases in dietary DHA are also known to modulate basal cortisol levels, however, they cannot prevent the increase in cortisol observed following a stress (Harel *et al.* 2001).

1.2.6 *Summary*

As the global population continues to grow, consistent sources of inexpensive dietary protein will become essential. In this regard, the progressive decline of wild fish stocks has opened the door for the expansion of the aquaculture industry and the development of new species for commercialization, including cold-water marine finfish. The successful and economically viable culture of Atlantic cod is possible, and is currently underway in Norway. The abundance of knowledge regarding the biology of this species has facilitated its commercialization; however, the production of large quantities of juvenile

fish for sea-cage stocking is still limited by a bottleneck in survival that occurs during the larval phase. Recent emphasis on larval research has improved the quality and quantity of juveniles being produced in intensive hatcheries in Norway, Scotland, Canada and the USA. Nevertheless, an understanding of the specific dietary requirements of larval cod is still incomplete, which in turn, has hindered the creation of a species-specific diet for this critical stage of development. Lipids, and more specifically HUFAs are essential for cellular membrane structure and function, neurological development, pigmentation, and many hormone systems, one of which is the release of cortisol via the HPI axis in response to stress. Consequently, this thesis is focused on linking the effect of dietary lipids to these biological processes with the aim of determining the optimal fatty acid content in live-feed enrichments that will maximize larval growth, survival and stress tolerance.

2 Ontogeny of the corticosteroid stress response in Atlantic cod and the validation of an enzyme-linked immunoassay (ELISA) for measuring corticosteroids in whole-body larval samples.

2.1 Abstract

The culture of Atlantic cod shows great potential, but expansion of current operations is hindered by a production bottleneck during the larval stage. Unfortunately much is still unknown about the physiology, including the stress response, of larvae during this critical phase. In the present study, the ontogeny of the corticosteroid stress response of Atlantic cod larvae was characterized by measuring the resting and stress-induced immunoreactive corticosteroid (IRC) concentrations in pooled samples at various stages of development with a focus on early growth and metamorphosis. A commercially available enzyme-linked immunoassay kit was validated for measuring IRC levels in extracted whole-body homogenates of Atlantic cod larvae. Measurable IRC was present in embryos at 2 days pre-hatch. Resting IRC levels showed only small fluctuations throughout development with slight increases occurring at 13 and 50 days post-hatch (dph) corresponding to complete yolk-sac absorption and the onset of metamorphosis, respectively. A significant corticosteroid response to an acute air exposure stress was observed at hatch (day 0) and likely indicates the functioning of the hypothalamus pituitary interrenal axis (HPI) and thus the endogenous production of cortisol by the larvae at this time. Although the larvae appeared to be responding to an acute stress as early as at hatch, there seemed to be a stress hyporesponsive period (SHRP) up to 30 dph during which the magnitude of the stress response was very small. Whole-body IRC levels in stressed larvae did not exceed 6.6 ng g^{-1} larval wet weight for the duration of

this period. High levels of circulating corticosteroids during this SHRP may have negative impacts on early neurological development. A second SHRP was observed at 50 dph, and likely corresponds to a critical point in metamorphosis when high levels of cortisol may have negative impacts on the development of the immune system.

2.2 Introduction

The Atlantic cod is an important commercial fish species for many countries surrounding the North Atlantic and Arctic oceans. Declining wild stocks in these areas and a complete moratorium on all commercial Atlantic cod fishing in Newfoundland, Canada, has put increased emphasis on the development of a viable cod aquaculture industry in recent years. Countries such as Norway, Iceland, Scotland, Canada and the United States have ongoing programs of research and development with the goal of maximizing the profitability of the fledgling industry. A major bottleneck in production occurs during the hatchery phase when mortality is high in early larval stages and throughout metamorphosis into the juvenile form. As a result, increased attention has been placed on understanding many of the biological processes that occur during this critical period.

Intensive hatchery rearing typically exposes young fish to multiple acute and chronic stressors including tank cleaning, grading, vaccination, crowding and changes in water quality (Barton and Iwama 1991, Schreck and Li 1991). One of the many physiological responses of fish to stress is an increase in the circulating levels of corticosteroids, the most common of which is cortisol (Barton 2002). In the short-term, the stress response is considered to be adaptive such that it allows the fish to cope with the stressful situation by maintaining its homeostatic state (Barton 2002). Repeated and/or prolonged exposure to a stress, however, can have severely negative consequences for growth, metabolic scope, reproductive capacity and immunocompetence and can ultimately result in death (Barton 1997). The cortisol stress response of cold-water marine finfish larvae is

considerably difficult to measure owing to their small size, and as a result, no studies were carried out on larval Altantic cod until very recently. King and Berlinsky (2006) were the first to measure corticosteroids in pooled samples of cod larvae using a radioimmunoassay (RIA) technique. These authors observed a measurable corticosteroid response to stress in larvae at 8 days post-hatch (dph). As of yet, changes in circulating corticosteroids in resting and stressed Atlantic cod larvae have not been measured during embryological development or metamorphosis.

The traditional method of hormone detection, including cortisol, in tissue and plasma samples is RIA. The majority of studies on larval teleosts, in which cortisol was measured in whole-body homogenates, have used variations of the RIA procedure validated for different ages and species (e.g. Takahashi *et al.* 1985, Young 1986, Pickering and Pottinger 1987, Brown *et al.* 1989, King and Berlinsky 2006). In recent years, however, there has been a shift toward faster methods of detection that do not involve the use of radioactive materials, one of which is the enzyme-linked immunoassay (ELISA). Barry *et al.* (1993) were able to validate an ELISA procedure for the detection of cortisol in the serum of rainbow (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*). Since then, however, very few studies have used an ELISA for cortisol quantification, especially in larval research when whole-body homogenates are generally required due to an extremely small blood volume. Nevertheless, these few studies were successful in validating the use of an ELISA for cortisol detection in a range of species including rainbow trout (Barry *et al.* 1995), tilapia (*Oreochromis mossambicus*: Rodgers

et al. 2003, Wu *et al.* 2005), white tail shiner (*Cyprinella galactura*: Sutherland 2003) and zebreafish (*Danio rerio*: Alsop and Vijayan 2008) larvae.

The principal objective of this study was to produce a detailed description of the ontogeny of the cortisol stress response in Atlantic cod larvae, with a focus on early development as well as metamorphosis. The present study also aimed to validate the use of a commercial ELISA kit for the quantification of cortisol in larval Atlantic cod.

2.3 Methods

2.3.1 *Cod Larvae*

A single batch of fertilized cod eggs was obtained from captive broodstock held at the Ocean Sciences Centre (Memorial University of Newfoundland, St. John's, Newfoundland, Canada) during the Spring/Summer (ambient) 2006 spawning event. The eggs were disinfected and incubated in a 250 L conical tank with natural seawater at a temperature of 5 - 6°C. Once >90% of the eggs had hatched (~76 degree days [dd]), the yolk-sac larvae were transferred into eight 250 L conical tanks at a density of 50 larvae L^{-1}. Water temperature at time of tank stocking was 8.2°C. Over the following three days, water temperature was increased by 2°C until an average temperature of 10°C was achieved. Water temperature throughout the remainder of the experiment ranged from 9.6 – 11.3°C. The inflow of water was initially set at 1 L min^{-1} and was gradually increased to 2 L min^{-1} throughout the course of the rearing period. A 24-h photoperiod was used in order to maximize growth and survival (Puvenendran and Brown 2002). Light intensity at the water surface ranged from 2000 lux during greenwater use (1-12 dph, see below) to 300 lux through weaning.

Larvae were fed rotifers (*Brachionus plicatilis*) enriched with AlgaMac 2000® (Aquafauna Bio-Marine Inc., Hawthorne, California, USA) three times daily. For the first twelve days following stocking, 2 L of live algal (*Nannochloropsis* sp.) culture was added to each tank in addition to the rotifer feedings. At 38 dph, the brine shrimp, *Artemia* sp., enriched with AlgaMac 3050® (Aquafauna Bio-Marine Inc., Hawthorne,

California, USA), was introduced to the larval diet at four feedings per day along with the rotifer feedings. The proportion of *Artemia* in the diet increased over the next 8 days, at which point rotifers were no longer provided. The larvae were then co-fed *Artemia* and particulate dry food (Gemma Micro, Skretting, Norway) for 4 days before the number of *Artemia* feedings was reduced to three, marking the beginning of weaning. Subsequently, every three days another feeding of *Artemia* was eliminated and replaced with dry food. At 59 dph the larvae were completely weaned.

2.3.2 *Stress Test and Sampling*

The development of the corticosteroid response to stress was characterized by subjecting groups of larvae to an acute stressor at predetermined age-intervals, which corresponded to changes in the rearing protocol (Table 2.1). Sampling began at 09:00 h, before the first daily live-food feeding, and remaining larvae were not fed until after sampling was complete. On each sampling day, control (resting) corticosteroid samples were taken from each tank by quickly netting several larvae and immediately euthanizing them with an overdose concentration (400 mg L^{-1}) of tricaine methane sulphonate (MS-222, Syndell Laboratories, Qualicum Beach, British Columbia, Canada). Once all larvae were immobilized (<30 s), they were removed, rinsed with sterilized seawater, placed in pre-weighed 2 mL cryovials and immediately frozen in liquid nitrogen. Following this, a second group of larvae from the same tank was netted, held out of the water for 30 s, and then gently released into a 5 L recovery tank. Larvae were re-netted and sampled, as

Table 2.1. Stress test sampling intervals and corresponding larval age (days post-hatch and degree days post-fertilization* or -hatch) as well as changes in rearing protocol and estimated changes in larval morphology.

Sampling number	Age (dph)	Age (dd)	Protocol	Morphology
1	-2	64.9*	Pre-hatch	Eyed egg
2	0	76.9*/0	Pre-transfer	Hatch
3	2	16.4	Pre-feeding	Open mouth but pre-exogenous feeding[1]
4	13	106.3	End of algae supplementation	Inflation of swim bladder and yolk sac completely absorbed[1]
5	30	280.1	Mid-rotifer feeding	Fin fold narrowing and fin differentiation started[1]
6	39	374.1	Introduction of *Artemia* to diet	
7	47	456.9	End of rotifers	
8	50	488.6	Start of weaning	Beginning of metamorphosis[2]
9	59	583.7	End of weaning	End of metamorphosis[2]

[1]From Hunt von Herbing *et al.* (1996). [2] From Pedersen and Falk-Petersen (1992).

described for the control fish, 1 h following the air exposure stress. The minimum required sample size for cortisol analysis was 50 mg wet weight (ww), and thus the number of larvae sampled at each interval decreased as fish grew, such that at hatch >100

larvae were taken per sample and 1-2 larvae were sufficient at the end of weaning. All samples were stored at –80°C until further analysis.

2.3.3 *Cortisol Extraction and ELISA*

The cortisol concentration of each larval sample was quantified using an ELISA kit (Neogen Corp., Detroit, Michigan, USA). Prior to analysis, extraction of the samples was carried out using a procedure modified from Hiroi *et al.* (1997). Briefly, samples were thawed on ice, weighed and aliquots of ~50 mg *ww* of pooled larvae were transferred into 1.5 mL Eppendorf tubes. Ice-cold phosphate-buffered saline (PBS: 0.1M PBS + 140 nM NaCl, pH 7.6) was added to each pooled sample to obtain a final tissue concentration of 50 mg *ww* mL^{-1} of PBS. The samples were then sonicated (Branson Sonifier S250A; Branson Ultrasonics Corp. Danbury, Connecticut, USA) on ice until completely liquefied, centrifuged and the supernatant pipetted into a clean Eppendorf tube. Aliquots of 250 µL from each sample were placed into 12 x 75 mm glass culture tubes, and double extracted with ether. Each extraction involved the addition of 1.5 mL of ether to the sample followed by 30 s of vigorous vortexing. The tubes were immersed in a dry ice-methanol bath for a further 30 s and then the ether layer was quickly and gently decanted into a fresh glass tube. Once this procedure was repeated, and the ether layers pooled, the tubes containing the extracted steroid dissolved in ether were evaporated under a gentle stream of nitrogen. Extracts were then resuspended in 150 µL or 300 µL of extraction buffer depending on the anticipated concentration of cortisol in the sample. Samples of

stressed and/or older (>30 dph) larvae were resuspended in the larger volume of extraction buffer since they typically contained higher amounts of cortisol.

The ELISA test procedures were performed as per the manufacturer's instructions. In short, serial dilutions of a 1 μg mL^{-1} cortisol standard (in methanol) were used for the production of a standard curve with seven data points (0.04, 0.1, 0.2, 0.4, 1, 2 and 10 ng mL^{-1}). Duplicate 50 μL aliquots of these standards and the extracted samples were added to an antibody (rabbit anti-cortisol) coated plate. A pair of wells on each plate remained blank in order to calculate maximum binding (see below). Using a repeater pipette, 50 μL of enzyme conjugate (cortisol horseradish peroxidase) was rapidly added to each well of the microplate. The plate was covered with plastic film, shaken gently and incubated in the dark at a constant temperature of 30°C for 1 h. Following incubation, the contents of the microplate were discarded and each well washed three times using a diluted wash buffer. Once more, a repeater pipette was used to rapidly add 150 μL of substrate (stabilized 3, 3', 5, 5' tetramethylbenzidine, TMB plus hydrogen peroxide, H_2O_2) to each well. The microplate was then re-covered, shaken and placed in the dark at 30°C for 30 min. After the second incubation, the plastic film was removed, and the bottom of the microplate was polished using a lint-free towel. The plate was shaken, and read on a microplate reader (DTX-880, Beckman Coulter Inc., Fullerton, California, USA) at a wavelength of 620 nm.

The two wells on each plate that received no addition of standard or sample were used to determine maximum binding (B) of the enzyme conjugate in the absence of free cortisol. The average reading from these two wells was considered to be the "zero standard" (B_0) and was used to calculate the percent of maximal binding ($\%B_x\ B_0^{-1}$) for each standard and sample absorbance (B_x). A standard dose-response curve was drawn by plotting the $\%B\ B_0^{-1}$ for each standard against the known concentrations of cortisol and then fitting the curve using a four parameter sigmoid equation (SigmaPlot 8, Systat Software Inc., San Jose, California, USA). The R^2 values for all standard curves were >90%.

2.3.4 *Validation of Extraction and ELISA*

Extraction efficiency was determined by spiking larval samples, which had undetectable cortisol levels (previously determined through ELISA procedure), with one of 5, 2, 1, 0.5 or 0.2 ng mL^{-1} concentrations of cortisol standard. Each concentration of standard was tested in at least two different larval samples. The spiked samples were homogenized, extracted and analyzed using the same procedures described above for all ontogenetic samples. The extraction procedure was also validated by analyzing serial dilutions of whole-body homogenates and comparing the differences in binding with the standard curve. Intra-assay variation was measured by analyzing the same extracted sample five times in a single microplate run and then calculating the coefficient of variation (CV) of the resulting cortisol concentrations. Interassay variation was also measured, in this case by analyzing the same extracted sample in three different microplate runs and then calculating the CV of the resulting cortisol concentrations.

2.3.5 *Statistical Analysis*

Prior to analysis, outliers were identified and removed from the data set using a Dean and Dixon test. A two-way analysis of variance (ANOVA) with time and stress treatment as explanatory variables was performed on the IRC concentration data. The interaction term was found to be highly significant ($F_{8,114}$, $p < 0.001$) and thus conclusions on the separate effects of time and stress treatment could not be made. Subsequently, a series of two-sample t-tests, between stressed and control values, were performed at each time interval. All data sets were tested for the assumptions of normality, heterogeneity and independence of residuals. When a data set was found to violate these assumptions and the resulting p-value of the t-test was approaching α, a randomization test of the data was performed using 300 iterations.

A one-way ANOVA was performed on the series of control (resting) IRC concentrations to determine the effect of sampling interval/age. Slight deviations in normality and independence of residuals was observed, however, replication number was large ($n = 64$) so the test was continued. Significant differences between intervals were identified using a Tukey-Kramer multiple comparison test.

A one-way ANOVA was also carried out on the data for the magnitude of the corticosteroid response, which was calculated as the ratio of each stress value to its corresponding control value. The coefficients of variation of the ratios were calculated

using the propagation of errors equation for the quotient of two measured values such that:

$$CV\ (x/y) \approx \sqrt{[CV(x)^2 + CV(y)^2]}, \text{ where}$$

$CV\ (x/y)$ is the Coefficient of Variation of the resulting ratio,

$CV(x)$ is the Coefficient of Variation of the stress corticosteroid values, and

$CV(y)$ is the Coefficient of Variation of the control corticosteroid values.

Significant differences between intervals were identified using a Tukey-Kramer multiple comparison test.

The one-way ANOVA comparing the magnitude of the corticosteroid stress response was performed using SigmaStat Version 2.03 (SPSS Inc., Chicago, Illinois, USA). All other analyses were carried out using Minitab 13.1 (Minitab Inc., State College, Pennsylvania, USA). A significance level of 5% ($\alpha = 0.05$) was used for all statistical tests. All data presented are in the form mean ± standard error (SE).

2.4 Results

2.4.1 *ELISA Validation*

Extraction efficiency, measured by the average recovery of exogenous cortisol from spiked whole-body homogenate samples, was 56.2 ± 2.39% (n = 14). A linear regression line fitted through the individual data points of 5, 2, 1, 0.5 and 0.2 ng mL^{-1} of spiked cortisol concentrations resulted in an R^2 value of 0.99 (Fig. 2.1). The equation of the regression line was y = 0.0259 + 0.52 * x, and the value for the intercept (0.0259) was not significantly different from 0 (t = 0.1592, p = 0.88). Values obtained from larval samples were not corrected for extraction efficiency. A typical dose-response cortisol standard curve generated using the ELISA procedure is shown in Fig. 2.2. Serial dilutions of extracted whole-body homogenates from larvae at two age classes (30 and 50 dph) exhibited binding parallel to the standard curve and all dilutions fell within the linear portion of the curve (Fig. 2.2).

The precision and reproducibility of the ELISA were evaluated by calculating the intra-assay and interassay variation of certain larval samples with a range of corticosteroid values. The intra-assay variation was found to differ depending on the concentration of corticosteroid contained within the sample. Samples with high concentrations, 0.93 ± 0.041 and 1.9 ± 0.089 ng mL^{-1} of corticosteroid had intra-assay CVs of 9.8 and 10.1%, respectively, in contrast to samples with lower concentrations, 0.49 ± 0.039 and 0.062 ± 0.0089 ng mL^{-1}, which had intra-assay CVs of 17.7 and 32.2%, respectively (n = 5).

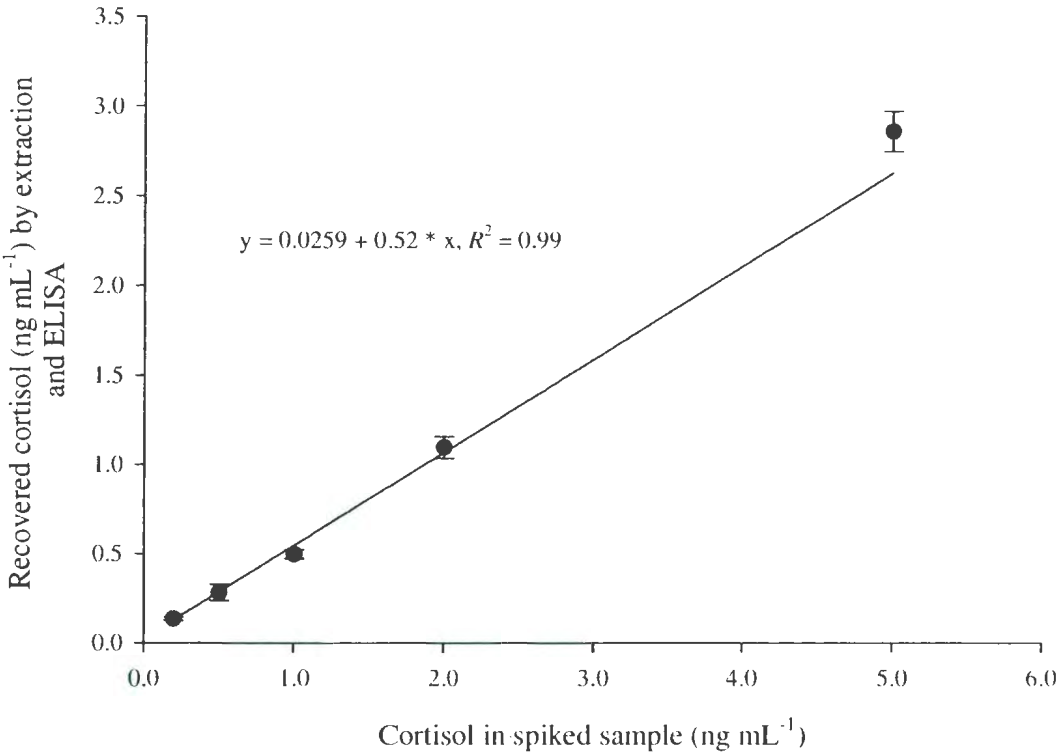


Figure 2.1. Regression of exogenous cortisol-spiked samples against recovery of cortisol from the same samples as measured by extraction and ELISA procedures Values are mean ± SE (n = 3 except for 0.2ng mL^{-1} where n = 2).

Interassay variation, on the other hand, showed less variability between coticosteroid concentrations, such that samples containing 0.32 ± 0.028 and 0.70 ± 0.057 ng mL^{-1} of corticosteroid had interassay CVs of 15.2 and 14.1%, respectively (n = 3).

The cross-reactivity of the anti-cortisol antibody with other steroids has been previously determined by the manufacturer (Neogen Corp., Detroit, Michigan, USA). Steroids potentially found in teleost samples, and which have cross-reactivities of greater than 0.1% include cortisone (15.77%), 11-deoxycortisol (15.00%), corticosterone (4.81%), 6β-

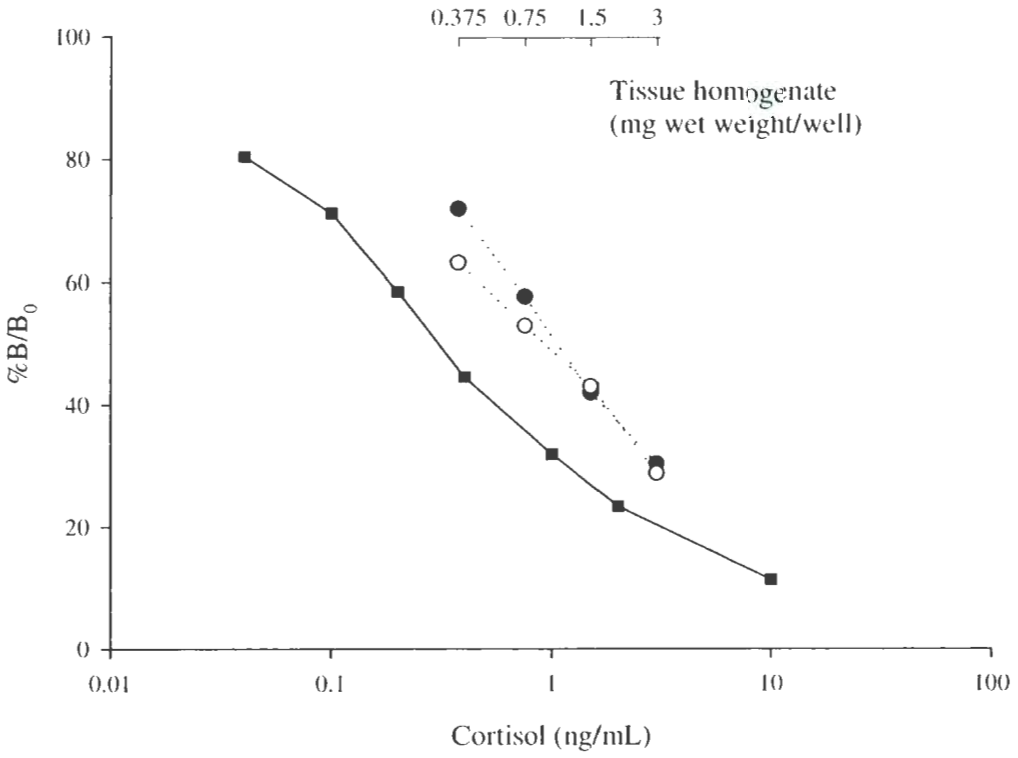


Figure 2.2. Dose-response curves of cortisol standards (■) and serial dilutions of whole-body homogenate extracts from larval Atlantic cod 30 (●) and 50 (○) days post-hatch. Each point represents the mean of duplicate measurements (n = 2). B = absorbance reading of standard or sample. B_0 = absorbance of zero standard.

hydroxycortisol (1.37%), 17-hydroxyprogesterone (1.36%), and deoxycorticosterone (0.94%). It is important to note however, that these cross-reactivities were determined for human samples, and therefore may not be directly applicable to teleost samples.

2.4.2 *Ontogeny*

Small amounts (1.3 ± 0.66 ng g^{-1} *ww*) of immunoreactive corticosteroids (IRC) were detected in cod embryos two days prior to hatching (Fig. 2.3). At hatch, resting IRC levels remained similarly low. A significant rise in resting IRC levels was not seen until

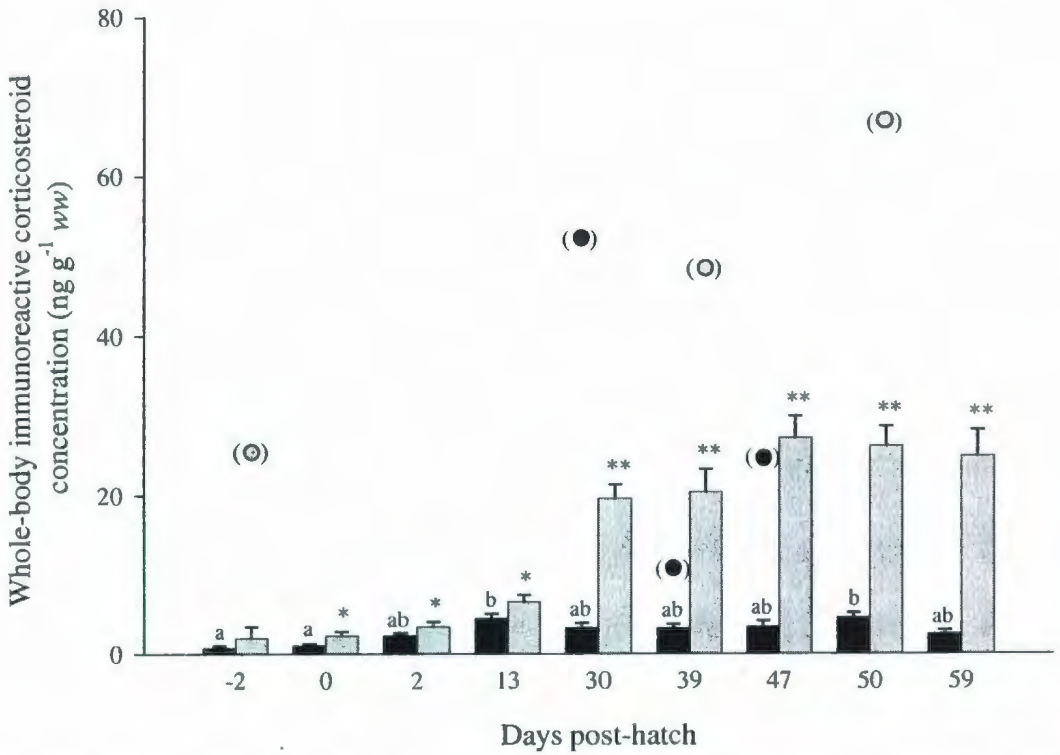


Figure 2.3. Effect of age on whole-body immunoreactive corticosteroid concentrations of Atlantic cod larvae before and 1 h following a 30 s air exposure stress. ■ = control; ■ = stressed. * or ** denotes significant difference between control and stressed value at same age interval at $p<0.05$ or $p<0.001$ respectively; means of control values with different letters were found to be significantly different ($p<0.05$). Values are mean ± SE (n = 5-8). (●) = outlier in control group; (●) = outlier in stressed group.

13 dph, corresponding to the end of live algae supplementation, at which point whole-body IRC levels were 4.5 ± 0.6 ng g^{-1} *ww* ($p < 0.05$). Between 30 and 47 dph, resting IRC levels remained fairly constant around 3.3 ng g^{-1} *ww*. At 50 dph, which marked the start of weaning, a second increase in resting IRC levels to 4.6 ± 0.59 ng g^{-1} *ww* occurred, but was only significantly different from pre-hatch and at hatch resting levels ($p < 0.05$). By the end of the experimental period, at 59 dph, resting IRC levels in the early juveniles was 2.5 ± 0.48 ng mg^{-1} *ww* (Fig. 2.3).

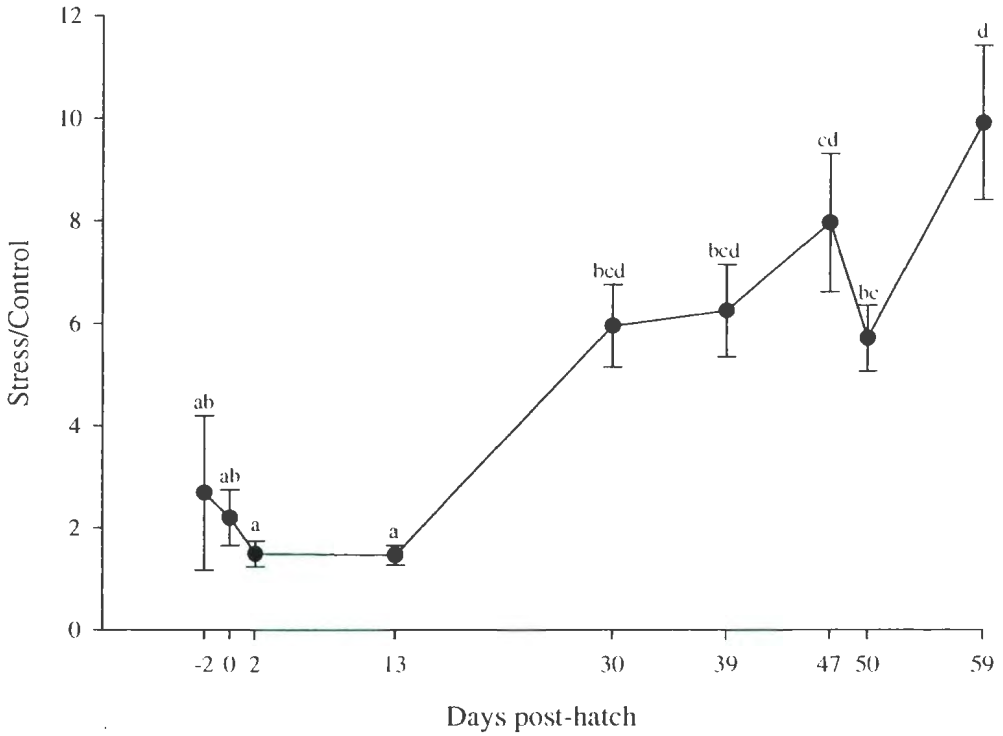


Figure 2.4. Magnitude of the immunoreactive corticosteroid stress response of Atlantic cod larvae at different ages expressed as the ratio of whole-body corticosteroid concentration of stressed *vs* control fish. Means with different letter superscripts are significantly different. Values are mean ± SE (n = 11-16).

Significant differences in IRC levels between control and stressed fish was observed as early as at hatch ($p < 0.001$, n = 12, Fig. 2.3). At 2 and 13 dph, significant increases in IRC levels of greater than 50% occurred 1 h post-stress ($p = 0.013$ and < 0.001 respectively, n = 16). Beginning with 30-day old larvae, major increases in post-stress IRC levels were seen. At 30 and 39 dph there was a 6-fold increase in IRC, while at 47 and 59 dph, IRC levels were 8 and 10 times higher than pre-stress levels, respectively ($p < 0.001$, n = 14-16, Fig. 2.4). It is interesting to note, however, that at 50 dph, the magnitude of the response decreased by 2 ratio units compared to 47 dph values (Fig.

2.4). Peaks in whole-body IRC concentration were between 19.6 ± 1.8 and 31.4 ± 5.5 ng g^{-1} ww.

2.5 Discussion

There are many advantages to using ELISAs over RIAs for steroid analysis, including faster analysis time, less consumable supplies, and no radioactive health hazards or disposal issues (Barry *et al.* 1993). To date, an ELISA has yet to be used for the determination of whole-body corticosteroid levels in larval Atlantic cod. The present study, however, was successful in validating the use of such a technique for this commercially important species.

ELISAs are extremely sensitive and as a result, intra-assay and interassay variation can be relatively high during early trials as researchers are becoming familiar with the procedure (Barry *et al.* 1993, Sutherland 2003). The intra-assay CVs of approximately 9-10% for concentrations of IRC greater than 1 ng mL^{-1} obtained in this study are consistent with other intra-assay CVs reported for both ELISA and RIA procedures using extracted whole-body samples (e.g. Stephens *et al.* 1997: 10.8%; Feist and Schreck 2002: 10%; Sutherland 2003: <10%). For samples with less than 1 ng mL^{-1}, and especially with less than 0.49 ng mL^{-1} IRC, the intra-assay variation was considerably higher. High lipid concentrations in samples are known to interfere with steroid RIAs (Rash *et al.* 1979, 1980) and ELISAs (Selby 1999). The source of this interference may be the formation of micelles (liposomes) by phospholipids and other amphipathic lipids in aqueous solution, within which non-polar steroids such as cortisol are trapped and effectively removed from the assay (Rash *et al.* 1980). Prior to analysis (using either RIA or ELISA), steroids are extracted using an organic solvent (such as diethyl ether in the present study), which

also results in the extraction of lipids from the sample. The solvent is removed by evaporation, and then the extract is re-suspended in an aqueous buffer. For samples with a high lipid content, such as whole-body homogenates, this procedure would cause the formation of many liposomes, and as a result, produce spurious cortisols values when read from a typical standard curve (Rash *et al.* 1980). The effect of lipaemia is not only dependent upon lipid content, but also steroid concentration. The negative bias caused by liposome formation becomes more pronounced as steroid concentration decreases (Rash *et al.* 1980); potentially resulting in a higher variability of assay results in samples with low steroid concentrations. The general increase in intra-assay CV with decreasing IRC concentrations seen in the present study may, at least partially, be explained by this phenomenon. Interassay variation can vary from ~5% (e.g. Jentoft *et al.* 2002) up to ~16% (e.g. de Jesus *et al.* 1991, Stephens *et al.* 1997). The interassay CVs of 14-15% obtained in this study are well within this range.

Regardless of the assay type, whole-body samples typically require homogenization and steroid extraction prior to analysis in order to eliminate cross-reactivity of non-cortisol molecules with the antibody. As a result, extraction efficiency must also be tested and verified in order to make quantitative comparisons between studies. Due to cortisol's high lipid solubility, large proportions of organic solvents such as diethyl ether and ethyl acetate ranging from 1:5 (homogenate:solvent, Hwang *et al.* 1992) up to 1:12 (de Jesus *et al.* 1991) are predominantly used for extraction. In order to be assayed, however, the solvent must first be evaporated, and the steroid precipitate dissolved and re-suspended in

an aqueous buffer. Unfortunately, cortisol is only slightly soluble in water (Budavari 1989). Consequently, the care and effort to which the steroid precipitate is resuspended will determine the amount of cortisol recovered from the sample and detected with the assay. As a result, extraction efficiencies, measured as the percent of exogenous cortisol recovered from a spiked sample, can range from the mid-60's (e.g. Hwang *et al.* 1992, Feist and Schreck 2002) to high 90's (e.g. Pottinger and Mosuwe 1994, Rogers *et al.* 2003). The extraction efficiency of 56% achieved in this study is unquestionably at the low end of the spectrum, and may be a result of the low homogenate:ether ratio of 1:6, or the effect of liposome formation (discussed above). Nevertheless, this low extraction efficiency was found to be consistent across a wide range of cortisol values, and thus qualitative comparisons remain valid. The IRC concentrations of between 1.0 – 2.3 ng g^{-1} *ww* for hatchlings measured in this study, when doubled, are comparable to those obtained by King and Berlinsky (2006) of ~5 ng g^{-1} *ww* for day 0 Atlantic cod using an RIA. As a result, quantitative comparisons are also possible when IRC values from this study are increased approximately two-fold.

One of the most widely used indicators of stress in fish is plasma cortisol levels (Wendelaar-Bonga 1997). Unfortunately, for most cold-water marine teleost species, including Atlantic cod, plasma extraction from larval and early juvenile stages is not feasible due to extremely small body size and/or an undeveloped circulatory system. Consequently, homogenates of whole-body or whole-tissue are used. In this case, steroids from all tissue compartments, not just the blood, are extracted and can potentially

contribute to the total immunoreactivity measured in an assay (Pottinger *et al.* 1992, Pottinger and Mosuwe 1994). Steroids, such as cortisol, are known to be removed from the body in part through the bile (Truscott 1979) and possibly accumulate within the liver or gallbladder at this time (Pottinger *et al.* 1992). As a result, whole-body IRC concentrations have the potential to vary significantly from circulating plasma cortisol levels. In the short term following a stress, however, IRC and circulating plasma cortisol levels in young rainbow trout (Pottinger and Mosuwe 1994) and juvenile Atlantic cod (King and Berlinsky 2006) showed similar dynamics of change for at least 1 h and up to 8 h post-stress. Furthermore, no difference in IRC levels was found in juvenile cod with intact or excised livers and gallbladders up to 6 h following a stress, suggesting that it takes longer than 6 h for biliary free IRC to accumulate (King and Berlinsky 2006). In this study, whole-body samples from stressed fish were collected just 1 h following an acute stress, and, therefore, it is safe to assume that most of the whole-body cortisol is in circulation and the changes seen are an accurate reflection of the stress response. It is important to note that the specific mechanism of cortisol metabolism in larval cod is not yet known; however, it most likely varies considerably from that of juvenile cod, and to make a definite assertion whether whole-body IRC concentrations are an accurate expression of the stress response, further research into elucidating this mechanism in larvae is required. Nevertheless, the range of resting whole-body IRC levels (1-5 ng g^{-1} *ww*) observed for larval Atlantic cod is comparable to the resting plasma cortisol levels found by Pérez-Casanova *et al.* (in press) for 10 g juvenile Atlantic cod (< 10 ng mL^{-1}) and Afonso *et al.* (2007) for another juvenile gadid, haddock (*Melanogrammus*

aeglefinus: < 5 ng mL^{-1}). These studies used similar assay protocols and identical ELISA kits as were used in the present study.

Cortisol (or IRC) has been found in the eggs of many teleost species including Japanese flounder (*Paralichthys olivaceus*: de Jesus *et al.* 1991), tilapia (Hwang *et al.* 1992), chum salmon (*Oncorhynchus keta*: de Jesus and Hirano 1992), rainbow trout (Pottinger and Mosuwe 1994, Barry *et al.* 1995a), Asian seabass (*Lates calcarifer*: Sampath-Kumar *et al.* 1995), chinook salmon (*Oncorhynchus tshawytscha*: Feist and Schreck 2002), yellow perch (*Perca flavescens*: Jentoft *et al.* 2002), and gilthead seabream (*Sparus aurata*: Szisch *et al.* 2005). It is presumed that this cortisol is maternally derived, and levels typically decline during development as the embryo metabolizes the hormone. However, an increase in IRC levels in eyed rainbow trout eggs was seen at 3 weeks prior to hatch, indicating that these embryos are capable of synthesizing (or converting) these steroids even before hatching (Pottinger and Mosuwe 1994). A similar increase was seen in the embryos of chum salmon 2 days prior to hatch suggesting that pre-hatch cortisol might not be exclusively of maternal origin in this species (de Jesus and Hirano 1992). In this study, it is unclear whether the IRC found in cod embryos 2 days prior to hatch is of maternal origin, embryological origin or both, and more studies are needed on the dynamics of steroids in Atlantic cod eggs from fertilization to hatch.

In most species, resting or basal cortisol levels increase around the time of hatching, which likely indicates the endogenous production of the hormone by the larvae (Hwang

et al. 1992, Barry *et al.* 1995a, Sampath-Kumar *et al.* 1995, Feist and Schreck 2002). In this study, a significant rise in basal IRC levels was not seen until 13 dph; however, an increase was observed in response to a 30 s air exposure stress at hatch. Although the progression of interrenal cell development in Atlantic cod larvae is not yet known, this increase in IRC likely indicates that corticosteroidogenesis is occurring at this time. The endogenous production of cortisol was seen in rainbow trout larvae even before the appearance of differentiated interrenal cells in the head kidney, and it was suggested that the enzymes necessary for cortisol biosynthesis are present even in the undifferentiated interrenal cells (Barry *et al.* 1995a). Further *in vitro* studies revealed that these undifferentiated interrenal cells are indeed capable of producing cortisol in response to stimulation with adrenocorticotrophic hormone (ACTH: Barry *et al.* 1995b). Additionally, studies on Asian seabass have revealed that larvae of this species have an intact HPI axis and the ability to synthesize cortisol *de novo* as early as 4 h post-hatch (Sampath-Kumar *et al.* 1997).

Cortisol in teleosts functions as both a glucocorticoid and mineralocorticoid affecting both metabolism, through gluconeogenesis, as well as osmoregulation. It has been suggested that a functioning HPI axis in teleosts or the analogous HPA (hypothalamus pituitary adrenal) axis in mammals, and thus the production of cortisol, in vertebrates is essential for maintaining glucose homeostasis during periods of fasting including the transition from an endogenous energy supply to exogenous feeding, which occurs during yolk-sac absorption in fish and parturition in mammals (Arai and Widmaier 1991, Barry

et al. 1995a). Consistent increases in basal IRC levels at the onset of first-feeding and/or complete yolk-sac absorption have been seen in chum salmon (de Jesus and Hirano 1992), rainbow trout (Barry *et al.* 1995a), yellowtail (*Seriola quinqueradiata*: Sakakura *et al.* 1998), silver seabream (*Sparus sarba*: Deane and Woo 2003), and gilthead seabream (Szisch *et al.* 2005) larvae. For Atlantic cod larvae reared at 10°C, which is comparable to the range of temperatures in the present study, complete yolk-sac depletion generally occurs around 10-17 dph (Hunt von Herbing *et al.* 1996). Consequently, the rise in resting IRC levels in cod larvae at 13 dph in the current study was presumably the result of a requirement for gluconeogenesis stimulated by a decrease in the endogenous food supply.

In terms of osmoregulation and the preservation of the hydro-mineral balance, cortisol is known to regulate the activity of the $Na^+ - K^+$ ATPase ion pump mechanisms in mitochondria-rich (MR) cells distributed over the larval epidermis and yolk-sac. High levels of cortisol were shown to increase the abundance and size of these MR cells in tilapia larvae (Ayson *et al.* 1995). A rise in activity of $Na^+ - K^+$ ATPase during the early development of silver seabream was also attributed to increased endogenous production of cortisol by the larvae (Deane and Woo 2003). At hatch, chloride cells, which are one of the two types of described MR cells, are present in the epidermis of cod larvae (Morrison 1993). It is possible that during the first two weeks of development, the observed increase in basal cortisol levels also plays a role in the stimulation of epidermal chloride cells and thus larval osmoregulation. Further studies on the ontogeny of chloride

cells and the activity of the $Na^+ - K^+$ ATPase ionic pump during early larval development in Atlantic cod are needed.

In addition to its key roles in regulating metabolism and the hydro-mineral balance, cortisol also plays a third and vital role in larval development, by acting in conjunction with thyroid hormones during the transition from larva to juvenile, a phase commonly referred to as metamorphosis. In species studied to date, basal cortisol levels have been found to increase gradually during early metamorphosis and reach a peak at metamorphic climax (de Jesus *et al.* 1991, Tanaka *et al.* 1995, Sakakura *et al.* 1997, Deane and Woo 2003, Szisch *et al.* 2005). Cortisol may also play a role in Atlantic cod metamorphosis given that a second increase in basal IRC levels was observed in the current study at 50 dph. Based on size data collected in Chapter 3, cod larvae at 50 dph have a total length of ~15 mm, which corresponds to the typical size at the start of metamorphosis (Pedersen and Falk-Petersen 1992). Since metamorphosis can involve changes to all aspects of an organism including its biochemistry, physiology, morphology and behaviour, it is typically accompanied by increased energy demands (Youson 1988). Consequently, the effects of cortisol on lipid and protein metabolism may be critical for cod metamorphosis in terms of the appearance of large numbers of chloride cells on the surface of well-developed gill lamellae (Morrison 1993) as well as the escalation of cannibalistic behaviour, which is highest during metamorphosis (Folkvord 1997). In the current study, it is unclear as to what specific point during metamorphosis the increase in IRC at 50 dph occurs, but it is interesting to note that the magnitude of the fluctuations in basal cortisol

levels are miniscule when compared to the increases of greater than 10-fold seen in Japanese flounder (de Jesus *et al.* 1991), plaice (Tanaka *et al.* 1995), silver sea bream (Deane and Woo 2003) and gilthead sea bream (Szisch *et al.* 2005) at metamorphosis. A study involving the detailed correlation of morphological landmarks during metamorphosis and IRC concentrations in Atlantic cod should be undertaken.

The transient increases in basal IRC levels at 13 and 50 dph also coincided with changes to the feeding protocol, which were the end of algae supplementation and the onset of weaning. In the absence of algae, light intensity increases throughout the water column, which has been shown to elicit a cortisol stress response in post-smolt Atlantic salmon (*Salmo salar*: Migaud *et al.* 2007); however, decreasing surface light intensity mitigates this effect. In the present study, light intensity on the tank surface was lowered to 1000 lux 4 h before cortisol sampling, and it is therefore unlikely that this change in lighting was the cause of the observed increase in basal IRC. The onset of weaning is marked by the removal of one daily *Artemia* feeding and its replacement with multiple feedings of an inert particulate diet. It is possible that larvae in the current experiment were stressed as a result of not recognizing the particulate matter as prey and ingesting no feed for 6 h; however, cortisol sampling occurred 24 h following the first *Artemia* replacement during which time the larvae received three full *Artemia* feedings. Overall, fluctuations in basal IRC did not appear to be directly influenced by adjustment of the feeding protocol, and were more likely caused by the changes in physiology discussed above.

Although fluctuations in basal IRC levels for Atlantic cod larvae were found to be relatively small and a significant rise was not seen until 13 dph, stress-induced cortisol production was observed as early as at hatch. In a previous study on Atlantic cod, a similar trend was observed in hatchlings, although the difference was not found to be statistically significant (King and Berlinksy 2006). In many of the teleost species studied to date, an endogenous production of cortisol in response to a stressor is not seen until 1-2 weeks post-hatch. An earlier response, however, could be possible in some species but was not detected in previous studies as a result of a long interval (5-7 days) between hatch and the first post-hatch sample (e.g. Szich *et al.* 2005, Pepels and Balm 2004, Deane and Woo 2003, Feist and Schreck 2002). Regardless of sampling interval, some salmonid species were found to have a prolonged lag in the expression of a cortisol stress response from hatch until at least 2 weeks (Barry *et al.* 1995a) and as late as 5 weeks post-hatch (Pottinger and Mosuwe 1994). Barry *et al.* (1995a) compared this post-hatch stress hyporesponsive period (SHRP) with a similar SHRP seen in some rodents after birth and which is attributed to a need for maintaining low levels of corticosteroids during a critical period of development when these hormones can have permanent deleterious effects on the growth and development of the central nervous system (Sapolsky and Meaney 1986). While a significant increase in IRC levels was observed in Atlantic cod larvae at hatch in the current study, the magnitude of the response was low, and large post-stress increases in IRC were not seen until 30 dph. This 2-3 week post-hatch period during which stress responsiveness is low was also seen in yellow perch larvae (Jentoft *et al.* 2002) and may potentially be explained by the presence of a SHRP.

During the development of larval Atlantic cod, a second period of lower corticosteroid stress response magnitude was found at 50 dph. As previously discussed, basal IRC levels were found to be elevated at this age, however, a correspondingly larger increase in post-stress IRC was not observed. The sampling intervals before and after this age, at 47 and 59 dph, display an increase in the magnitude of the IRC stress response; however, it suddenly decreased at 50 dph. Thus, it appears that perhaps a second SHRP occurs during the early stages of metamorphosis. Feist and Schreck (2002) found a similar decrease in the magnitude of the cortisol stress response in young chinook salmon at 4 weeks post-hatch. Since high levels of cortisol are known to have a deleterious effect on immune system function (Maule *et al.* 1986), they suggested that the proper development of the salmonid immune system at this age requires consistently low levels of circulating corticosteroids as well as a decrease in the ability to produce them during stress. In Atlantic cod, the lymphoid organs develop gradually during the transition to the juvenile stage and do not become fully functional until after the end of metamorphosis (Schroeder *et al.* 1998). The overall immune system is not considered to be wholly competent until 2-3 months post-hatch (Schroeder *et al.* 1998, Magnadottir *et al.* 2004). As a result, the potential developmental events occurring around the time of metamorphosis in cod that would require a down-regulation of the corticosteroid stress response could be the initiation and/or early proliferation of the immune system.

The maximum whole-body IRC values of ~30 ng g^{-1} *ww* in stressed cod larvae between 47 and 59 dph are considerably lower than the plasma cortisol concentrations of acutely

stressed juvenile Atlantic cod (~195 ng mL^{-1}: Pérez-Casanova *et al.* in press). This six-fold disparity may be a factor of the difference between whole-body and plasma samples. The concentration of cortisol in whole-body samples is expressed relative to the weight of the entire larvae while plasma concentrations are expressed relative to the volume of a specific bodily fluid in which cortisol is found. This causes the absolute value of the whole-body concentration to appear smaller. Increases in larval weight over time may have a similar effect on whole-body IRC concentrations between larvae of different ages. To allow for comparisons, corticosteroid values are standardized against larval weight; however, as larvae grow the cell and tissue types determining larval weight do not go unchanged. The differentiation of organ systems and the ossification of bones cause the factors influencing the weight of older larvae to be vastly different from newly hatched larvae. As a result, corticosteroid levels in older larvae are standardized against an inflated larval weight and appear lower than if all values were standardized against an unchanging factor such as number of larvae. Unfortunately, the small size of young larvae precludes the counting of individual larvae in early samples. The development of a very sensitive assay for low cortisol concentrations; however, would decrease the number of larvae required per sample and make the standardization against larval number possible.

In summary, it is possible to use commercially available ELISA kits for the determination of immunoreactive corticosteroid levels in homogenates of larval Atlantic cod. Extra attention should be paid to steroid extraction as well as the precision of analytical

techniques. Small amounts of corticosteroids are present in cod embryos prior to hatch, but the origin of this corticosteroid could not be confirmed in this study. Basal IRC levels increase at two points during larval development, firstly at the time of yolk-sac absorption and then again at the onset of metamorphosis. Endogenous corticosteroidogenesis in response to an acute stress occurs as early as at hatch, and becomes clearly evident at 30 dph. It is possible that a stress hyporesponsive period is present for 2 weeks following hatch, during which time larvae are only able to produce small amounts of corticosteroids in order to avoid negative impacts on early neurological development. A secondary SHRP at the onset of metamorphosis may also be present in order to allow for the proper development of the immune system. Given that the ability of cod larvae to adequately respond to external stressors through the production of corticosteroids is reduced during certain critical periods of early development, potentially stressful aquaculture protocols such as changing light regime/intensity and grading should be avoided at these times.

3 The effect of differentially enriched live food on growth, lipid composition and response to handling stress in hatchery reared Atlantic cod larvae.

3.1 Abstract

Although great progress has been made in recent years regarding the specific dietary requirements of larval Atlantic cod under intensive hatchery conditions, much is still unknown about the impact of varying ratios of highly unsaturated fatty acids (HUFAs) on vital physiological processes including the stress response. In the present study, three different live-food enrichment protocols, using a variety of commercially available enrichment products, were tested for their effect on growth and stress resistance. The protocols tested included the enrichment of rotifers and *Artemia* with: (1) AquaGrow® Advantage, (2) Selco® products, and (3) a combination of AlgaMac® products and *Pavlova* sp. algae paste. Differential enrichment affected the lipid and fatty acid composition of rotifers, *Artemia* and the developing larvae. Larvae from the AlgaMac+*Pavlova* treatment had a consistently smaller percentage of eicosapentaenoic acid (EPA) and larger percentages of arachidonic (AA), docosahexaenoic acid (DHA) and docosapentaenoic (ω6DPA) acids compared with larvae from other treatments. Differences in growth parameters were seen as early as 7 days post-hatch (dph). Larvae fed with AlgaMac+*Pavlova* enriched live feed were significantly bigger than larvae in both other treatments, a trend that persisted throughout the rearing period and into metamorphosis and weaning. When challenged with an acute 15 s air exposure and tank transfer stress, 41 dph larvae in the AlgaMac+*Pavlova* treatment had maximal post-stress survivorship. A second air exposure stress at 48 dph, revealed the kinetics of post-stress

corticosteroid release. Larvae in all treatments responded to the second stress with a rapid increase in whole body corticosteroid concentration. By 2 h post-stress, larvae in the Advantage and Selco treatments exhibited decreasing corticosteroid levels, while the level in the AlgaMac+*Pavlova* larvae remained elevated. In addition, these larvae appeared to have an increased sensitivity toward repeated sampling resulting in a transient and variable corticosteroid increase in the unstressed group. These results suggest that an elevated proportion of AA in the diet sensitizes and prolongs the corticosteroid response of larval cod to an acute stress; however, this prolonged response is not associated with increased mortality. These findings also emphasize the importance of supplying high dietary DHA, AA and ω6DPA during the early stages of development in order to promote future larval and juvenile performance.

3.2 Introduction

In recent years, the intensive production of Atlantic cod has become the focus of much attention, due, primarily, to an ongoing decline in wild stocks alongside an increase in demand and price for fresh fish on the world market (Svåsand *et al.* 2004). Unfortunately, expansion of the industry has been hampered by high production costs and a lack of juvenile fish on account of a large bottleneck in survival during the larval phase (Svåsand *et al.* 2004). One of the reasons for this high mortality is an incomplete understanding of the specific dietary needs of larval cod at this early stage in development, especially for highly unsaturated fatty acids (HUFAs). The importance of two HUFAs, namely, docosahexaenoic acid (DHA; 22:6ω3), and eicosapentaenoic acid (EPA; 20:5ω3), in the nutrition of fish has long been recognized (Sargent *et al.* 1989). Arachidonic acid (AA; 20:4ω6), on the other hand, has only recently been established as an essential fatty acid (EFA) for marine teleosts (Izquierdo *et al.* 2000, Bell and Sargent 2003). There exists an abundance of literature concerning the effects of DHA, EPA and AA on larval development. These studies have highlighted several significant roles of these three fatty acids in membrane structure and function, neurological development (Furuita *et al.* 1998), pigmentation (Estévez *et al.* 1999, Villalta *et al.* 2004), physical deformities (Gapasin *et al.* 1998, Gapasin and Duray 2001) as well as various hormone systems, including the release of the stress hormone cortisol via the hypothalamus-pituitary interrenal axis (HPI: Harel *et al.* 2001, Koven *et al.* 2003).

The extremely small size and undifferentiated digestive system of larval Atlantic cod necessitates the use of live food, typically rotifers, *Brachionus plicatilis*, and the brine shrimp *Artemia* sp., during the hatchery phase. Although these two organisms are relatively inexpensive and readily available in large quantities, they are nutritionally deficient, especially in HUFAs, when compared to the wild copepods on which cod larvae would naturally be feeding (Rainuzzo *et al.* 1989, Dehert 1999, Hamre *et al.* 2002). Consequently, rotifers and *Artemia* are enriched with some type of emulsion, typically high in both lipid and protein, before being fed to the larvae. There is currently a wide range of commercially available enrichment products, and each differs in its total lipid content as well as fatty acid composition. Insight into the specific dietary needs of cod during the larval phase will determine the choice of enrichment product to maximize both growth and survival. Recently, several studies have focused on elucidating the specific nutritional needs of larval cod for lipids and EFAs during rotifer and *Artemia* feeding (Zheng *et al.* 1996, Cutts *et al.* 2006, O'Brien MacDonald *et al.* 2006, Park *et al.* 2006, Garcia *et al.* 2007a,b). Results from these studies point toward a high dietary requirement for DHA and AA during the early stages of development. Garcia *et al.* (2007b) also found a positive correlation between larval size and tissue content of another ω6 fatty acid: docosapentaenoic acid (ω6DPA). Currently, ω6DPA is not considered to be an EFA for marine fish, however, mounting evidence to the contrary has emerged in recent years (see Parrish *et al.* 2007).

The dietary requirement for a single specific HUFA cannot meaningfully be considered in isolation due to the competitive interactions known to occur between these molecules, their precursors and end products (Sargent *et al.* 1999). One of the most recognized biochemical interactions is the competition between EPA and AA for eicosanoid biosynthesis. Eicosanoid is a generic term for a family of biologically active derivatives of C_{20} PUFAs (eicosapolyenoic acids), and includes, amongst others, prostaglandins (PG), thromboxanes and leukotrienes (Mustafa and Srivastava 1989). These hormone-like compounds are produced by nearly every tissue in the body, and, as stated by Sargent *et al.* (1999) in general terms, are produced in response to stressful situations, both at a cellular and whole body level. In fish, as in mammals, AA is the preferred precursor for eicosanoid synthesis; however, EPA is also readily converted into its own discrete suite of eicosanoids, which are typically less biologically active (Tocher 2003). Moreover, *in vitro* studies on gill homogenates and brain astroglial cells of juvenile turbot (*Scophthalmus maximus*) have shown that an increased cellular concentration of EPA competitively inhibits the formation of PGs from AA (Henderson *et al.* 1985, Bell *et al.* 1994). At a whole body level, dietary supplementation with ω3 HUFAs, also significantly reduced the production of AA derived eicosanoids in Atlantic salmon smolts (*Salmo salar*: Bell *et al.* 1993). The stimulus for eicosanoid biosynthesis is typically independent of nutritional status; however, the diet influences the relative amounts of EPA and AA in cellular membranes, and thus the pool of available substrates. In this way, the fatty acid composition of the diet has a considerable effect on the rate and intensity of the AA-derived eicosanoid response, once a stimulus is perceived (Lands 1991).

Prostaglandins, including those derived from AA, are known to be involved in the release of cortisol through the activation of the HPI axis in fish (Gupta *et al.* 1985, Van Anholt *et al.* 2003). Further studies of mammalian systems have also shown the direct involvement of EPA and AA-derived eicosanoids in the modulation of glucocorticoid release from the analogous hypothalamus-pituitary-adrenal (HPA) axis. As a result, the ratio of EPA:AA in tissue is most likely involved in the proper regulation of cortisol at rest and during a stress response. Numerous studies on different species and developmental stages of larval teleosts have shown that dietary supplementation with AA results in better survival following the application of an acute stress (Furuita *et al.* 1998, Koven *et al.* 2001, Harel *et al.* 2001, Koven *et al.* 2003, Willey *et al.* 2003). For gilthead sea bream larvae (*Sparus aurata*), this better survival may be associated with a concurrent attenuation of the cortisol response following the stress event (Van Anholt *et al.* 2004). The effects of increased dietary AA on cortisol following a stress event are, however, varied and its beneficial character seems to be related not only to the extent of AA supplementation and developmental stage of the larvae but also to the type and duration of the stress (Harel *et al.* 2001, Koven *et al.* 2003, Van Anholt *et al.* 2004).

To date and to the best of my knowledge, no studies have quantified the effects of different dietary HUFA ratios on the stress response of larval Atlantic cod. Therefore, the objectives of the present study were to evaluate the effect of different commercially available live-food enrichment products on the growth performance and stress resistance

of Atlantic cod larvae reared under intensive conditions, and to assess the effect of dietary and tissue DHA/EPA/AA on the release of cortisol following an acute air exposure stress using whole-body corticosteroid samples.

3.3 Methods

3.3.1 *Larviculture and Feeding*

Fertilized cod eggs were obtained from captive broodstock kept at the Ocean Sciences Centre (Memorial University of Newfoundland, St. John's, Newfoundland, Canada) during the Spring/Summer 2005 spawning event. The eggs were disinfected and then incubated in 250 L conical tanks at a temperature of 6°C until >90% had hatched (~95 degree days [dd]). At this point, the yolk-sac larvae from four incubators were pooled and transferred to six 3000 L first-feeding tanks at a density of 50 larvae L^{-1}. Constant aeration was provided by multiple airstones. Water flow was increased periodically from 2.0 L min^{-1} to a maximum of 14.5 L^{-1} during the six week rearing phase. Light intensity was kept at 2000 lux for the first twelve days after which it was gradually decreased to 600 lux (Monk *et al.* 2006). A 24-h light photoperiod was used throughout the duration of the experiment to maximize growth and survival (Puvenendran and Brown 2002). Water temperature ranged from 9.3 – 12.9°C.

From day 0 to day 12, 100 L of algae (*Nannochloropsis* sp.) was steadily siphoned into each tank in order to maintain an absorbance of 0.02 (measured at 630 nm wavelength). Starting at 3 days post-hatch (dph), 20 mL of Instant Algae Nanno 3600® (Reed Mariculture, Campbell California, USA) paste was added to each tank at 1:00 a.m. in order to maintain a high density of algal cells during the overnight hours. Larvae were fed enriched rotifers (*Brachionus plicatilis*) three times daily to maintain a prey density of 333 rotifers L^{-1}, which increased up to 5333 rotifers L^{-1} during the first 21 dph. Before

feeding, prey density from each individual tank was determined, and additional rotifers were then added to achieve the desired prey density (data not shown). Prey density was re-sampled 10 min after rotifers were fed to each tank, and any deficiencies in the prey density were supplemented.

At 27 dph (~289 dd), before the addition of *Artemia* nauplii to the diet, larval density was homogenized among the six tanks. An estimate of the initial density was achieved by slowly lowering the water level in each tank until approximately 1000 L remained, after which ten random 1 L samples were taken and the larvae counted in each. An average number of larvae per litre was calculated and used to estimate the number of larvae in the entire tank (data not shown). The tank with the least number of larvae was used as the target density. Larvae were removed from the five remaining tanks based on their calculated numbers of larvae L^{-1} until the target density was reached. The target tank received the same treatment as the other five tanks with the exception that no larvae were removed.

Immediately following the equalization of the tank densities, approximately one-quarter of the diet was replaced with enriched *Artemia* nauplii. The proportion of *Artemia* in the diet increased over the following 6 days, after which rotifers were no longer provided, and continued to increase until an *Artemia* prey-density of 2500 L^{-1} was reached. *Artemia* were fed to the larvae four times daily using the same prey density calculation and supplementation procedure described above for rotifers.

At 41 dph, larvae that were not transferred to experimental glass aquaria (Fig. 3.1) but remained in the 3000 L rearing tanks were gradually weaned onto an inert particulate diet (Gemma Micro, Skretting, Norway). The number of daily *Artemia* feedings was immediately reduced to three. Every following third day another feeding of *Artemia* was eliminated and replaced with dry food. At 50 dph the larvae were completely weaned.

3.3.2 *Growth*

Growth, in terms of standard length and myotome height, was measured every 80 dd (~7 days) using a microscope fitted with a digital camera (Pixera Professional, Pixera Corp., San Jose, California, USA). Digital images were subsequently analysed using Matrox Inspector Version 3.0 (Matrox Electronic Systems Inc., Dorval, Quebec, Canada). Condition factor and length specific growth rate were calculated as per Puvanendran and Brown (1999):

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

Length Specific Growth Rate = $[\mathrm{Ln}(SLt_2) - \mathrm{Ln}(SLt_1) / t_2\text{-}t_1]$, where

SLt_2 is the Standard Length (mm) at time interval 2,

SLt_1 is the Standard Length (mm) at time interval 1, and

$t_2\text{-}t_1$ is the difference (days) between the two time intervals.

Standard length, myotome height and condition factor of fully weaned juveniles at 50 dph (~564 dd) from the original 6 treatment tanks were also assessed using the methods described above.

3.3.3 *Live-food Enrichment*

Enrichment products Aquagrow® Advantage (Advanced Bionutrition Corp., Columbia, Maryland, USA), Protein Selco Plus®/Gadi Selco® (INVE Aquaculture, Dendermonde, Belgium,), AlgaMac 2000®/3050® (Aquafauna Bio-Marine Inc., Hawthorne, California, USA,) and Instant Algae® (*Pavlova* sp. algae paste, Reed Mariculture, Campbell California, USA) were used alone or in combination to enrich rotifers and/or *Artemia* for a total of 3 different dietary treatments. The three treatments tested were (1) AquaGrow® Advantage for both rotifers and *Artemia* (hereafter referred to as Advantage), (2) Protein Selco Plus® for rotifers and Gadi Selco® for *Artemia* (hereafter referred to as Selco) and (3) AlgaMac 2000® + Instant Algae® for rotifers and AlgaMac 3050® + Instant Algae® for *Artemia* (hereafter referred as AlgaMac +*Pavlova*). Each dietary treatment was tested in duplicate.

Rotifers were cultured in 3000 L tanks with an initial stocking density of approximately 150 mL^{-1}. Rotifers were fed Culture Selco 3000® (INVE Aquaculture, Dendermonde, Belgium) for three days after which the rotifers were washed, concentrated and placed in 300 L conical enrichment tanks at a density of 500,000 L^{-1}. For treatment 1, rotifers were enriched with 50 mg L^{-1} of Advantage at 01:00 and 05:00 h. Rotifers for treatment 2 were enriched with 125 mg L^{-1} of Selco at 03:00 and 06:00 h. Finally, for treatment 3, 150 mg L^{-1} of AlgaMac was added to the rotifers at 21:00 and 03:00 h, as well as 1.5 mL L^{-1} of *Pavlova* sp. at 03:00 h. Enriched rotifers were harvested at 09:00 h, concentrated,

rinsed and put into 20 L buckets. Approximately 25 L of ambient seawater (~4°C) was then added to the rotifers in order to lower their temperature and prevent them from settling on the bottom of the larval tanks.

Artemia cysts (Embryon®, INVE Aquaculture, Dendermonde, Belgium) were hydrated decapsulated and hatched as per the manufacturer's instructions. The hatched nauplii were placed in 300 L conical enrichment tanks with aerated seawater at 30°C. For treatment 1, *Artemia* were stocked at a density of 200,000 nauplii L^{-1} and enriched with 300 mg L^{-1} of Advantage at 17:00 and 01:00 h. *Artemia* for treatment 2 were also stocked at a density of 200,000 nauplii L^{-1} and enriched with 100 mg L^{-1} of Selco at 13:00 and 23:00 h. The enrichment of *Artemia* for treatment 3 involved a stocking density of 100,000 nauplii L^{-1} and the addition of 200 mg L^{-1} of AlgaMac at 21:00 and 03:00 h along with 0.75 mL L^{-1} of *Pavlova* sp. also at 03:00 h. Enriched *Artemia* were harvested, concentrated, and rinsed at 09:00 h. Approximately 25 L of ambient seawater (~4°C) was then used to dilute the *Artemia* before feeding to the larvae.

Rotifers and *Artemia* for afternoon and evening feedings were stored in insulated coolers supplied with multiple air stones and ice packs in order to maintain a temperature below 7°C and minimize nutritional loss.

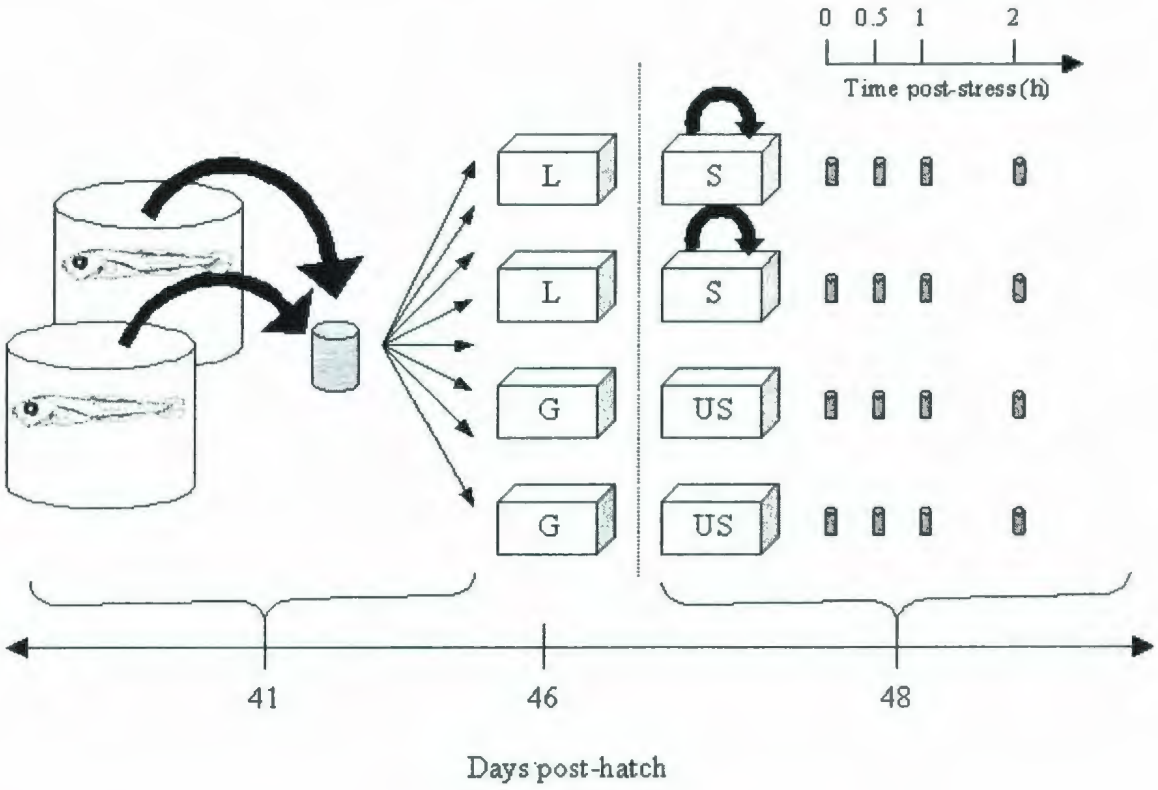


Figure 3.1. Diagrammatic representation of the experimental set-up and sampling protocol for a single dietary treatment (see Methods section for full description). Groups of 41 day-old cod larvae reared in duplicate 3000 L treatment tanks were subjected to a 15 s air-exposure stress, pooled into a bucket (represented by the light grey cylinder), and used to stock eight 37 L glass aquaria (represented by the rectangular prisms). Post-stress survival was evaluated 24 and 48 h following transfer. At 46 dph, larvae from 2 aquaria (*L*) were sampled for lipid content and larvae from another 2 aquaria (*G*) were measured for growth parameters. Larvae in the remaining 4 aquaria (*S* and *US*) were used for corticosteroid sampling (represented by the small dark grey cylinders) at 48 dph. Time zero (0) samples were taken from all 4 aquaria, and then larvae from 2 aquaria (*S*) were given a 15 s air exposure (represented by the curved black arrows). Repeated samples were taken from stressed (*S*) aquaria at 0.5, 1 and 2 h post-stress and unstressed (*US*) aquaria at 0.5, 1 and 2 h post-time zero sampling. *NB* not to scale.

3.3.4 *Acute Air-exposure and Transfer Stress*

A diagrammatic representation of the experimental set-up and sampling protocol is given in Fig. 3.1. At 41 dph (~441 dd), eight days after starting an *Artemia*-only diet, random groups of larvae were netted from each tank and held out of the water for 15 s before

being placed in a bucket of clean seawater. Stressed larvae from the duplicate tanks in each dietary treatment were pooled into one bucket and used to stock eight 37 L (10 gallon) glass aquaria at a density of 13 larvae L^{-1}. Inside each aquarium was a large black plastic box with a mesh (500 μm pore size) bottom and side inserts. Larvae continued to be fed *Artemia* from their respective dietary treatment four times daily at a prey density of 2500 *Artemia* L^{-1}. Post-transfer survival was evaluated every 24 h by removing and counting all dead larvae from each aquarium. Disturbance to the remaining larvae was minimized by using a small siphon to remove mortalities from the bottom of the aquaria.

At 7 days post-transfer (48 dph, ~524 dd), 2 aquaria from each dietary treatment (6 aquaria total) were subjected to a second 15 s air exposure by lifting the black mesh box, containing the larvae, out of the aquarium. A further 2 aquaria from each treatment were not subjected to the additional air exposure stress. These aquaria served as controls and were used to assess the effects of repeated sampling. After this point none of the larval aquaria were given any food. Larvae in the remaining 4 tanks were used for growth and lipid sampling.

3.3.5 *Corticosteroid Sampling and Analysis*

Before the application of the second air exposure stress, groups of larvae from four aquaria of each treatment (2 tanks to be stressed and 2 unstressed aquaria) were quickly netted and placed into an anaesthetic bath containing 400 mg L^{-1} tricaine methane sulphonate (MS-222, Syndel Laboratories, Qualicum Beach, British Columbia, Canada).

Immobilized larvae were divided into three groups of 20, rinsed with sterilized seawater, placed into 2 mL cryovials and immediately flash frozen by immersion into liquid nitrogen. The entire process from netting to freezing took less than 1 min. Directly following the procurement of the time zero (0) samples, the two stressed aquaria from each treatment were subjected to the second 15 s air exposure. Samples were then taken from the stressed aquaria using the same procedure outlined above at 30 min, 1 h, and 2 h post-stress. Concurrent samples from the control aquaria were taken at identical time intervals to determine any effect of repeated sampling. All cortisol samples were stored at –80°C until the time of analysis.

Whole body corticosteroid concentration of the larval samples was quantified using an enzyme-linked immunoassay (ELISA) kit (Neogen Corp., Detroit, Michigan, USA). Prior to analysis, samples were thawed on ice, weighed and aliquots of >100 mg wet weight (*ww*) of pooled larvae (3-4 larvae) were transferred into 1.5 mL Eppendorf tubes. Ice-cold phosphate-buffered saline (PBS: 0.1 M PBS + 140 nM NaCl, pH 7.6) was added to each pooled sample in order to obtain a final tissue concentration of 50 mg *ww* mL^{-1}. The samples were then sonicated (Branson Sonifier S250A; Branson Ultrasonics Corp. Danbury, Connecticut, USA) on ice until completely liquefied, centrifuged and the supernatant pipetted into a clean Eppendorf tube. Aliquots of 250 µL from each sample were placed in 12 x 75 mm glass culture tubes, in duplicate, and double extracted with ether. Each extraction involved the addition of 1.5 mL of ether to the sample followed by 30 s of vigorous vortexing. The tubes were immersed in a dry ice-methanol bath for a

further 30 s and then the ether layer was quickly and gently decanted into a fresh glass tube. Once this procedure was repeated, and the ether layers pooled, the tubes containing the extracted steroid dissolved in ether were evaporated under a gentle stream of nitrogen. Extracts were then resuspended in 300 µL of extraction buffer.

The ELISA test procedures were as per the manufacturer's instructions. In short, serial dilutions of a 1 µg mL^{-1} cortisol standard (in methanol) were used for the production of a standard curve with seven data points (0.04, 0.1, 0.2, 0.4, 1, 2 and 10 ng mL^{-1}). Duplicate 50 µL aliquots of these standards and the extracted samples were added to an antibody (rabbit anti-cortisol) coated plate. Using a repeater pipette, 50 µL of enzyme conjugate (cortisol horseradish peroxidase) was rapidly added to each well of the microplate. The plate was covered with plastic film, shaken gently and incubated in the dark at a constant temperature of 30°C for 1 h. Following incubation, the contents of the microplate were discarded and each well washed three times using a diluted wash buffer. Once more, a repeater pipette was used to rapidly add 150 µL of substrate (stabilized 3, 3', 5, 5' tetramethylbenzidine, TMB plus hydrogen peroxide, H_2O_2) to each well. The microplate was then re-covered, shaken and placed in the dark at 30°C for 30 min. After the second incubation, the plastic film was removed, and the bottom of the microplate was polished using a lint-free towel. The plate was shaken, and read on a microplate reader (DTX-880, Beckman Coulter Inc., Fullerton, California, USA) at a wavelength of 620 nm.

3.3.6 *Lipid Sampling and Analysis*

Triplicate samples of unenriched and enriched rotifers and *Artemia* nauplii from each dietary treatment were taken for dry weight, lipid class and major fatty acid analyses. A sample of rotifers or *Artemia* was first filtered through a fine mesh screen (50 μm pore size) to remove the culture water, and then placed in a 1 L volume of UV sterilized seawater. The density of the zooplankton was then determined by direct counting of the number of individuals in multiple sub-sample volumes. For each individual lipid sample, an amount of >50,000 rotifers or >5,000 *Artemia* was taken. Samples for dry weight were collected using 10% of the quantity collected for lipid analysis.

Triplicate larval samples were taken at hatch (day 0), before transfer to the glass aquaria (39 dph), before the second exposure stress (47 dph), and after weaning onto dry food (50 dph) for dry weight measurements and lipid analysis. At hatch, 150 larvae were sampled to obtain sufficient tissue for analysis, and the number of larvae required decreased over time as the fish grew. For day of hatch samples, larvae were pooled from the same four incubators used to stock the six larval rearing tanks. Samples were taken directly from the incubators before the larvae were exposed to any exogenous food source, and indicate only the lipid content of the newly hatched larvae and attached yolk sac. Two days before the transfer of larvae to the glass aquaria, samples of pooled larvae from each of the pairs of treatment tanks were taken. Samples of larvae at 47 dph were taken out of two aquaria from each treatment, which were not involved in the subsequent acute air exposure stress

test. Larvae were starved for a period of 24 h before each sampling to ensure empty digestive tracts and prevent any contribution of prey items to the lipid profile.

All samples for lipid analysis were collected on pre-combusted glass microfibre filters, placed directly in chloroform and stored under nitrogen at -20°C. Dry weight samples were collected on smaller pre-weighed glass microfibre filters, rinsed with 3% ammonium formate to remove salt, and then dried at 75°C for 48 h before being re-weighed using a microbalance. Total lipids were cold-extracted in chloroform/methanol following Parrish (1999) using a modified Folch procedure (Folch *et al.* 1957).

Thin layer chromatography with flame ionization detection (TLC/FID) by means of an MARK V Iatroscan (Iatroscan Laboratories, Tokyo, Japan) was used to determine specific amounts of lipid classes contained in each sample as described by Parrish (1987). Extracts were spotted on silica gel-coated Chromarods and a three-stage development system was used to separate lipid classes. The resulting three chromatograms were combined using T-data scan software (RSS, Bemis, Tennessee, USA). Lipid classes were identified against known standards (Sigma-Aldrich Corp., Oakville, Ontario, Canada). Crude lipid extracts were also subjected to transesterification using 14% boron trifluoride (BF_3) in methanol to produce fatty acid methyl esters (FAMEs) following a procedure based on Morrison and Smith (1964) and outlined in Parrish (1999). Analysis of the resultant FAMEs was carried out using an HP 6890 model GC equipped with an HP 7683 autosampler (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). Fatty-

acid peaks were integrated using HP Enhanced Chemstation G1701BA Version B.00.00 (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada) and identified against known standards (PUFA 1, PUFA 3, BAME, and a Supelco 37-component FAME mix, Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada).

3.3.7 *Statistical Analysis*

All data sets were examined to verify normality, independence and homogeneity of variance before further analysis was undertaken. Differences in standard length (SL), myotome height (MyH), condition factor (CF) and length-specific growth rate (LSGR) between treatments of the same dietary treatment were assessed using an analysis of covariance (ANCOVA). There was no significant tank effect ($p>0.2$) on any of these variables and thus the data from duplicate tanks in each of the three treatments were combined for further analysis. Growth characteristics of 46 dph larvae in aquaria and 50 dph fully weaned juveniles were analysed using a one-way analysis of variance (ANOVA).

Differences in dry weight, total lipid, lipid classes and fatty acid composition of rotifers, *Artemia* and 39 dph (~418 dd) larvae were analysed using a one-way ANOVA. For 46 dph (~512 dd) larvae and 50 dph (~564 dd) juveniles, measurements from duplicate tanks were assessed using a t-test. Where significant differences existed between tanks, these values were excluded from further statistical analysis; otherwise the data from both tanks was combined and used in subsequent one-way ANOVAs.

A logistic ANCOVA was used to determine the effect of treatment and sampling time on the post-stress survival of larvae at 41 dph (~441 dd). Whole-body corticosteroid concentrations of 48 dph (~524 dd) were analysed using a three-way ANOVA. The tank effect was not significant ($p>0.18$) and thus values from duplicate tanks were combined for further analysis. A significant interaction between time and stress level ($p=0.001$) prevented interpretation of individual effects. The data was then broken up into multiple two-way ANOVAs comparing the progression of unstressed and then stressed values over time between treatments as well as the difference in unstressed and stressed values between treatments at each sampling time.

Where significant differences were obtained, a post-hoc multiple comparison test (Tukey HSD) was performed. Growth and whole-body corticosteroid data were analysed using Minitab Version 13.1 (Minitab Inc., State College, Pennsylvania, USA), lipid data was analysed using SigmaStat Version 2.03 (Systat Software Inc., San Jose, California, USA), and the logistic ANCOVA on post-stress survival was performed using R Version 2.4.1 (R Foundation for Statistical Computing, Vienna, Austria). All data are expressed as means ± standard error (SE) and a significance value (α) of 5% was used for all tests.

3.4 Results

3.4.1 *Growth*

Standard length and myotome height of the larvae increased in all three dietary treatments over the course of the experimental period. After 160 dd of feeding, a difference in standard length was seen between two of the dietary treatments with the AlgaMac+*Pavlova* combination yielding larger fish compared to the Advantage treatment ($p=0.032$). By 320 dd, which followed the addition of *Artemia* to the diet, a clear variation in length was seen between all three treatments such that the AlgaMac+*Pavlova* larvae were significantly ($p<0.01$) longer than both the Advantage and Selco larvae (Fig. 3.2 A). A similar pattern was seen in myotome height, such that as early as 80 dd, larvae fed either AlgaMac+*Pavlova* or Selco had larger myotome heights than larvae fed Advantage. The myotome heights of AlgaMac+*Pavlova*-fed larvae remained elevated compared to either one or both of the other treatments up through 320 dd. By the end of the rearing period (400 dd); however, no significant differences in terms of myotome height were present between treatments ($p=0.08$: Fig. 3.2 B).

Larval condition mirrored the trend observed for myotome height. Early in rearing, larvae fed either AlgaMac+*Pavlova* or Selco had significantly higher condition factors compared to larvae fed Advantage. By 240 dd, AlgaMac+*Pavlova* larvae had the highest condition factor of all treatments, and remained significantly elevated along with the Selco larvae at 320 dd. As was seen with myotome height, by the end of the rearing period, no differences in condition factor were observed between treatments (Fig. 3.3 A).

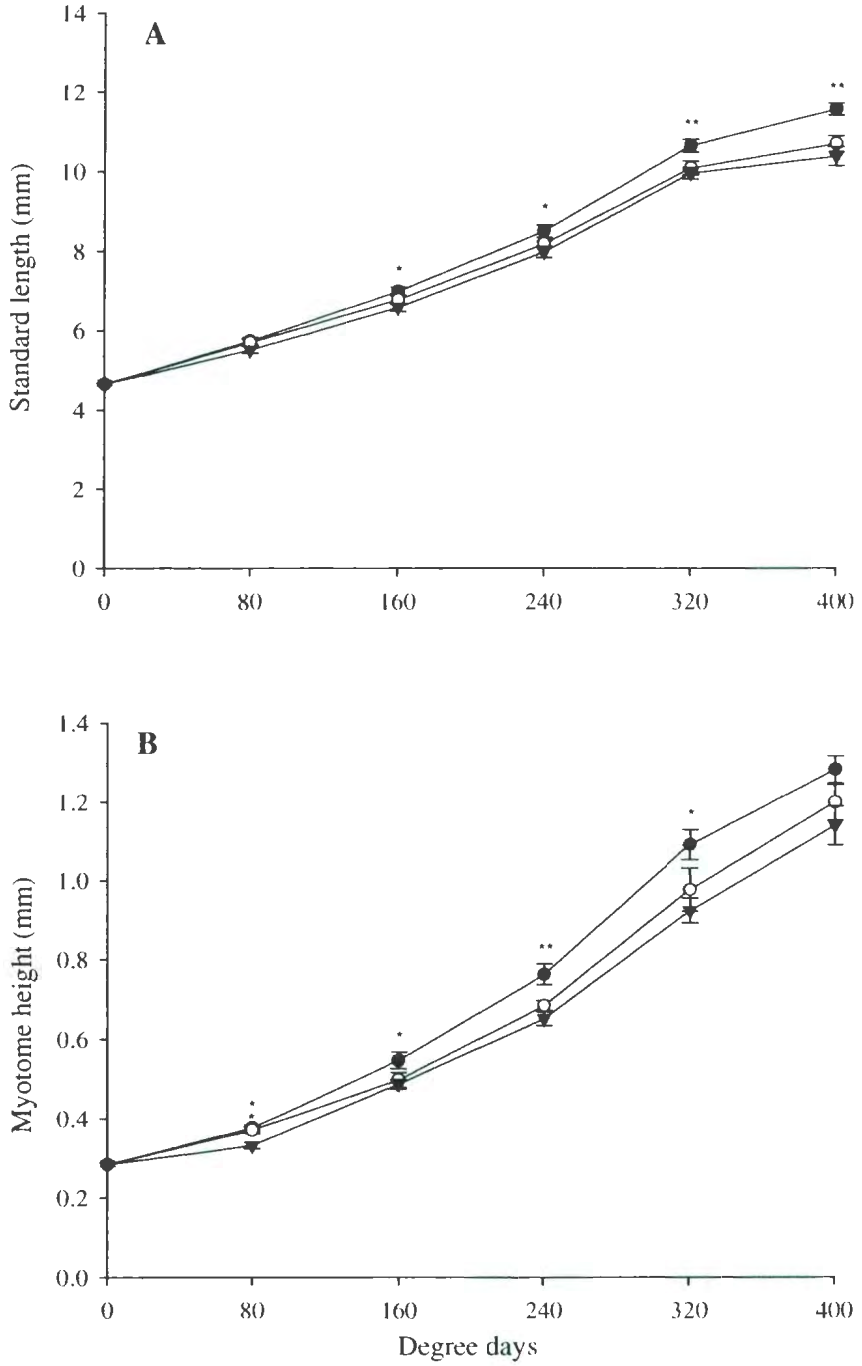


Figure 3.2. Standard length (A) and myotome height (B) of Atlantic cod larvae over time among three dietary treatments; AlgaMac® + *Pavlova* sp. (●), Selco® (○) and AquaGrow® Advantage (▼). * denotes significant difference from Advantage treatment ($p<0.05$). ** denotes significant difference from both Selco® and AquaGrow® Advantage treatments ($p<0.01$). Values are mean ± SE (n=20).

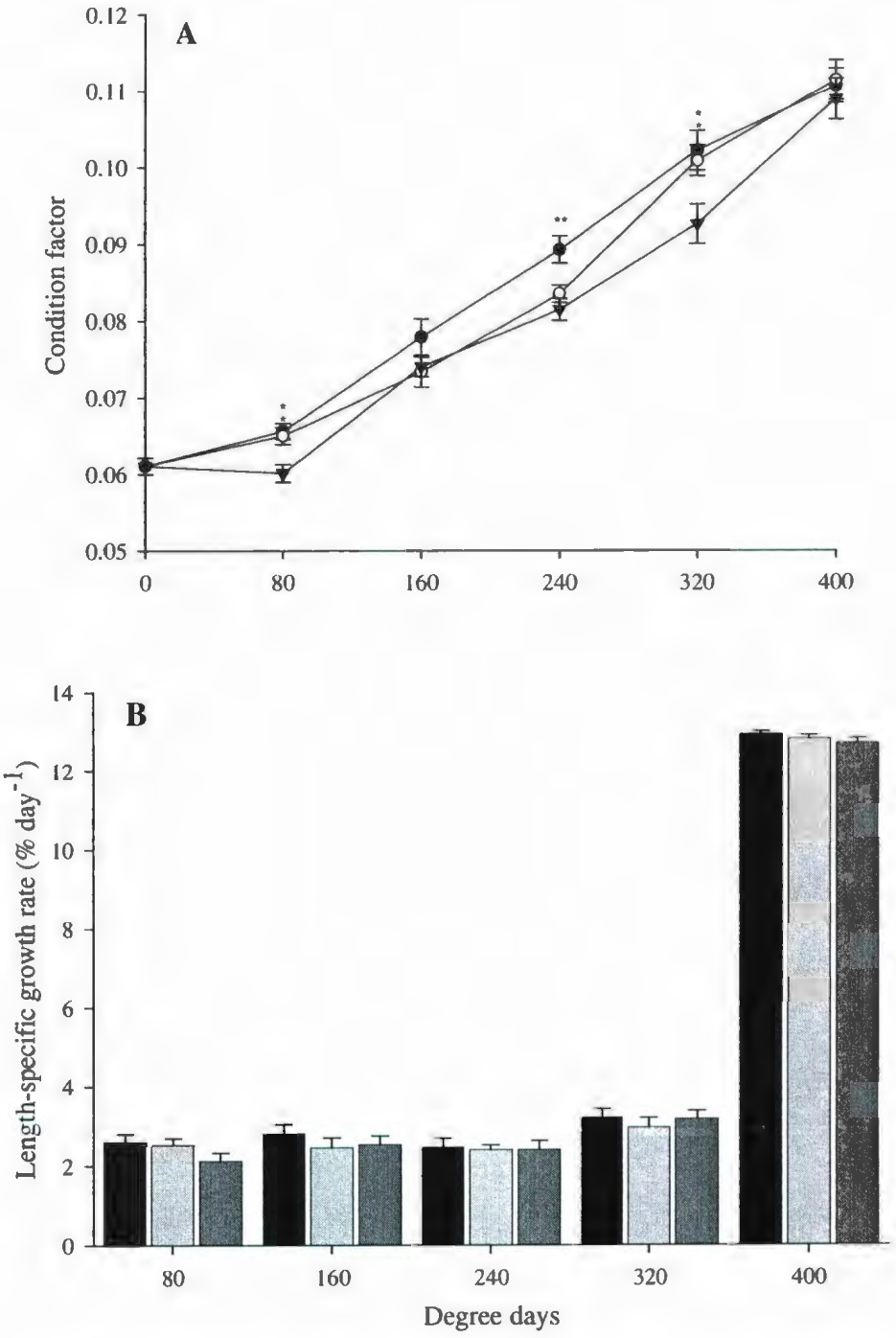


Figure 3.3. Condition factor (A) and length-specific growth rate (B) of Atlantic cod larvae over time among three dietary treatments; AlgaMac® + *Pavlova* sp. (●/■), Selco® (○/□) and AquaGrow® Advantage (▼/■). * denotes significant difference from Advantage treatment ($p<0.05$). ** denotes significant difference from both Selco® and AquaGrow® Advantage treatments ($p<0.01$). Values are mean ± SE ($n=20$).

There were no significant differences in length-specific growth among larvae from all treatments throughout the rearing period (Fig. 3.3 B).

Differences in larval dry weight between dietary treatments at 39 and 46dph (pre- and post-aquarium transfer) mirror the trend observed for SL and MyH (Tables 3.3 and 3.4). Larvae in the AlgaMac+*Pavlova* treatment were consistently heavier than larvae in the Advantage treatment. It is interesting to note; however, that no significant differences were seen in larval SL, MyH or CF at 46 dph between treatments ($p>0.31$, data not shown).

After larvae from all treatments were fully weaned onto the same inert particulate diet at ~564 dd, differences in growth persisted. Larvae that were fed live food enriched with a combination of AlgaMac+*Pavlova* were significantly larger in terms of standard length ($p<0.001$) and muscle myotome height ($p<0.001$) than the larvae from both other treatments (Fig. 3.4), as well as heavier in terms of dry weight ($p=0.03$) than larvae in the Advantage treatment (Table 3.5). No differences in condition factor or length-specific growth rate were seen.

3.4.2 *Lipid Analysis*

Rotifers enriched with Selco contained 42.5 ± 5.1 mg g^{-1} *dw* total lipid, which was significantly less than unenriched rotifers and rotifers enriched with the two other dietary treatments (Table 3.1). This pattern in total lipid content; however, did not correspond

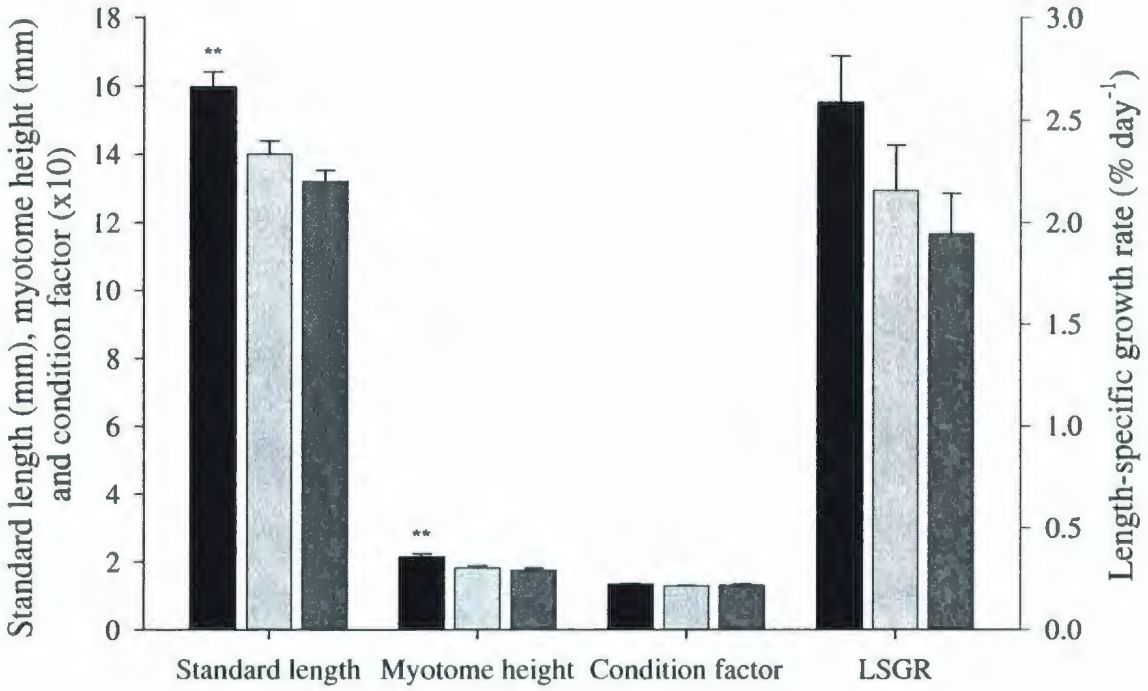


Figure 3.4. Growth parameters of fully weaned Atlantic cod larvae at 50 days post-hatch (~564 degree days) among three live-food dietary treatments. AlgaMac® + *Pavlova* sp. (■), Selco® (■), and AquaGrow® Advantage (■). ** denotes significant difference from both other treatments ($p<0.001$). Values are means ± SE (n=40, except for the Selco® treatment where n=39).

with the differences in rotifer size (ng *dw* rotifer^{-1}) between enrichment treatments, and therefore likely indicates differences in total lipid content of the enrichment products themselves. Minor differences in lipid class makeup (as % of total lipid) of rotifers in terms of free fatty acids and sterols occurred between treatments. Conversely, the fatty acid composition of differentially enriched rotifers varied greatly. The main differences were in monounsaturated fatty acids (MUFA, as % of total fatty acid) and polyunsaturated fatty acids (PUFA), which showed a slight decrease and a 2-fold increase

Table 3.1. Lipid class and fatty acid composition of unenriched rotifers and three experimental diets consisting of differentially enriched rotifers.

	Unenriched	AlgaMac 2000® + *Pavlova* sp.	Protein Selco Plus®	AquaGrow® Advantage
***Dry weight* (ng) rotifer^{-1}**	71.6 ± 13.4 ^{c}	280.5 ± 1.3 ^{a}	188.1 ± 7.5 ^{b}	181.8 ± 5.0 ^{b}
Total lipid (mg g^{-1} *dry weight*)	80.9 ± 4.6 ^{a}	67.6 ± 0.7 ^{a}	42.5 ± 5.1 ^{b}	67.1 ± 7.9 ^{a}
Lipid classes (% of total lipid)				
Ethyl esters	1.7 ± 1.1	2.0 ± 0.1	3.1 ± 0.5	2.3 ± 1.0
Methyl esters	1.5 ± 1.0	1.4 ± 0.1	2.8 ± 0.4	1.4 ± 0.9
Triacylgylcerols	23.7 ± 13.4	36.3 ± 0.5	25.2 ± 1.6	30.3 ± 16.9
Free fatty acids	14.2 ± 0.4 ^{a}	7.4 ± 0.3 ^{b}	6.3 ± 1.2 ^{b}	9.9 ± 2.5 ^{a}
Sterols	2.3 ± 0.3 ^{ab}	2.9 ± 0.1 ^{ab}	3.1 ± 0.1 ^{a}	1.8 ± 0.5 ^{b}
Acetone mobile polar lipids	12.1 ± 8.8	7.6 ± 0.4	6.4 ± 0.8	18.8 ± 11.1
Phospholipids	42.4 ± 6.0	42.2 ± 1.2	52.1 ± 2.4	34.8 ± 10.6
Fatty acid composition (% of total fatty acid)				
14:0	2.1 ± 0.1 ^{c}	3.8 ± 0.3 ^{b}	2.3 ± 0.2 ^{c}	10.1 ± 0.1 ^{a}
16:0	21.6 ± 0.9	20.8 ± 0.7	19.9 ± 1.5	20.2 ± 1.1
18:0	8.5 ± 0.3 ^{a}	5.0 ± 0.2 ^{bc}	5.9 ± 0.4 ^{b}	3.8 ± 0.5 ^{c}
Sum SFA	35.3 ± 1.9	31.3 ± 1.1	30.0 ± 2.7	35.6 ± 1.5
16:1ω7	9.9 ± 0.0 ^{a}	7.5 ± 0.4 ^{b}	9.0 ± 0.6 ^{ab}	4.6 ± 0.3 ^{c}
18:1ω7	4.2 ± 0.0 ^{ab}	5.3 ± 0.2 ^{a}	4.9 ± 0.3 ^{a}	3.1 ± 0.3 ^{b}
18:1ω9	19.0 ± 0.3 ^{a}	11.1 ± 0.4 ^{b}	17.4 ± 1.1 ^{a}	13.3 ± 0.5 ^{b}
18:1ω11?	2.1 ± 0.0 ^{a}	2.1 ± 0.0 ^{a}	2.2 ± 0.1 ^{a}	1.0 ± 0.2 ^{b}
20:1ω9	3.3 ± 0.0 ^{a}	2.4 ± 0.1 ^{b}	3.2 ± 0.4 ^{a}	1.7 ± 0.1 ^{b}
22:1ω11(13)	1.5 ± 0.1 ^{a}	0.6 ± 0.0 ^{b}	0.8 ± 0.1 ^{b}	0.7 ± 0.1 ^{b}
24:1	0.8 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	1.6 ± 0.6
Sum MUFA	44.1 ± 0.2 ^{a}	32.6 ± 1.0 ^{b}	41.4 ± 2.5 ^{a}	27.9 ± 1.1 ^{b}
16:3ω4?	1.4 ± 0.3 ^{ab}	1.5 ± 0.1 ^{ab}	1.8 ± 0.0 ^{a}	0.9 ± 0.1 ^{b}
18:2ω6	5.7 ± 0.0 ^{ab}	5.5 ± 0.4 ^{ab}	7.0 ± 1.0 ^{a}	2.5 ± 0.2 ^{b}
18:4ω3	0.1 ± 0.0 ^{b}	1.6 ± 0.1 ^{a}	0.2 ± 0.0 ^{b}	–
20:4ω6 (AA)	0.6 ± 0.0 ^{bc}	1.5 ± 0.2 ^{a}	1.4 ± 0.4 ^{ab}	0.4 ± 0.0 ^{c}
20:5ω3 (EPA)	1.7 ± 0.0 ^{ab}	2.8 ± 0.3 ^{ab}	3.5 ± 1.0 ^{a}	1.0 ± 0.1 ^{b}
22:5ω3	1.0 ± 0.2	1.3 ± 0.3	1.8 ± 0.5	0.8 ± 0.0
22:5ω6 (DPA)	0.3 ± 0.2 ^{b}	3.3 ± 0.2 ^{a}	0.3 ± 0.0 ^{b}	0.1 ± 0.0 ^{b}
22:6ω3 (DHA)	3.0 ± 0.2 ^{c}	11.0 ± 0.8 ^{b}	4.9 ± 1.1 ^{bc}	27.8 ± 3.0 ^{a}
Sum PUFA	17.9 ± 1.9 ^{b}	34.2 ± 2.1 ^{a}	26.2 ± 5.0 ^{b}	35.3 ± 2.7 ^{a}
Sum C_{20} PUFA	3.3 ± 0.0 ^{ab}	6.4 ± 0.7 ^{ab}	6.9 ± 1.8 ^{a}	2.2 ± 0.1 ^{b}

Table 3.1 cont.

	Unenriched	AlgaMac 2000® + *Pavlova* sp.	Protein Selco Plus®	AquaGrow® Advantage
Sum C_{22} PUFA	5.6 ± 1.6 [c]	17.0 ±0.9 [b]	7.6 ± 1.8 [c]	28.8 ± 3.2 [a]
Sum ω3	7.4 ± 0.6 [c]	20.0 ± 1.4 [ab]	12.9 ± 3.1 [bc]	30.5 ± 2.9 [a]
Sum ω6	8.2 ± 1.0	11.5 ± 0.8 [a]	9.9 ± 1.7 [a]	3.6 ± 0.0 [b]
ω3/ ω6	0.9 ± 0.0 [b]	1.7 ± 0.0 [b]	1.3 ± 0.1 [b]	8.6 ± 0.8 [a]
DHA/EPA	1.8 ± 0.1 [b]	4.1 ± 0.2 [b]	1.5 ± 0.1 [b]	27.0 ± 5.0 [a]
EPA/AA	2.9 ± 0.1 [a]	1.8 ± 0.1 [b]	2.6 ± 0.0 [a]	2.8 ± 0.1 [a]
DHA/DPA	11.6 ± 5.4 [bc]	3.3 ± 0.1 [c]	18.1 ± 1.5 [b]	290.0 ± 4.3 [a]

Values are mean of triplicate measurements ± SE. Different letter superscripts indicate significant differences ($p<0.05$).
Only lipid classes and fatty acids contributing >1.5% in at least one treatment are reported.
Other identified lipid classes were hydrocarbons, steryl/wax esters, ethyl ketones, alcohols and diacylglycerols.
Other identified fatty acids were *i*15:0, *ai*15:0, 15:0, *i*16:0, *ai*16:0(?), 17:0, *i*17:0, *ai*17:0(?), 19:0, 20:0, 21:0, 22:0, 23:0, 24:0, 14:1, 16:1ω5, 16:1ω9(?), 16:1ω11(?), 17:1, 18:1ω5(?), 20:1ω7(?), 20:1ω11(?), 22:1ω7, 22:1ω9, 16:2ω4, 18:2, 18:2ω4, 20:2ω6, 18:3ω3, 18:3ω4, 18:3ω6, 20:3ω3, 20:3ω6, 16:4ω1, 16:4ω3(?), 18:4ω1(?), 20:4ω3, 22:4ω3(?), 22:4ω6(?), 21:5ω3(?), trimethyltridecanoic acid (TMTD), and pristanic acid(?).
– non-detectable.

(respectively) in rotifers enriched with AlgaMac+*Pavlova* or Advantage. The MUFA and PUFA contents of Selco-enriched rotifers, on the other hand, remained unchanged from unenriched values. The sum of saturated fatty acids (SFA) was unaltered by enrichment, and ranged from 30-36.5% (Table 3.1). Enrichment of rotifers with either AlgaMac+*Pavlova* or Selco resulted in an ~2-fold increase in the 20-carbon PUFAs AA (20:4ω6) and EPA (20:5ω3). Feeding rotifers with Advantage, on the other hand, increased their DHA (22:6ω3) content by a remarkable 32-fold compared with unenriched rotifers. The EPA content in Advantage rotifers; however, remained comparable to unenriched values and was significantly lower than the EPA content of Selco rotifers. AlgaMac+*Pavlova* also significantly increased rotifer-DHA content, and

was the only enrichment to be a source of another 22-carbon PUFA, ω6DPA (22:5ω6). Differential enrichment also markedly affected the levels of total ω3, ω6, 20- and 22-carbon PUFAs (C_{20} and C_{22}) as well as the DHA/EPA, EPA/AA and DHA/DPA ratios (Table 3.1).

The enrichment of *Artemia* nauplii with similar, and in the case of Advantage, the same, products as those used for rotifers, resulted in wholly different lipid profiles. *Artemia* enriched with Advantage had the lowest total lipid content at 59.3 ± 12.5 mg g^{-1} *dw*, which was significantly lower than the 216.8 ± 46.9 mg g^{-1} *dw* of the AlgaMac 3050+*Pavlova*-enriched *Artemia* (Table 3.2). There were no significant differences in nauplii size (μg *dw* $rotifer^{-1}$) between treatments (p=0.52). All enrichment treatments increased the free fatty acid content of *Artemia* (3-6 fold) compared to unenriched nauplii. Advantage-enriched *Artemia* had less triacylglycerols, while Gadi Selco-enriched *Artemia* had decreased quantities of both acetone mobile polar lipids and phospholipids. With regards to fatty acid composition, total SFA was uniform between treatments, as was seen for rotifers; however, total MUFA and total PUFA were also consistent among *Artemia* enrichments (Table 3.2). A very clear contrast in DHA-content was seen between rotifers and *Artemia* enriched with Advantage. While Advantage-enriched rotifers had the highest DHA content, Advantage-enriched *Artemia* had the lowest DHA content, which was on par with that of unenriched *Artemia* (Table 3.2). Conversely, DHA content increased considerably in AlgaMac+*Pavlova* and Selco-fed *Artemia*; values 87-

Table 3.2. Lipid class and fatty acid composition of unenriched *Artemia* sp. and three experimental diets consisting of differentially enriched *Artemia* sp.

	Unenriched	AlgaMac 3050® + *Pavlova* sp.	Gadi Selco®	AquaGrow® Advantage
***Dry weight* (µg) *Artemia*$^{-1}$**	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.5 ± 0.1
Total lipid (mg g^{-1} *dry weight*)	147.1 ± 14.8 ^{ab}	216.8 ± 46.9 ^{a}	172.4 ± 17.6 ^{ab}	59.3 ± 12.5 ^{b}
Lipid classes (% of total lipid)				
Ethyl esters	–	0.5 ± 0.5 ^{b}	1.1 ± 0.1 ^{ab}	2.6 ± 0.6 ^{a}
Methyl esters	–	1.6 ± 0.6 ^{b}	1.7 ± 0.2 ^{b}	4.7 ± 0.5 ^{a}
Triacylgylcerols	59.7 ± 1.4 ^{ab}	57.8 ± 5.8 ^{ab}	66.8 ± 0.5 ^{a}	48.2 ± 2.4 ^{b}
Free fatty acids	1.4 ± 0.8 ^{c}	4.1 ± 0.8 ^{bc}	8.8 ± 1.2 ^{a}	5.8 ± 0.6 ^{ab}
Sterols	3.9 ± 0.5	3.3 ± 1.0	2.0 ± 0.2	4.7 ± 1.0
Diacylglycerols	1.8 ± 0.3	1.0 ± 0.4	1.3 ± 0.1	1.0 ± 0.3
Acetone mobile polar lipids	6.4 ± 0.7 ^{ab}	8.3 ± 2.4 ^{ab}	3.7 ± 0.4 ^{b}	9.9 ± 0.7 ^{a}
Phospholipids	25.7 ± 1.1 ^{a}	23.1 ± 2.6 ^{a}	14.2 ± 0.3 ^{b}	22.2 ± 2.4 ^{ab}
Fatty acid composition (% of total fatty acid)				
14:0	0.9 ± 0.0 ^{c}	1.9 ± 0.1 ^{a}	1.7 ± 0.1 ^{a}	1.3 ± 0.0 ^{b}
16:0	11.2 ± 0.4 ^{b}	14.7 ± 0.9 ^{a}	15.3 ± 0.5 ^{a}	11.7 ± 0.1 ^{b}
18:0	5.8 ± 0.3	6.5 ± 0.4	7.0 ± 0.1	5.0 ± 2.5
Sum SFA	20.2 ± 0.7	25.4 ± 1.5	26.7 ± 0.8	20.2 ± 2.5
16:1ω7	5.8 ± 0.1 ^{a}	3.9 ± 0.1 ^{b}	5.2 ± 0.5 ^{a}	4.6 ± 0.1 ^{ab}
18:1ω7	12.0 ± 0.3 ^{ab}	10.8 ± 0.6 ^{bc}	9.7 ± 0.4 ^{c}	13.1 ± 0.3 ^{a}
18:1ω9	21.5 ± 0.6	15.6 ± 0.5	13.8 ± 5.2	20.8 ± 0.1
Sum MUFA	42.6 ± 1.0	32.6 ± 1.3	33.4 ± 4.4	41.8 ± 0.5
18:2ω6	5.7 ± 0.2 ^{a}	3.5 ± 0.2 ^{b}	5.6 ± 0.4 ^{a}	5.0 ± 0.4 ^{a}
18:3ω3	18.1 ± 1.1 ^{a}	10.4 ± 0.7 ^{c}	11.7 ± 1.4 ^{bc}	16.5 ± 1.5 ^{ab}
18:4ω3	1.7 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
20:4ω6 (AA)	0.9 ± 0.1 ^{b}	2.2 ± 0.2 ^{a}	1.5 ± 0.1 ^{b}	1.1 ± 0.1 ^{b}
20:5ω3 (EPA)	2.9 ± 0.3 ^{a}	4.8 ± 0.5 ^{ab}	7.3 ± 0.8 ^{b}	4.5 ± 0.6 ^{a}
22:5ω6 (DPA)	–	3.7 ± 0.3 ^{a}	0.2 ± 0.0 ^{b}	–
22:6ω3 (DHA)	0.1 ± 0.0 ^{c}	10.3 ± 0.8 ^{a}	5.1 ± 0.5 ^{b}	2.0 ± 0.8 ^{c}
Sum PUFA	32.6 ± 1.9	38.9 ± 2.9	36.7 ± 3.5	33.5 ± 2.3
Sum C_{20} PUFA	2.3 ± 0.5 ^{b}	8.0 ± 0.8 ^{ab}	9.8 ± 1.1 ^{a}	6.2 ± 0.8 ^{ab}
Sum C_{22} PUFA	0.2 ±0.0 ^{c}	14.3 ± 1.1 ^{a}	6.0 ± 0.6 ^{b}	2.1 ± 0.8 ^{c}
Sum ω3	23.2 ± 1.6	27.4 ± 2.1	26.6 ± 2.9	24.9 ± 1.7
Sum ω6	7.4 ± 0.4 ^{b}	10.4 ± 0.7 ^{a}	8.1 ± 0.5	6.8 ± 0.6 ^{b}
ω3/ ω6	3.1 ± 0.1 ^{ab}	2.7 ± 0.0 ^{b}	3.3 ± 0.1 ^{a}	3.7 ± 0.2 ^{a}

Table 3.2 cont.

	Unenriched	AlgaMac 3050® + *Pavlova* sp.	Gadi Selco®	AquaGrow® Advantage
DHA/EPA	–	2.2 ± 0.1^{a}	0.7 ± 0.0^{b}	0.5 ± 0.2^{bc}
EPA/AA	3.3 ± 0.0^{c}	2.1 ± 0.0^{d}	5.0 ± 0.1^{a}	4.0 ± 0.0^{b}
DHA/DPA	–	2.8 ± 0.0^{b}	21.5 ± 0.5^{a}	–

Values are mean of triplicate measurements ± SE. Different letter superscripts indicate significant differences ($p<0.05$).
Only lipid classes and fatty acids contributing >1.5% in at least one treatment are reported.
Other identified lipid classes were hydrocarbons, steryl/wax esters, and alcohols.
Other identified fatty acids were *i*15:0, *ai*15:0, 15:0, *i*16:0, *ai*16:0(?), 17:0, *i*17:0, *ai*17:0, 19:0, 20:0, 21:0, 22:0, 23:0, 24:0, 14:1, 15:1, 24:1, 16:1ω5, 16:1ω9(?), 16:1ω11(?), 17:1, 18:1ω5(?), 20:1ω7, 20:1ω11(?), 22:1ω7, 22:1ω9, 22:1ω11(13), 24:1, 16:2ω4, 18:2, 18:2ω4, 20:2ω6, 16:3ω4(?), 18:3ω4, 18:3ω6, 20:3ω3, 20:3ω6, 16:4ω1, 18:4ω1(?), 20:4ω3, 22:4ω3(?), 22:4ω6(?), 21:5ω3(?), 22:5ω3, trimethyltridecanoic acid (TMTD), and pristanic acid (?).
– non-detectable

and 43-fold higher than unenriched *Artemia*. Some similarities, however, were found between the rotifer and *Artemia* enrichment treatments, and included differences in the fatty acid content of three notable EFAs, AA, EPA and DPA. As was the case for rotifers, feeding *Artemia* AlgaMac+*Pavlova* resulted in a 2.5-fold increase in AA as well as the only significant increase in ω6DPA (Table 3.2). Selco-fed *Artemia*, on the other hand, showed a 2.5-fold enrichment in EPA, which was also significantly higher than the EPA content of Advantage-fed *Artemia*. Little variation was observed in the PUFA compositions of unenriched and Advantage-enriched *Artemia*, in which high levels of the 18-carbon ω3 and ω6 PUFAs, linolenic acid (LNA: 18:3ω3) and linoleic acid (LA: 18:2ω6), were preserved (Table 3.2).

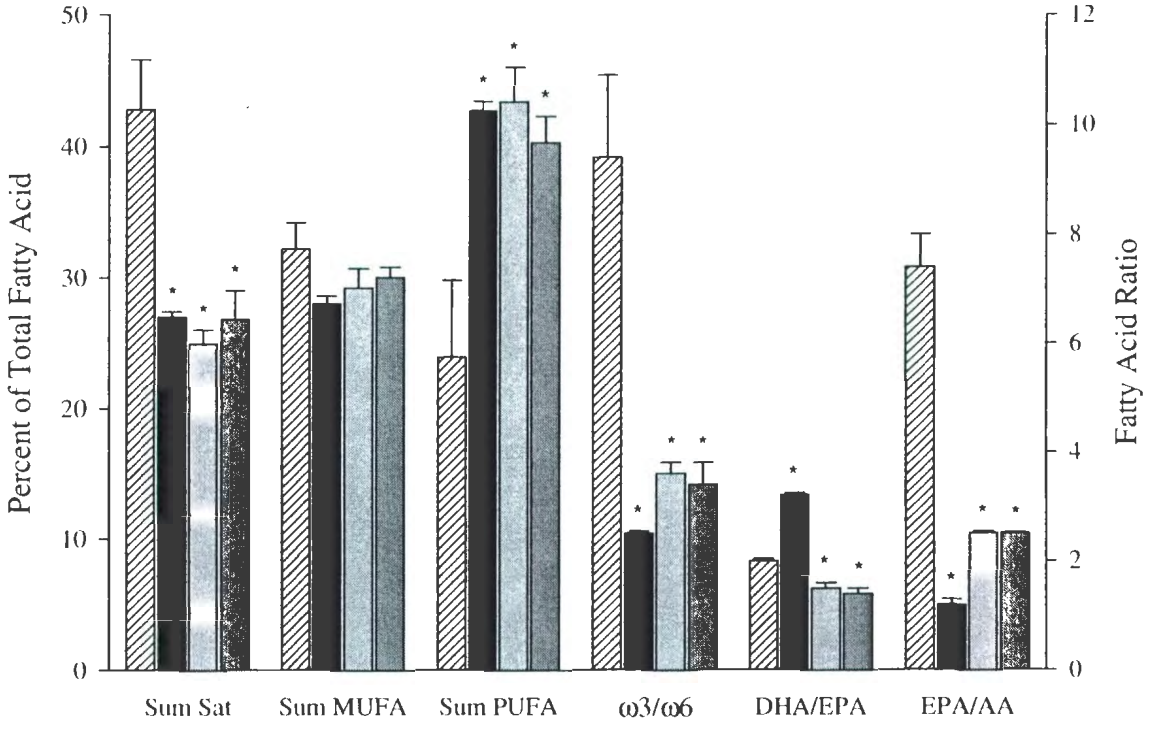


Figure 3.5. Select fatty acid compositions of Atlantic cod larvae at hatch (▨) and 39 dph (~ 418 dd) fed one of three different dietary treatments; AlgaMac® + *Pavlova* sp. (■), Selco® ( ) and AquaGrow® Advantage (■). * denotes significant difference from value at hatch ($p<0.05$). Values are means of triplicates ± SE.

The fatty acid levels in 39 dph (~418dd) Atlantic cod larvae, compared with those of yolk-sac larvae, largely reflect the switch from an endogenous food source to exogenous feeding on enriched rotifers and *Artemia*. Fatty acid composition has shifted from being largely dominated by saturates to having a high percentage of PUFA, with MUFA-content remaining fairly uniform (Fig. 3.5). Increased levels of ω6 PUFAs, mainly LA, AA, and DPA (in the case of AlgaMac+*Pavlova*), in the diet have greatly decreased the ω3/ω6 and EPA/AA ratios compared with levels at hatch (Fig. 3.5).

Differences were seen in the lipid/fatty acid profiles of 39 dph larvae among dietary treatments in terms of total lipid content, LA, AA, EPA, DPA and DHA (Table 3.3). The Selco-fed larvae had the highest total lipid content at 136.9 ± 8.5 mg g^{-1} *dw*, a result in contrast to the total lipid content of Selco-enriched rotifers and *Artemia*, which did not have the largest total lipid values (Tables 3.1 and 3.2). The observed differences in larval fatty acid composition between treatments largely parallel those in the enriched rotifer and *Artemia* profiles. Larvae fed live food enriched with a combination of AlgaMac+*Pavlova* had elevated AA content compared with larvae fed Advantage-enriched live food. DHA content also appeared slightly larger in AlgaMac+*Pavlova* larvae, but this difference was not significant (p=0.054). They did, however, have significantly higher DPA and reduced EPA levels compared with both Advantage and Selco larvae (Table 3.3). As a consequence, ω3/ω6 and EPA/AA ratios in AlgaMac+*Pavlova* larvae were considerably lower while the DHA/EPA ratio was elevated. Suprisingly, larvae fed Advantage-enriched live food had a high level of EPA, which was comparable to that in Selco larvae, despite Advantage rotifers and *Artemia* having the lowest EPA content. The DHA/DPA ratio of cod larvae varied greatly between all three dietary treatments with AlgaMac+*Pavlova* larvae having the lowest ratio, followed by Selco and then Advantage with the highest. It is also interesting to note that the C_{20} and C_{22} PUFA amounts were altered by dietary treatment. Larvae fed AlgaMac+*Pavlova*-enriched live feed had significantly lower C_{20} PUFA compared with larvae in the other two treatments and their C_{22} PUFA content was elevated compared to

Table 3.3. Lipid class and fatty acid composition of Atlantic cod larvae 39 days post-hatch (~418 degree days); two days before 15 s air exposure stress, transfer to aquaria and subsequent survival measurement.

	AlgaMac® + *Pavlova* sp.	Selco®	AquaGrow® Advantage
***Dry weight* (mg) larvae^{-1}**	2.0 ± 0.1 [a]	1.5 ± 0.1 [b]	1.2 ± 0.0 [c]
Total lipid (mg g^{-1} *dry weight*)	85.5 ± 4.0 [b]	136.9 ± 8.5 [a]	107.7 ± 6.9 [b]
Lipid classes (% of total lipid)			
Ethyl ketones	1.8 ± 1.5	0.7 ± 0.1	0.5 ± 0.3
Triacylgylcerols	7.0 ± 1.2	5.8 ± 0.9	7.9 ± 1.0
Free fatty acids	1.3 ± 0.5	2.6 ± 0.0	1.5 ± 0.4
Sterols	15.4 ± 0.5	14.2 ± 0.4	17.0 ± 1.1
Acetone mobile polar lipids	3.9 ± 0.1 [a]	1.4 ± 0.4 [b]	1.6 ± 0.7 [b]
Phospholipids	68.8 ± 2.2	75.1 ± 0.9	70.0 ± 2.0
Fatty acid composition (% of total fatty acid)			
16:0	16.8 ± 0.3	15.2 ± 0.7	16.5 ± 1.2
18:0	7.8 ± 0.1	7.3 ± 0.3	7.9 ± 0.7
Sum SFA	27.0 ± 0.4	24.9 ± 1.1	26.8 ± 2.2
16:1ω7	2.0 ± 0.1	2.3 ± 0.2	2.5 ± 0.2
16:1ω9?	1.0 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
18:1ω7	6.1 ± 0.2	7.9 ± 0.4	6.5 ± 2.4
18:1ω9	12.5 ± 0.1	14.5 ± 0.9	16.3 ± 1.5
20:1ω9	0.7 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
24:1	2.6 ± 0.3	1.1 ± 0.5	1.1 ± 0.5
Sum MUFA	28.0 ± 0.6	29.2 ± 1.5	30.0 ± 0.8
18:2ω6	2.6 ± 0.0 [b]	3.7 ± 0.1 [a]	4.1 ± 0.3 [a]
18:3ω3	5.3 ± 0.0	5.8 ± 0.2	5.4 ± 2.7
20:4ω6 (AA)	4.4 ± 0.1 [a]	3.7 ± 0.3 [ab]	3.4 ± 0.1 [b]
20:5ω3 (EPA)	5.3 ± 0.1 [b]	9.1 ± 0.6 [a]	8.3 ± 0.3 [a]
22:5ω3	0.6 ± 0.3 [b]	2.2 ± 0.2 [a]	1.7 ± 0.1 [a]
22:5ω6 (DPA)	3.7 ± 0.2 [a]	0.5 ± 0.1 [b]	0.2 ± 0.0 [b]
22:6ω3 (DHA)	16.9 ± 0.4	13.6 ± 1.8	12.0 ± 0.7
Sum PUFA	42.7 ± 0.7	43.4 ± 2.6	40.3 ± 2.0
Sum C_{20} PUFA	11.2 ± 0.3 [b]	14.3 ± 0.9 [a]	13.4 ± 0.5 [ab]
Sum C_{22} PUFA	21.4 ± 0.4 [a]	16.8 ± 2.0 [ab]	14.7 ± 1.0 [b]
Sum ω3	30.0 ± 0.5	32.1 ± 2.5	29.1 ± 2.5
Sum ω6	11.8 ± 0.3 [a]	9.0 ± 0.2 [b]	8.8 ± 0.5 [b]
ω3/ ω6	2.5 ± 0.0 [b]	3.6 ± 0.2 [a]	3.4 ± 0.4 [a]

Table 3.3 cont.

	AlgaMac® + *Pavlova* sp.	Selco®	AquaGrow® Advantage
DHA/EPA	3.2 ± 0.0^{a}	1.5 ± 0.1^{b}	1.4 ± 0.1^{b}
EPA/AA	1.2 ± 0.0^{b}	2.5 ± 0.0^{a}	2.5 ± 0.1^{a}
DHA/DPA	4.6 ± 0.1^{c}	26.7 ± 1.0^{b}	51.9 ± 1.5^{a}

Values are mean of triplicate measurements ± SE of pooled samples from duplicate treatment tanks. Different letter superscripts indicate significant differences between treatments ($p<0.05$).
Only lipid classes and fatty acids contributing >1.5% in at least one treatment are reported.
Other identified lipid classes were hydrocarbons, steryl/wax esters, methyl esters, methyl ketones, alcohols and diacylglycerols.
Other identified fatty acids were 14:0, *i* 5:0, *ai*15:0, 15:0, *i*16:0, *ai*16:0(?), 17:0, *i*17:0, *ai*17:0(?), 19:0, 20:0, 21:0, 22:0, 23:0, 24:0, 16:1ω5, 16:1ω11(?), 17:1, 18:1ω5(?), 18:1ω11(?), 20:1ω7(?), 20:1ω11(?), 22:1ω7, 22:1ω9, 22:1ω11(13), 16:2ω4, 18:2, 18:2ω4, 20:2ω6, 16:3ω4(?), 18:3ω4, 18:3ω6, 20:3ω3, 20:3ω6, 16:4ω3(?), 18:4ω1(?), 18:4ω3, 20:4ω3, 22:4ω3(?), 22:4ω6(?), 21:5ω3(?), trimethyltridecanoic acid (TMTD), and pristanic acid(?).

Advantage larvae, with the value for Selco larvae falling midway between the two (Table 3.3). The combined sum of all C_{20} and C_{22} PUFAs, however, did not vary significantly with dietary treatment ($p=0.296$, data not shown).

The lipid profiles of 46 dph (~512 dd) larvae were found to be very comparable to those of 39 dph larvae with only a few differences. Larvae fed Selco-enriched live food were surpassed by the Advantage-fed larvae in terms of total lipid content, however, this result should be interpreted with caution since a significant tank effect was discovered in the Selco treatment and no further statistical analysis was undertaken. AlgaMac+*Pavlova* larvae continued to have significantly lower EPA and higher DPA levels than larvae in the other two treatments (Table 3.4). Similar to 39 dph larvae, elevated levels of AA, DHA and C_{22} PUFA were observed in AlgaMac+*Pavlova* larvae, but unlike 39 dph larvae, these were significantly different compared with both other treatments. The trends

Table 3.4. Lipid class and fatty acid composition of Atlantic cod larvae 46 days post-hatch (~512 degree days); one day before 15 s air exposure stress and subsequent corticosteroid sampling.

	AlgaMac® + *Pavlova* sp.	Selco®	AquaGrow® Advantage
***Dry weight* (mg) larvae^{-1}**	2.7 ± 0.3 [a]	2.4 ± 0.1 [ab]	2.0 ± 0.3 [b]
Total lipid (mg g^{-1} *dry weight*)	76.7 ± 5.1 [b]	88.6	107.6 ± 9.5 [a]
Lipid classes (% of total lipid)			
Triacylgylcerols	3.9	4.4 ± 0.8	2.7 ± 0.2
Free fatty acids	1.5 ± 0.3	1.4 ± 0.4	1.2 ± 0.1
Sterols	18.9 ± 0.4 [a]	16.9 ± 1.1 [ab]	15.6 ± 0.7 [b]
Acetone mobile polar lipids	3.8	2.1 ± 0.4	1.9 ± 0.5
Phospholipids	70.0	73.8 ±1.6	77.6 ± 1.0
Fatty acid composition (% of total fatty acid)			
16:0	16.5 ± 0.8	15.4 ± 0.2	15.0 ± 0.7
18:0	8.4 ± 0.3	7.5 ± 0.1	7.5 ± 0.3
Sum SFA	26.9 ± 1.2	25.1 ± 0.3	24.8 ± 0.1
16:1ω7	1.6 ± 0.1 [b]	2.0 ± 0.0 [a]	1.8 ± 0.1 [a]
18:1ω7	7.8 ± 0.4	8.3 ± 0.1	8.3 ± 0.3
18:1ω9	12.5 ± 0.5 [b]	14.5 ± 0.2 [a]	14.5 ± 0.5 [a]
24:1	1.9 ± 0.4	1.7 ± 1.1	1.7 ± 1.2
Sum MUFA	26.3 ± 0.9	29.3 ± 0.6	29.0 ± 1.3
18:2ω6	2.3	3.3 ± 0.0	3.4 ± 0.1
18:3ω3	4.4	5.8 ± 0.1 [b]	6.7 ± 0.3 [a]
20:4ω6 (AA)	4.9 ± 0.2 [a]	3.9 ± 0.1 [b]	3.8 ± 0.2 [b]
20:5ω3 (EPA)	5.1 ± 0.3 [b]	9.5 ± 0.3 [a]	9.4 ± 0.7 [a]
22:5ω3	0.8 ± 0.0 [c]	2.0 ± 0.1 [a]	1.6 ± 0.1 [b]
22:5ω6 (DPA)	4.3 ± 0.3 [a]	0.5 ± 0.0 [b]	0.2 ± 0.0 [b]
22:6ω3 (DHA)	18.7 ± 0.5 [a]	13.3 ± 0.4 [b]	14.2 ± 1.1 [b]
Sum PUFA	44.6 ± 2.0	43.1 ± 0.9	43.8 ± 2.4
Sum C_{20} PUFA	11.3 ± 0.4 [b]	14.9 ± 0.4 [a]	14.8 ± 1.0 [a]
Sum C_{22} PUFA	24.4 ± 1.7 [a]	16.0 ± 0.5 [b]	16.2 ± 1.3 [b]
Sum ω3	30.4 ± 1.7	32.4 ± 0.8	33.7 ± 2.2
Sum ω6	12.4 ± 0.4 [a]	8.8 ± 0.1 [b]	8.6 ± 0.2 [b]
ω3/ ω6	2.4 ± 0.1 [b]	3.7 ± 0.1 [a]	3.9 ± 0.2 [a]
DHA/EPA	3.6 ± 0.2 [a]	1.4 ± 0.0 [b]	1.5 ± 0.0 [b]
EPA/AA	1.1 ± 0.0 [b]	2.5 ± 0.0 [a]	2.5 ± 0.1 [a]
DHA/DPA	4.3 ± 0.1 [c]	28.3 ± 0.6 [b]	65.7 ± 2.5 [a]

Table 3.4 cont.
Values are mean of triplicate measurements from two replicate treatment aquaria ± SE (n=2). Mean values recorded without error, indicate where a significant difference between replicate aquaria was found ($p<0.05$); these values were excluded from further statistical analysis. Different letter superscripts indicate significant differences between treatments ($p<0.05$). Only lipid classes and fatty acids contributing >1.5% in at least one treatment are reported. Other identified lipid classes were hydrocarbons, ethyl ketones, methyl ketones, alcohols and diacylglycerols.
Other identified fatty acids were 14:0, *i*15:0, *ai*15:0, 15:0, *i*16:0, *ai*16:0(?), 17:0, *i*17:0, *ai*17:0(?), 19:0, 20:0, 21:0, 22:0, 23:0, 24:0, 15:1, 16:1ω5, 16:1ω9(?), 16:1ω11(?), 17:1, 18:1ω5(?), 18:1ω11(?), 20:1ω7(?), 20:1ω9, 20:1ω11(?), 22:1ω7, 22:1ω9, 22:1ω11(13), 16:2ω4, 18:2, 18:2ω4, 20:2ω6, 16:3ω4(?), 18:3ω4, 18:3ω6, 20:3ω3, 20:3ω6, 16:4ω3(?), 18:4ω1(?), 18:4ω3, 20:4ω3, 22:4ω3(?), 22:4ω6(?), 21:5ω3(?), trimethyltridecanoic acid (TMTD), and pristanic acid(?).

observed among treatments for C_{20} PUFA content as well as the ω3/ω6, DHA/EPA, EPA/AA and DHA/DPA ratios were comparable to those in 39 dph larvae (Tables 3.2 and 3.3). The sum of all C_{20} and C_{22} PUFAs, was again not significantly different between treatments ($p=0.339$, data not shown).

Total lipid content evened out among dietary treatments following the gradual weaning of the cod larvae onto the same microparticulate diet. In contrast, differences in fatty acid proportions persisted, most notably AA, EPA, DHA, DPA, C_{20} PUFA, C_{22} PUFA content as well as ω3/ω6, DHA/EPA, EPA/AA and DHA/DPA ratios (Table 3.5). Thus, juveniles that were fed live food enriched with AlgaMac+*Pavlova* during the larval phase continued to differ significantly in terms of fatty acid composition compared with juveniles that were fed either Selco or Advantage-enriched live food. The total lipid content of the microparticulate diet is 201 g kg^{-1}, while the DHA/EPA and EPA/AA ratios are 1.25:1 and 12:1 respectively (Curnow *et al.* 2006).

Table 3.5. Lipid class and fatty acid composition of fully weaned Atlantic cod larvae 50 days post-hatch (~564 degree days).

	AlgaMac® + *Pavlova* sp.	Selco®	AquaGrow® Advantage
***Dry weight* (g) larvae^{-1}**	5.5 ± 0.2 [a]	5.1 ± 0.4 [ab]	4.2 ± 0.3 [b]
Total lipid (mg g^{-1} *dw*)	93.1 ± 9.4	92.6 ± 9.8	70.0 ± 10.2
Lipid classes (% of total lipid)			
Triacylgylcerols	4.4 ± 0.5 [a]	4.0 ± 0.3 [a]	2.2 ± 0.2 [b]
Free fatty acids	2.9 ± 0.6	3.4	1.7 ± 0.3
Sterols	13.7 ± 0.5	14.5 ± 0.4	18.7
Acetone mobile polar lipids	1.6 ± 0.4	1.0 ± 0.3	2.3
Phospholipids	75.4 ± 1.8	74.9 ± 0.7	72.6
Fatty acid composition (% of total fatty acid)			
16:0	15.7	14.3 ± 0.1	14.1 ± 0.1
18:0	6.6 ± 1.1	6.3 ± 0.1	6.7 ± 0.1
Sum SFA	23.8	22.4 ± 0.1	22.6 ± 0.1
16:1ω7	1.5 ± 0.0 [b]	1.7 ± 0.0 [a]	1.6 ± 0.0 [b]
18:1ω7	6.0 ± 0.1 [c]	6.4 ± 0.1 [b]	7.2 ± 0.1 [a]
18:1ω9	11.2 ± 0.1 [c]	12.8 ± 0.1 [b]	13.3 ± 0.1 [a]
Sum MUFA	22.6 ± 0.3 [b]	25.0 ± 0.3 [a]	26.2 ± 0.4 [a]
18:2ω6	4.8 ± 0.1 [a]	4.7 ± 0.2 [a]	4.0 ± 0.1 [b]
18:3ω3	2.6 ± 0.8 [b]	4.8 ± 0.1 [a]	6.0 ± 0.1 [a]
20:4ω6 (AA)	4.6 ± 0.0 [a]	4.1 ± 0.1 [b]	4.2 ± 0.1 [b]
20:5ω3 (EPA)	7.8 ± 0.2 [b]	11.7 ± 0.0 [a]	11.3 ± 0.1 [a]
22:5ω3	1.1 ± 0.0 [c]	2.1 ± 0.0 [a]	1.5 ± 0.0 [b]
22:5ω6 (DPA)	4.0 ± 0.1 [a]	0.6 ± 0.0 [b]	0.3 ± 0.0 [c]
22:6ω3 (DHA)	22.2 ± 0.5 [a]	17.5 ± 0.3 [b]	16.7 ± 0.2 [b]
Sum PUFA	51.9 ± 0.5 [a]	50.7 ± 0.4 [ab]	49.2 ± 0.5 [b]
Sum C_{20} PUFA	13.9 ± 0.2 [b]	17.4 ± 0.1 [a]	17.2
Sum C_{22} PUFA	28.3 ± 0.6 [a]	20.9 ± 0.4 [b]	19.4 ± 0.4 [b]
Sum ω3	35.4 ± 0.5 [b]	38.1 ± 0.1 [a]	37.5 ± 0.3 [a]
Sum ω6	14.6 ± 0.2 [a]	10.6 ± 0.2 [b]	9.7 ± 0.1 [c]
ω3/ ω6	2.4 ± 0.0 [c]	3.6 ± 0.1 [b]	3.9 ± 0.0 [a]
DHA/EPA	2.8 ± 0.0 [a]	1.5 ± 0.0 [b]	1.5 ± 0.0 [b]
EPA/AA	1.7 ± 0.0 [c]	2.8 ± 0.0 [a]	2.7 ± 0.0 [b]
DHA/DPA	5.6 ± 0.1 [c]	31.8 ± 0.5 [b]	61.8 ± 2.1 [a]

Table 3.5 cont.

Values are mean of triplicate measurements from two replicate treatment tanks ± SE (n=2). Mean values recorded without error, indicate where a significant difference between replicate tanks was found ($p<0.05$); these values were excluded from further statistical analysis. Different letter superscripts indicate significant differences between treatments ($p<0.05$).

Only lipid classes and fatty acids contributing >1.5% in at least one treatment are reported.

Other identified lipid classes were hydrocarbons, ethyl ketones, methyl ketones, alcohols and diacylglycerols.

Other identified fatty acids were 14:0, *i*15:0, *ai*15:0, 15:0, *i*16:0, *ai*16:0(?), 17:0, *i*17:0, *ai*17:0(?), 19:0, 20:0, 21:0, 22:0, 23:0, 24:0, 14:1, 15:1, 16:1ω5, 16:1ω9(?), 16:1ω11(?), 17:1, 18:1ω5(?), 20:1ω7(?), 20:1ω9, 20:1ω11(?), 22:1ω7, 22:1ω9, 22:1ω11(13), 16:2ω4, 18:2, 18:2ω4, 20:2ω6, 16:3ω4(?), 18:3ω4, 18:3ω6, 20:3ω3, 20:3ω6, 16:4ω1, 16:4ω3(?), 18:4ω1(?), 18:4ω3, 20:4ω3, 22:4ω3(?), 22:4ω6(?), 21:5ω3(?), trimethyltridecanoic acid (TMTD), and pristanic acid(?).

3.4.3 *Post-stress Survival and Cortisol*

Following a 15 s exposure stress and tank transfer at 41 dph (~441 dd), larvae in all three treatments had very high survival between 82 – 90 %. Larvae that were fed live food enriched with AlgaMac+*Pavlova* had significantly higher survival ($p=0.04$: 90.6%) than Advantage-fed larvae (84.5%) at 24 h post-stress (Fig. 3.6). While this pattern was repeated at 48 h post-stress, the differences in survival between treatments were not significant ($p=0.086$).

Baseline (time 0) corticosteroid values of 48-day-old (~524 dd) larvae ranged between 2 – 4 ng g^{-1} *ww*. There was no effect of treatment or stress group on baseline values ($p>0.34$). Corticosteroid values in unstressed larvae did not vary significantly with time ($p>0.267$) indicating that there was no effect of repeated sampling on whole-body corticosteroid concentration. Larvae in all treatments responded to the 15 s air exposure stress with a rapid increase in corticosteroid concentration that was significant after 30 minutes and remained elevated compared to baseline and control values after 1 h (Fig. 3.7). At 2 h post-stress, corticosteroid concentrations in Selco and Advantage larvae had decreased to baseline values, while concentrations in AlgaMac+*Pavlova* larvae remained significantly elevated compared with baseline ($p<0.001$) and unstressed values ($p<0.001$: Fig. 3.7C). The peak in corticosteroid concentration of 11.7 ng g^{-1} *ww* in stressed AlgaMac+*Pavlova* larvae at 2 h was also significantly higher than the 2.8 and 5.9 ng g^{-1} *ww* concentrations observed in unstressed ($p<0.001$) and stressed ($p=0.05$) Selco larvae respectively, at the same time interval.

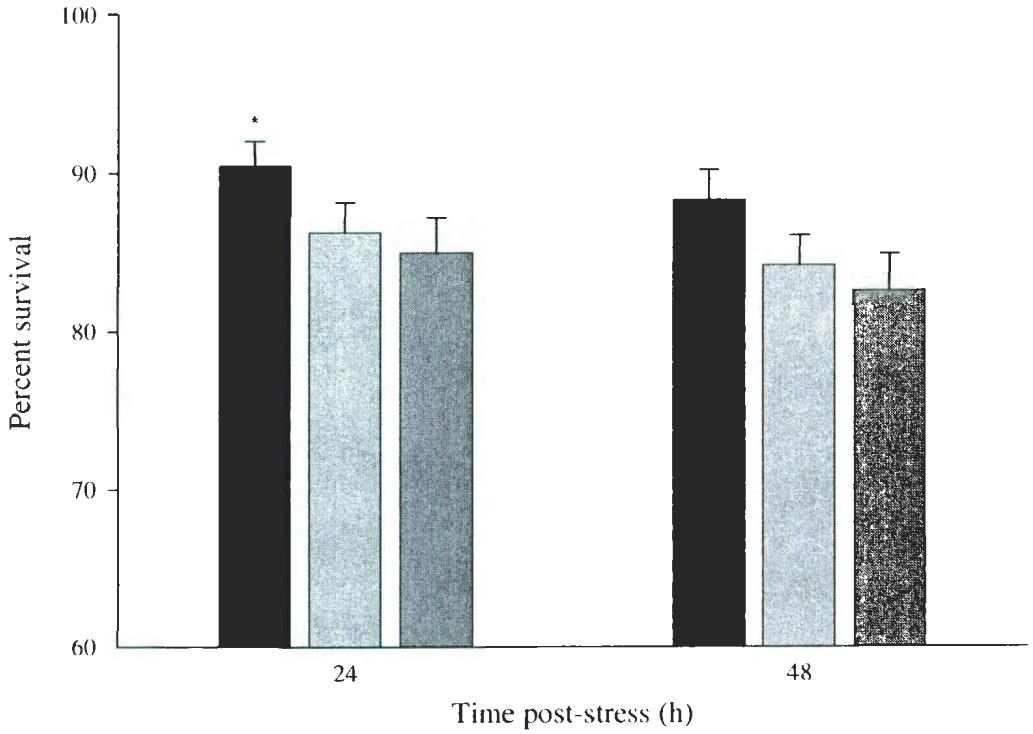
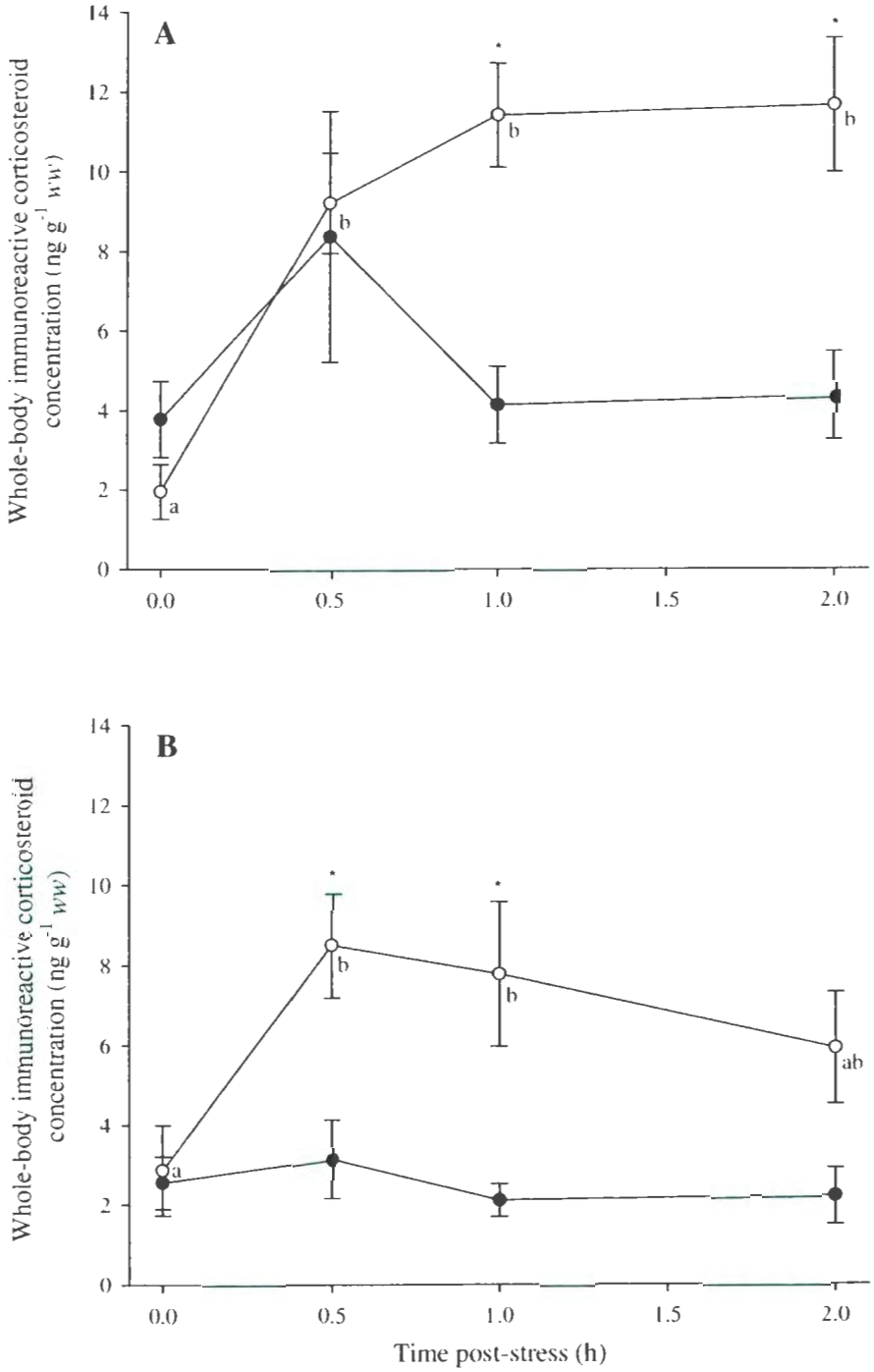


Figure 3.6. The effect of dietary treatment on survival of Atlantic cod larvae at 41 dph (~ 441 dd) following a 15 s air exposure and tank transfer stress. AlgaMac® + *Pavlova* sp. (■), Selco® (▒), and AquaGrow® Advantage (▓). * denotes significant difference from AquaGrow® Advantage ($p<0.05$). Values are means ± SE (n=8).


A
B
Whole-body immunoreactive corticosteroid concentration (ng g^{-1} ww)
Time post-stress (h)
0
2
4
6
8
10
12
14
0.0
0.5
1.0
1.5
2.0
a
b
b
b
b
ab
*

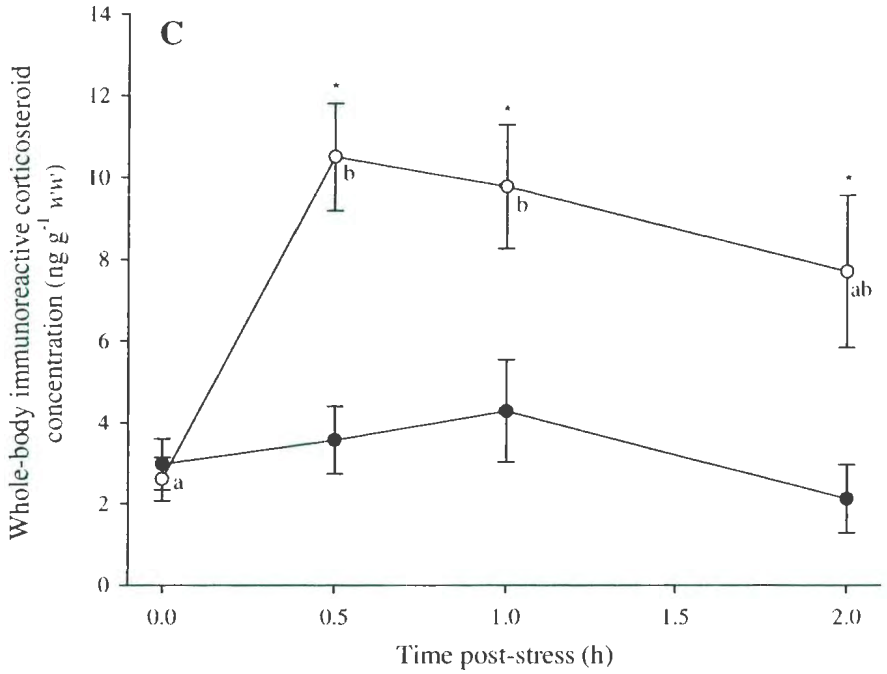


Figure 3.7. Time course of whole-body immunoreactive corticosteroid concentration following a 15 s air exposure stress in 48 dph (~524 dd) Atlantic cod larvae fed one of three different dietary treatments. (A) AlgaMac® + *Pavlova* sp., (B) Selco®, and (C) AquaGrow® Advantage. (●) Unstressed larvae, (○) stressed larvae. * denotes significant difference between stressed and unstressed values within a sampling interval ($p<0.05$). Different letters indicate significant differences between stressed values over time ($p<0.05$). Values are mean of triplicate measurements from two replicate aquaria ± SE.

3.5 Discussion

Atlantic cod larvae fed live-food organisms enriched with a combination of AlgaMac 2000® and *Pavlova* sp. had the best overall growth. Differences in myotome height and standard length between treatments were seen throughout the rotifer feeding stage and as early as 7 dph (80 dd). Larvae in the AlgaMac+*Pavlova* treatment were consistently larger than those in the Advantage treatment. In a study using similar enrichments, Garcia *et al.* (2007b) also found that Atlantic cod larvae fed a combination of rotifers enriched with AlgaMac 2000® and *Pavlova* sp. grew fastest and were largest in terms of standard length and dry weight. Several recent studies concerning the effect of rotifer lipid and fatty acid content on the early growth and survival of Atlantic cod have all emphasized the importance of a high dietary DHA/EPA ratio (O'Brien MacDonald *et al.* 2006, Park *et al.* 2006, Garcia *et al.* 2007b). Both larval dry weight and growth rate in cod have been positively correlated with tissue DHA content, as well as the tissue content of the ω6 fatty acid, AA (Cutts *et al.* 2006, Garcia *et al.* 2007b). In addition, dietary supplementation of AA in gilthead seabream was shown to enhance growth during early (21-30 dph) stages of larval development (Bessonart *et al.* 1999, Van Anholt *et al.* 2004). Rotifers in the present study that were enriched with Advantage showed no significant increase in AA compared with the unenriched sample, while those enriched with AlgaMac+*Pavlova* had the highest level of AA as well as another ω6 fatty acid docosapentaenoic acid (DPA). A similarly large amount of AA was also found in rotifers enriched with Protein Selco Plus; however, larval growth in this treatment was significantly lower than that observed for AlgaMac+*Pavlova*. This may be a result of a lower DHA/EPA ratio in the Protein Selco

Plus (1.5) rotifers compared with those enriched with AlgaMac+*Pavlova* (4.1). Bessonart *et al.* (1999) found that the positive effects of high dietary AA on growth were masked by a low dietary DHA/EPA ratio in gilthead seabream larvae. It is worth mentioning that rotifers enriched with Advantage had an exceptionally high DHA/EPA ratio, but that this did not result in optimal larval growth. These results are in agreement with the study by Garcia *et al.* (2007b), where rotifers enriched with AquaGrow Advantage had the highest DHA/EPA ratio, but resulted in the lowest larval growth and survival. These authors suggested that a low AA percentage and consequently a high EPA/AA ratio in Advantage-enriched rotifers was the cause of this poor larval performance. Competitive interactions between DHA, EPA, AA, their precursors and products occur at many physiological and biochemical levels, and thus the dietary requirement for one of these HUFAs cannot be meaningfully considered in isolation from the others (Sargent *et al.* 1999). This highlights a growing opinion in larval nutrition research that although the importance of providing adequate, and oftentimes high, levels of DHA to developing marine fish is well known and accepted, other EFAs, particularly EPA and AA, must also be provided in sufficient quantities, and that the ratio of these HUFAs to one another is what needs to be considered above all when determining optimum diets.

Comparable to the trends observed in the differentially enriched rotifers, *Artemia* enriched with AlgaMac+*Pavlova* had the largest percentages of AA and DHA, as well as the highest DHA/EPA ratio compared with both other enrichment treatments. Selco and Advantage *Artemia* had DHA/EPA ratios of less than 1, which was mostly the result of

significantly lower amounts of DHA, and in the case of the Selco treatment, a high proportion of EPA. Surprisingly, the exceptionally high level of DHA characteristic of the Advantage rotifers was not seen in the Advantage *Artemia*, although the same product was used to enrich both kinds of live-food organisms. Harel *et al.* (2002) observed that rotifers were more efficient than *Artemia* at absorbing DHA from the enrichment diet. It is also well known that during the enrichment process, *Artemia* are capable of changing both the lipid class and fatty acid composition of the supplied diet (McEvoy *et al.* 1996, Coutteau and Mourente 1997) in particular, DHA is readily retroconverted into EPA (Barclay and Zeller 1996, Navarro *et al.* 1999, Han *et al.* 2001). Navarro *et al.* (1999) also found that during periods of starvation, enriched *Artemia* showed a marked reduction in DHA content, which was attributed to a preferential oxidization of this fatty acid. In the present study, *Artemia* enriched with Advantage had the lowest total lipid content, indicating a possible 'starvation' situation, which in turn might account for the low DHA percentage. In the case of the Selco-enriched *Artemia*, the low DHA content was most likely the result of retroconversion to EPA, since this enrichment treatment was the only one that showed a significant increase in this particular fatty acid compared with unenriched *Artemia*.

Once enriched *Artemia* were added to the larval diet (25 dph, ~289 dd), the divergence in standard length between larvae in the AlgaMac+*Pavlova* treatment and both other treatments strengthened. Again, this could be attributed to high dietary AA and DHA. Improved growth in later stages of development has been seen in yellowtail (*Limanda*

ferruginea: Copeman *et al.* 2002), Japanese flounder (*Paralichthys olivaceus*: Kanazawa 1997), and red sea bream (*Pagrus major*: Furuita *et al.* 1996) larvae fed a diet enriched with DHA and in striped bass (*Morone saxatilis*: Harel *et al.* 2001) and summer flounder (*Paralichthyus dentatus*: Willey *et al.* 2003) larvae fed *Artemia* enriched with AA. The divergence in size in the present study, however, was not consistent across all growth parameters, and moreover, at the end of the rearing period, there were no differences in myotome height, condition factor or growth rate between larvae in each treatment. DHA is known to be a major component of eyes, brain and nervous tissues, and high levels of dietary DHA are required during periods of rapid development, such as early larval stages. Consequently, the requirements of developing marine fish larvae for highly unsaturated fatty acids of the ω3 type are typically higher than those of juveniles and pre-adults (Sargent *et al.* 2002). Before becoming juveniles, Atlantic cod larvae undergo a period of metamorphosis, which typically coincides with weaning and the end of *Artemia* feeding (Stoss *et al.* 2004). This may explain why at the end of the rearing period in the present study, larvae fed *Artemia* with a high DHA content (AlgaMac+*Pavlova*) did not show an overwhelmingly better growth capacity compared with the other dietary treatments. The effectiveness of AA enrichment on growth promotion may also decline with larval age. Willey *et al.* (2003) found no significant difference in dry weight amongst larval summer flounder fed *Artemia* enriched with various amounts of AA. They did, however, observe a significant effect of elevated rotifer AA content on larval growth through the *Artemia* stage and regardless of *Artemia* AA content, suggesting that the effectiveness of AA may be more critical during the early progression of larval

development. Even though high dietary DHA and AA may have less of a growth promoting effect during advanced larval stages in Atlantic cod, it is important to note that the differences in size between dietary treatments were carried through, and perhaps even exaggerated, in fully weaned juvenile fish. Juveniles at 50 dph that were fed live food enriched with AlgaMac+*Pavlova* in the larval phase continued to be the largest fish in terms of standard length, myotome height and dry weight. In addition, the fatty acid composition of all juveniles remained reflective of the live-food enrichments on which they fed during the larval stage, such that those in the AlgaMac+*Pavlova* treatment had higher proportions of DHA and AA as well as the highest DHA/EPA ratio. These results re-emphasize the importance of supplying adequate amounts of dietary EFAs during larval development, so as to promote optimal growth in juvenile and adult stages. It is also possible that supplying high levels of some fatty acids, namely DHA and AA, in early development serve to biologically prime larvae, resulting in an enhanced larval condition, which persists throughout later development, metamorphosis and weaning.

Late in the larval stage, Atlantic cod acquire cannibalistic behaviour, which can result in extremely high mortality, and is typically prevented by periodic fish grading. The process of grading, however, can be intensely stressful; it generally involves subjecting the young larvae/juveniles to netting, brief air exposure and transfer to a new tank, and some degree of mortality is usually observed. The frequency and duration of the stress will ultimately determine the extent of grading-related mortality; however, larval nutrition may also play a vital role. Supplementation of DHA in the larval diet was shown to increase vitality

(survival following an acute air exposure) in yellowtail (Furuita *et al.* 1996), red sea bream (Kanazawa 1997), and Japanese flounder (Furuita *et al.* 1998) larvae. Furthermore, Harel *et al.* (2001) found that striped bass larvae with high levels of tissue DHA had better survivorship following a hypersaline immersion, but that larvae with high levels of both DHA and AA had maximal survivorship. The positive effect of AA on larval acute stress resistance has also been seen in Japanese flounder (Furuita *et al.* 1998), gilthead sea bream (Koven *et al.* 2001, 2003), and summer flounder (Willey *et al.* 2003). Therefore, it is not surprising that in the present study, the treatment with the highest survival following a 15 s air exposure and tank transfer stress was AlgaMac+*Pavlova*. At 24 h post-stress, survivorship of larvae in the AlgaMac+*Pavlova* treatment was significantly better than the Advantage treatment, and was likely a result of less AA and a lower proportion of DHA/EPA in the tissue of Advantage larvae. Survival at 24 h in the Selco treatment was not statistically different from either of the other treatments, which is consistent with the finding of an intermediate amount of tissue AA in Selco larvae. On the other hand, no differences in tissue DHA were observed between treatments, and the DHA/EPA ratio of Selco larvae (1.5) was almost identical to that of Advantage larvae (1.4), but significantly lower than AlgaMac+*Pavlova* larvae (3.2). This suggests that in contrast to growth, the stress resistance benefits of high AA are not concealed by a low DHA/EPA ratio, and that AA may play a more important role than DHA in the survival of Atlantic cod following an acute stress such as grading. Although the survival differences at 48 h post-stress were not statistically significant, the pattern was highly

comparable to the one observed at 24 h, the p-value (0.08) was approaching α, and consequently the effect of dietary treatment should not be discounted as transitory.

Eicosanoids are known to play many roles in several physiological processes, and, as stated by Sargent *et al.* (1999), in a broad sense, they are produced in response to stressful situations. Thus, the complexity of the relationship between AA, stress and survival, stems from the involvement of AA in the production of these highly active molecules. Larvae in all three treatments showed a marked increase in AA over the rearing period, resulting in a 3-fold, and in the case of AlgaMac+*Pavlova*, a 6-fold, decrease in the EPA:AA ratio compared with the value at hatch. Feeding rotifers and *Artemia* enriched with AlgaMac+*Pavlova* not only resulted in an increased AA content of the larvae, but also a decreased EPA content in comparison to the other two treatments. This is most likely the result of competition between AA and EPA for a limited number of esterification sites in membrane phospholipids (Lands 1991, Bell *et al.* 1995, Bessonart *et al.* 1999). These two HUFAs also compete for the same enzymes involved in eicosanoid synthesis, such that if there is more EPA present in the tissue, an abundance of prostaglandins of the three series (PGE_3 and PGF_3) will be produced, thereby inhibiting the synthesis of the more biologically active prostaglandins (PGE_2 and PGF_2) from AA (Henderson *et al.* 1985, Bell *et al.* 1995). The specific functions of these two types of PGs in the stress response of fish are not completely understood, however, PGs are known to modulate the sensitivity of the HPI axis and ultimately the release of cortisol, which is the main corticosteroid in fish (Gupta *et al.* 1985, Van Anholt *et al.* 2003).

Furthermore, evidence from mammalian studies suggest that AA and other HUFA-derived eicosanoids regulate steroidogenesis, the release of hormones from the hypothalamus and pituitary, as well as the binding of cortisol to intracellular receptors (for review see Lands 1991, Sumida 1995). In a previous study on the ontogeny of the stress response (Chapter 2), it was observed that although Atlantic cod larvae are able to respond to an acute stress by means of an increase in corticosteroid levels as early as at hatch, the magnitude of this response is significantly lower during the first 2 weeks post-hatch compared with the response in older larvae (>30 dph). This period of decreased sensitivity is referred to as the stress hyporesponsive period (SHRP), and the complete mechanism responsible has yet to be elucidated (Yoshiumura *et al.* 2003). The high EPA/AA ratio found at hatch (~7.5/1), in the current study, however, might well account for the SHRP seen in early developmental stages of larval cod. Once larvae begin exogenous feeding, they readily start incorporating AA into cellular membranes, thereby decreasing the tissue EPA/AA ratio. When more AA is available, the potential for a stronger production of AA-derived eicosanoids increases, such that once a stress is perceived, the stimulation of the HPI axis would occur faster and with more intensity, resulting in a larger release of corticosteroids. A temporary increase in circulating cortisol is considered an adaptive response to stress, which enhances survival by facilitating the mobilization of energy stores, while simultaneously maintaining homeostasis (Wendelaar Bonga 1997). Therefore, supplying high levels of dietary AA to Atlantic cod larvae during first feeding is not only important in terms of promoting growth, but is also crucial to the proper development of the adaptive stress response.

In all dietary treatments, Atlantic cod larvae at 46 dph (~512 dd) responded to a 15 s air exposure with a rapid increase in whole-body corticosteroid levels within 30 minutes of the stress. It has been suggested that cod larvae have a functioning HPI axis at hatch (Chapter 2), and as a result, this increase is most likely the result of *de novo* synthesis of cortisol by the larvae. While larval corticosteroid levels in the Selco and Advantage treatments began to decrease 2 h following the stress, peak levels (11.7 ng g^{-1} *ww*) persisted in the AlgaMac+*Pavlova* larvae. This contrasts with the attenuation of the cortisol response observed in gilthead sea bream larvae fed *Artemia* enriched with a high level of AA (Van Anholt *et al.* 2004). The moderating effect of cortisol, however, was found to be less pronounced in older post-metamorphosis larvae (Van Anholt *et al.* 2004). As stated previously, cod larvae at the end of the present study were most likely in the early stages of metamorphosis; this may, in part, account for the absence of attenuation in the corticosteroid response of larvae in the AlgaMac+*Pavlova* treatment. Another unexpected result was the high variability in the corticosteroid response of unstressed AlgaMac+*Pavlova* larvae at the second sampling interval (30 min). Variation of this degree was not seen at any other sampling interval or in either of the other treatments. Although the mean corticosteroid concentration at this time (8.4 ng g^{-1} *ww*) for this group was not significantly elevated compared with the value at time 0 (3.8 ng g^{-1} *ww*), it was also not statistically different from the value of the stressed group at 30 min (9.2 ng g^{-1} *ww*). These observations suggest that AlgaMac+*Pavlova* larvae not subjected to an air exposure, nevertheless reacted to a single repeated sampling event by

means of the corticosteroid stress response. This increased sensitivity to a mild stress may well be explained by the low tissue EPA:AA in AlgaMac+*Pavlova* larvae. Given that AA is the preferred substrate for eicosanoid biosynthesis in fish (Tocher 2003), a decrease in EPA and its competitive dampening effects would thus increase the availability of free AA for eicosanoid production and the subsequent stimulation of the HPI axis. It is important to note that a prolonged corticosteroid stress response in AlgaMac+*Pavlova* larvae was not associated with a decrease in post-stress survival. As previously discussed, larvae from this treatment had the best survival following an air exposure and tank transfer stress. It is, therefore conceivable that part of the adaptive response to stress in Atlantic cod larvae is the requirement of a sustained elevation in circulating corticosteroids for at least 2 h post-stress. Higher mortality observed in the Selco and Advantage treatments may have been caused by a rapid depression in AA-derived eicosanoid production by means of a greater proportion of EPA in the tissue, which resulted in an inability to maintain high circulating corticosteroid levels for an adequate period of time following the stress.

Resting whole-body corticosteroid levels of <10 ng g^{-1} *ww* in 48 dph cod larvae are comparable to resting plasma cortisol levels (ng mL^{-1}) in 10 g juvenile cod. In contrast, peak corticosteroid levels of 8-12 ng g^{-1} *ww* in acutely stressed cod larvae are 20-fold lower than plasma cortisol levels in acutely stressed juvenile cod (Pérez-Casanova *et al.* in press). There is a lack of correspondence between whole-body and plasma cortisol samples on account of whole-body samples being expressed relative to the weight of an

entire larvae or multiple larvae while plasma concentrations are expressed relative to the volume of a specific bodily fluid in which cortisol is found. This results in plasma samples being more concentrated per unit volume and thus appearing bigger as an absolute value compared with whole-body samples. For further discussion on the differences between whole-body corticosteroids and plasma cortisol see Chapter 2.

Recent studies have highlighted the importance of ω6DPA in the nutrition of marine organisms (Milke *et al.* 2006, Parrish *et al.* 2007, Garcia *et al.* 2007a,b). At present, it is not considered to be an EFA for marine fish, however, mounting evidence suggests otherwise. Parrish *et al.* (2007) demonstrated that larvae of Atlantic cod do not synthesize ω6DPA from other fatty acids, and when it is made available in the diet, it is readily taken up without biotransformation. In addition, cod larvae fed rotifers and *Artemia* enriched with high amounts of this particular fatty acid showed significantly higher growth rates and the best rearing survival (Garcia *et al.* 2005, 2007a). In the present study, best overall growth and post-stress survival was seen in the same dietary treatment, AlgaMac+*Pavlova*, which was followed by Selco and then Advantage. It is interesting to note that the only fatty acid parameter measured in larval tissue (at both 39 and 46 dph) that followed this same trend was the ratio of DHA/DPA. For most other parameters (ie. DHA/EPA, EPA/AA, ω3/ω6, etc.) there was overlap between the treatments, especially between Selco and Advantage. Larvae fed Selco-enriched live feed, however, had generally better growth and a tendency toward better post-stress survival than larvae in the Advantage treatment. These results confirm the positive effects of ω6DPA on growth

in larval cod, suggest an important role for this particular fatty acid in stress tolerance, and further strengthen the argument for including ω6DPA in marine teleost nutrition research.

Despite significant variation in tissue content of C_{20} PUFA, C_{22} PUFA, LNA, LA, AA, EPA, DHA and DPA, the sum of C_{20} and C_{22} PUFAs as well as total PUFA remained unchanged between larvae of different dietary treatments. This indicates that on a cellular level, cod larvae require a specific proportion of C_{20}+C_{22} PUFA as well as total PUFA, but that there is a degree of plasticity in terms of specific fatty acids that make up this proportion. It appears that PUFA may be able to substitute for each other and maintain phospholipid membrane structure, provided that a certain proportion of total PUFA is conserved and that a specific amount of this total PUFA contains C_{20} or C_{22} chains.

In summary, Atlantic cod larvae fed live food enriched with AlgaMac+*Pavlova* were found to have the best overall growth and highest resistance to an acute air exposure stress. The DHA/EPA/AA contents of rotifers and *Artemia* enriched with AlgaMac+*Pavlova* sp. were 7.3/1.9/1 and 4.6/2.1/1 respectively. These ratios are in agreement with those found by Garcia *et al.* (2007a,b) who suggested optimal ratios for larval cod of 11/1.5/1 in rotifers and 7/2/1 in *Artemia*. These ratios also reflect the requirement of cod larvae for high proportions of dietary DHA and AA early in development, and that these requirements, especially for DHA, most likely decrease with age. In contrast, live food enriched with Advantage (69.5/2.8/1 in rotifers, 1.8/4/1 in

Artemia) or Selco (3.5/2.5/1 in rotifers, 3.5/5/1 in *Artemia*) had consistently lower proportions of AA and, with the exception of Advantage-enriched rotifers, DHA. Larvae in these dietary treatments showed reduced growth and higher stress-related mortality. High dietary AA and a low tissue EPA:AA ratio in AlgaMac+*Pavlova* larvae did not result in an attenuation of the corticosteroid stress response, as was expected. Nevertheless, post-stress survival in this treatment was highest overall and was statistically better than the survival of larvae in the Advantage treatment. It is therefore possible that cod larvae require a prolonged elevation in circulating corticosteroids in order to effectively cope with an acute stressor, and that larvae with high proportions of tissue EPA are unable to sustain this response due to a decrease in AA-derived eicosanoid production. Further research regarding the effect of fatty acid nutrition on the corticosteroid response and survival of cod larvae following a stress is required, especially for younger, pre-metamophosing stages as well as for different acute and chronic stressors relevant in intensive cod culture.

Concluding Remarks and Suggestions for Future Research

The commercially viable mass rearing of Atlantic cod in North America is possible. Successful European operations should be looked upon as models; however, their procedures and protocols require adaptation to a different set of abiotic conditions as well as potential broodstock populations. Intensive research into all aspects of cod culture has generated a plethora of data, but there is still much to be understood, especially with regards to the critical larval phase. Results from the present studies have revealed new information regarding the ontogeny of the corticosteroid stress response and how fatty acid nutrition can influence this response in a mass-rearing situation. Also, a practical enzyme-linked immunoassay (ELISA) for the detection of immunoreactive corticosteroids (IRC) was validated for use with whole body samples of larval cod.

A significant increase in IRC levels in response to a stress was observed in hatchling Atlantic cod, suggesting that the *de novo* synthesis of cortisol was occurring. In order to verify this assertion, further research must be directed into establishing the presence of functional interrenal cells at this age. The magnitude of the corticosteroid stress response during the first two weeks post-hatch was reduced compared with older larvae. This indicated a potential stress hyporesponsive period (SHRP), during which the release of cortisol is minimized in order to mitigate its potentially negative effects on neurological development. The complete mechanism behind the SHRP in fish as well as in mammals has not been completely elucidated. The relatively high EPA/AA ratio found at hatch may partially explain the SHRP in cod, such that a high amount of tissue EPA would

minimize the synthesis of AA-derived eicosanoids thereby limiting their stimulatory effect on the HPI axis, and the subsequent release of cortisol following a stress. A secondary SHRP may also be present during metamorphosis in cod. A more detailed study regarding changes to the corticosteroid stress response of cod during metamorphosis, as well as the potential effect of high circulating corticosteroids on the development of the immune system is required.

With regards to the dietary requirements of cod larvae for specific fatty acids, the results from the present study underline the ubiquitous need for high dietary DHA in developing marine teleosts. High levels of DHA, AA and ω6DPA in live food enrichments appear to promote growth and survival throughout the larval phase and these benefits may have the potential to carry on into juvenile stages. It would be very interesting to follow a cohort of cod from hatch, through the larval phase, during which groups would be fed differently enriched live food, past metamorphosis, onto maturity, and see how far these effects of larval nutrition are passed. A tissue EPA/AA ratio of ~1/1 seems to promote survival following an acute air-exposure. The beneficial effects of high AA in terms of stress tolerance, however, are almost certainly affected by numerous conditions including larval age and type (ie. salinity, temperature, crowding) and duration (acute *vs* chronic) of the stress. Additional studies where these parameters are varied, especially to reflect potential culture conditions, are needed in order to determine the optimal dietary level of AA.

Although a low ratio of EPA/AA resulted in maximal survivorship following an acute stress, it was not associated with a decrease in the corticosteroid response. Consequently, it is possible that cod larvae require a prolonged elevation in corticosteroid levels post-stress in order to maintain homeostasis. A high tissue EPA/AA ratio would abbreviate the response, resulting in increased mortality. Very few studies have focused on determining the specific effects of fatty acid nutrition on the release of cortisol in response to a stress. In the present study, it is difficult to ascertain whether the differences in the corticosteroid response were solely the result of differences in the EPA/AA ratio, and thus a study in which the dietary content of AA is specifically manipulated, is merited.

References

Afonso, L.O.B., Hosoya, S., Osborne, J., Gamperl, A.K., Johnson, S., 2007. Lack of glucose and hsp70 in haddock *Melanogrammus aeglefinus* (L.) subjected to handling and heat shock. J. Fish Biol. 71, 1-11.

Alsop, D., Vijayan, M.M., 2008. Development of the cortiocosteroid stress axis and receptor expression in zebrafish. Am. J. Physiol. Regul. Integr. Comp. Physiol. (in press)

Arai, M. and Widmaier, E.P., 1991. Activation of the pituitary-adrenocortical axis in day-old rats by insulin-induced hypoglycemia. Endocrinology. 129, 1505-1512.

Ayson, F.G., Kaneko, T., Hasegawa, S., Hirano, T., 1995. Cortisol stimulates the size and number of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia (*Oreochromis mossambicus*) *in vitro* and *in vivo*. J. Exp. Zool. 272, 419-425.

Barclay, W. and Zeller, S., 1996. Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. J. World Aquacult. Soc. 27, 314-322.

Barry, T.P., Lapp, A.F., Kayes, T.B., Malison, J.A., 1993. Validation of a microtitre plate ELISA for measuring cortisol in fish and comparison of stress responses of rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*). Aquaculture 117, 351-363.

Barry, T.P., Malison, J.A., Held, J.A., Parrish, J.J., 1995a. Ontogeny of the cortisol stress response in larval rainbow trout. Gen. Comp. Endocrinol. 97, 57-65.

Barry, T.P., Ochiai, M., Malison, J.A., 1995b. *In vitro* effects of ACTH on interrenal corticosteroidogenesis during early larval development in rainbow trout. Gen. Comp. Endocrinol. 99, 382-387.

Barton, B.A., 1997. Stress in finfish: Past, present and future - a historical perspective. Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Cambridge University Press, Cambridge, pp. 1-33.

Barton, B.A. and Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. 1, 3-26.

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integ. Comp. Bio. 42, 517-525.

Baskerville-Bridges, B. and Kling, L.J., 2000a. Early weaning of Atlantic cod (*Gadus morhua*) larvae onto a microparticulate diet. Aquaculture 189, 109-117.

Baskerville-Bridges and Kling, 2000b. Development and evaluation of microparticulate diets for early weaning of Atlantic cod *Gadus morhua* larvae. Aquacult. Nutr. 6, 171-182.

Bell, J.G. and Sargent, J.R., 2003. Arachidonic acid in aquaculture feeds: Current status and future opportunities. Aquaculture 218, 491-499.

Bell, J.G., Dick, J.R., McVicar, A.H., Sargent, J.R., Thompson, K.D., 1993. Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar*). Pros. Leuk. Ess. Fatty Acids 49, 665-673.

Bell, J.G., D R Tocher, J R Sargent, 1994. Effect of supplementation with 20:3(*n*-6), 20:4(*n*-6) and 20:5(*n*-3) on the production of prostaglandins E and F of the 1-, 2- and 3-series in turbot (*Scophthalmus maximus*) brain astroglial cells in primary culture. Bioch. et Biophys. Acta 1211, 335-42.

Bell, J.G., Castell, J.D., Tocher, D.R., MacDonald, F.M., Sargent, J.R., 1995. Effects of different dietary arachidonic acid:docosahexaenoic acids ratios on phospholipid fatty acid compositions and prostaglandin production in juvenile turbot, *Scophthalmus maximus*. Fish Physiol. Biochem. 14, 139-151.

Brett, J.R., 1958. Implications and assessment of environmental stress. In: Larkin, P.A. (Ed.), The Investigation of Fish-Power Problems. MacMillan Lectures in Fisheries, Vancouver, pp. 69-83.

Brown, J.A., Edwards, D., Whitehead, C., 1989. Cortisol and thyroid hormone responses to acid stress in the brown trout, *Salmo trutta* L. J. Fish Biol. 35, 73-84.

Brown, J.A., Minkoff, G., Puvanendran, V., 2003. Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems. Aquaculture 227, 357-372.

Budavari, S., 1989. #4710. In: Merck Index. Merck & Co. Inc., Rahway, NJ.

Cahu, C. and Infante, J.Z., 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture. 200, 161-180.

Charmandari, E., Tsigos, C., Chrousos, G., 2005. Endocrinology of the stress response. Annu. Rev. Physiol. 67, 259-284.

Copeman, L.A., Parrish, C.C., Brown, J.A., Harel, M., 2002. Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. Aquaculture 210, 285-304.

Curnow, J., King, J., Partridge, G., Kolkovski, S., 2006. Effects of two commercial microdiets on growth and survival of barramundi (*Lates calcarifer* Bloch) larvae within various early weaning protocols. Aquacult. Nutr. 12, 247-255.

Cutts, C.J., Sawanboonchun, J., Mazorra de Quero, C., Bell, J.G., 2006. Diet-induced differences in the essential fatty acid (EFA) compositions of larval Atlantic cod (*Gadus morhua* L.) with reference to possible effects of dietary EFAs on larval performance. ICES J. Mar. Sci. 63, 302-310.

de Jesus, E.G.T. and Hirano, T., 1992. Changes in whole body concentrations of cortisol, thyroid hormones, and sex steroids during early development of the chum salmon, *Oncorhynchus keta*. Gen. Comp. Endocrinol. 85, 55-61.

de Jesus, E.G.T., Hirano, T., Inui, Y., 1991. Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder *Paralichthys olivaceus*. Gen. Comp. Endocrinol. 82, 369-376.

De Silva, S.S. and Anderson, T.A., 1995. Fish Nutrition in Aquaculture. Chapman and Hall, London.

Deane, E.E. and Woo, N.Y.S., 2003. Ontogeny of thyroid hormones, cortisol, hsp10 and hsp90 during silver sea bream larval development. Aquaculture 72, 805-818.

Dehert, P., 1999. Culture and manipulation techniques in live food production. In: Lee, C.-S., O'Bryen, P. (Eds.), Advanced Bio-technology in hatchery production. Proceedings of an Aquaculture Interchange Program Workshop, 6-9 December 1999, Honolulu, Hawaii, USA.

Estévez, A., McEvoy, L.A., Bell, J.G., Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in arachidonic and eicosapentaenoic acids. Aquaculture 180, 321-343.

Feist, G. and Schreck, C.B., 2002. Ontogeny of the stress response in chinook salmon, *Oncorhynchus tshawytscha*. Fish Physiol. Biochem. 25, 31-40.

Folkvord, A., 1997. Ontogeny of cannibalism in larval and juvenile fishes with special emphasis on Atlantic cod. In: Chambers, R.C. and Trippel, E.A. (Eds.), Early Life History and Recruitment in Fish Populations. Chapman & Hall, London, pp. 251-278.

Fraser, A.J., Gamble, J.C., Sargent, J.R., 1988. Changes in lipid content, lipid class composition and fatty acid composition of developing eggs and unfed larvae of cod *(Gadus morhua)* . Mar. Biol. 99, 307-319.

Furuita, H., Takeuchi, T., Toyota, M., Watanabe, T., 1996. EPA and DHA requirements in early juvenile red sea bream using HUFA enriched *Artemia* nauplii. Fish. Sci. 62, 246-251.

Furuita, H., Takeuchi, T., Uematsu, K., 1998. Effects of eicosapentaenoic and docosahexaenoic acids on growth, survival and brain development of larval Japanese flounder (*Paralichthys olivaceus*). Aquaculture 161, 269-279.

Gadek-Michalska, A., Bugajski, J., Bugajski, A.J., Glod, R., 2002. Effect of adrenergic antagonists and cyclooxygenase inhibitors on the nicotine-induced hypothalamic-pituitary-adrenocortical activity. J. Phys. Pharm. 53, 275-287.

Gapasin, R.S.J. and Duray, M.N., 2001. Effects of DHA-enriched live food on growth, survival and incidence of opercular deformities in milkfish (*Chanos chanos*). Aquaculture 193, 49-63.

Gapasin, R.S.J., Bombeo, R., Lavens, P., Sorgeloos, P., Nelis, H., 1998. Enrichment of live food with essential fatty acids and vitamin C: effects on milkfish (*Chanis chanos*) larval performance. Aquaculture 162, 269-286.

Garcia, A.S., Parrish, C.C., Brown, J.A., 2005. Effect of different live food enrichments on early growth and lipid composition of Atlantic cod larvae (*Gadus morhua*). European Aquaculture Society. Special Pub. 36, 164-167.

Garcia, A.S., Parrish, C.C., Brown, J.A., 2007a. Growth and lipid composition of Atlantic cod (*Gadus morhua*) larvae in response to differently enriched *Artemia franciscana*. Fish Physiol. Biochem. (in press).

Garcia, A.S., Parrish, C.C., Brown, J.A., 2007b. A comparison among differently enriched rotifers (*Brachionus plicatilis*) and their effect on Atlantic cod (*Gadus morhua*) larvae early growth, survival and lipid composition. Aquacult. Nutr. (in press).

Gupta, O.P., Lahlou, B., Botella, J., Porthe-Nibelle, J., 1985. *In vivo* and *in vitro* studies on the release of cortisol from interrenal tissue in trout. I. effects of ACTH and prostaglandins. Exp. Biol. 43, 201-212.

Hamre, K., 2006. Nutrition in cod (*Gadus morhua*) larvae and juveniles. ICES J. Mar. Sci. 63, 267-274.

Hamre, K., Opstad, I., Espe, M., olbakken, J., Hemre, G.I., Pittman, K., 2002. Nutrient composition and metamorphosis success of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae fed natural zooplankton or *Artemia*. Aquacult. Nutr. 8, 139-148.

Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and subsequently starved *Artemia franciscana* nauplii enriched with different essential fatty acids. Aquaculture 199, 93-105.

Harel, M. and Place, A.R., 1999. The nutritional quality of live feeds for fish larvae. Bull. Aquacult. Assoc. Canada 98, 6-11.

Harel, M., Gavasso, S., Leshin, J., Gubernatis, A., Place, A.R., 2001. The effect of tissue docosahexaenoic and arachidonic acids levels on hypersaline tolerance and leucocyte

composition in striped bass (*Morone saxatilis*) larvae. Fish Physiol. Biochem. 24, 113-123.

Harel, M., Koven, W.M., Lein, I., Barr, Y., Behrens, P., Stubblefield, J., Zohar, Y., Place, A.R., 2002. Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. Aquaculture 213, 347-362.

Henderson, R.J., Bell, M.V., Sargent, J.R., 1985. The conversion of polyunsaturated fatty acids to prostaglandins by tissue homogenates of the turbot, *Scophthalmus maximus* (L.). J. Exp. Mar. Biol. Ecol. 85, 93-99.

Herbert, N.A., Steffensen, J.F., 2005. The response of Atlantic cod, *Gadus morhua*, to progressive hypoxia: fish swimming speed and physiological stress. Mar. Biol. 147, 1403-1412.

Hirai, A., Tahara, K., Tamura, Y., Saito, H., Terano, T., Yoshida, S., 1985. Involvement of 5-lipoxygenase metabolites in ACTH-stimulated corticosteroidogenesis in rat adrenal glands. Prostaglandins 30, 749-767.

Hiroi, J., Sakakura, Y., Tagawa, M., Seikai, T., Tanaka, M., 1997. Developmental changes in low-salinity tolerance and responses of prolactin, cortisol and thyroid hormones to low-salinity environment in larvae and juveniles of Japanese flounder, *Paralichthys olivaceus*. Zool. Sci. 14, 987-992.

Holt, G.J., 1993. Feeding larval red drum on microparticulate diets in a closed recirculating water system. J. World Aquacult. Soc. 24, 225-230.

Hunt von Herbing, I., Boutilier, R.G., Miyake, T., Hall, B.K., 1996. Effects of temperature on morphological landmarks critical to growth and survival in larval Atlantic cod (*Gadus morhua*). Mar. Biol. 124, 593-606.

Hwang, P., Wu, S., Lin, J., Wu, L., 1992. Cortisol content of eggs and larvae of teleosts. Gen. Comp. Endocrinol. 86, 189-196.

Iguchi, K., Ogawa, K., Nagae, M., Ito, F., 2003. The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*). Aquaculture 220, 515-523.

Iwama, G.K., Vijayan, M.M., Forsyth, R.B., Ackerman, P.A., 1999. Heat shock proteins and physiological stress in fish. Amer. Zool. 39, 901-909.

Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish larvae. Aquacult. Nutr. 2, 183-191.

Izquierdo, M.S., Socorro, J., Arantzamendi, L., Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem. 22, 97-107.

Jentoft, S., Held, J.A., Malison, J.A., Barry, T.P., 2002. Ontogeny of the cortisol stress response in yellow perch (*Perca flavescens*). Fish Physiol. Biochem. 26, 371-378.

Kanazawa, A., 1997. Effects of docosahexaenoic acid and phospholipids on stress tolerance of fish. Aquaculture 155, 129-134.

Kanazawa, A., Koshio, S., Teshima, S., 1989. Growth and survival of larval red sea bream *Pagrus major* and Japanese flounder *Paralichthys olivaceus* fed microbound diets. J. World Aquacult. Soc. 20, 31-37.

King, W. and Berlinsky, D.L., 2006. Whole-body corticosteroid and plasma cortisol concentrations in larval and juvenile Atlantic cod *Gadus morhua* L. following acute stress. Aquacult. Res. 37, 1282-1289.

Kolkovski, S., Koven, W., Tandler, A., 1997. The mode of action of *Artemia* in enhancing utilization of microdiet by gilthead seabream *Sparus aurata* larvae. Aquaculture 155, 193-205.

Koven, W.M., Barr, Y., Lutzky, S., Ben Atia, I., Weiss, R., Harel, M., Behrens, P., Tandler, A., 2001. The effect of dietary arachidonic acid (20:4*n* - 6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture 193, 107-122.

Koven, W.M., Van Anholt, R.D., Lutzky, S., Ben Atia, I., Nixon, O., Ron, B., Tandler, A., 2003. The effect of dietary arachidonic acid on growth, survival, and cortisol levels in different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or daily salinity change. Aquaculture 228, 307-320.

Lands, W.E.M., 1991. Biosynthesis of prostaglandins. Annu. Rev. Nutr. 11, 41-60.

Lankford, S.E., Adams, T.E., Miller, R.A., Cech Jr., J.J., 2005. The cost of chronic stress: impacts of a nonhabituating stress response on metabolic variables and swimming performance in sturgeon. Physiol. Biochem. Zool. 78, 599-609.

Leger, P., Bengtson, D.A., Simpson, K.L., Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source. In: Barnes, M. (Ed.), Marine Biology and Oceanography: An Annual Review. Aberdeen Univ. Press, pp. 521-623.

Magnadottir, B., Lange, S., Steinarsson, A., Gudmundsdottir, S., 2004. The ontogenic development of innate immune parameters of cod (*Gadus morhua* L.). Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 139, 217-224.

Maule, A., Tripp, R., Kaattari, S., Schreck, C., 1989. Stress alters immune function and disease resistance in chinook salmon (*Oncorhynchus tshawytscha*). J Endocrinol 120, 135-142.

Migaud, H., Cowan, M., Taylor, J., Ferguson, H.W., 2007. The effect of spectral composition and light intensity on melatonin, stress and retinal damage in post-smolt Atlantic salmon, *Salmo salar*. Aquaculture 270, 390-404.

Milke, L., Bricelj, V.M., Parrish, C., 2006. Comparison of early life history stages of the bay scallop, *Argopecten irradians*: effects of microalgal diets on growth and biochemical composition. Aquaculture 260, 272-289.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211-268.

Monk, J., Puvanendran, V., Brown, J.A., 2006. Do different light regimes affect the foraging behaviour, growth and survival of larval cod (*Gadus morhua* L.)? Aquaculture 257, 287-293.

Morrison, C.M., 1993. Histology of the Atlantic cod, *Gadus morhua*: an atlas. Part four. Eleutheroembryo and larva. Can. Spec. Publ. Fish. Aquat. Sci. 119, 496.

Morrison, W.R. and Smith, L.M., 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J. Lipid Res. 5, 13-23.

Mourente, G., Vásquez, R., 1995. Changes in the content of total lipid, lipid classes and their fatty acids of developing eggs and unfed larvae of the Senegal sole, *Solea senegalensis* Kaup. Fish Physiol. Biochem. 15, 221-235.

Mourente, G., Rodríguez, A., Grau, A., Pastor, E., 1999. Utilization of lipids by *Dentex dentex* L. (Osteichthyes, Sparidae) larvae during lecitotrophia and subsequent starvation. Fish Physiol. Biochem. 21, 45-58.

Mustafa, T. and Srivastava, K.C., 1989. Prostaglandins (eicosanoids) and their role in ectothermic organisms. In: Gilles, R. (Ed.), Advances in Comparative and Environmental Physiology. Springer-Verlag, Berlin, pp. 157-207.

Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversions during enrichment of *Artemia*. Aquaculture 174, 155-166.

O'Brien-MacDonald, K., Brown, J.A., Parrish, C.C., 2006. Growth, behaviour, and digestive enzyme activity in larval Atlantic cod (*Gadus morhua*) in relation to rotifer lipid. ICES J. Mar. Sci. 63, 275-284.

Olsen, Y., van der Meeren, T., Reitan, K.I., 2004. First feeding technology. In: Moksness, E., Kjorsvik, E., Olsen, Y. (Eds.), Culture of Cold-Water Marine Fish. Blackwell Publishing Ltd., Oxford, pp. 279-333.

Park, H.G., Puvanendran, V., Kellett, A., Parrish, C.C., Brown, J.A., 2006. Effect of enriched rotifers on growth, survival and composition of larval Atlantic cod (*Gadus morhua*). ICES J. Mar. Sci. 63, 285-295.

Parrish, C.C., 1987. Separation of aquatic lipid classes by chromarod thin-layer chromatography with measurement by iatroscan flame detection. Can. J. Fish. Aquat. Sci. 44, 722-731.

Parrish, C.C., 1999. Determination of total lipid, lipid classes and fatty acids in aquatic samples. In: Arts, M.T. and Wainman, B.C. (Eds.), Lipids in Freshwater Ecosystems. Springer-Verlag New York Inc, New York, pp. 4-20.

Parrish, C.C., Whiticar, M., Puvanendran, V., 2007. Is ω6 docosapentaenoic acid an essential fatty acid during early ontogeny in marine fauna? Limnol. Oceanogr. 52, 476-479.

Pedersen, T. and Falk-Petersen, I.B., 1992. Morphological changes during metamorphosis in cod (*Gadus morhua* L.), with particular reference to the development of the stomach and pyloric caeca. J. Fish Biol. 41, 449-461.

Pérez-Casanova, J.C., Murray, H.M., Gallant, J.W., Ross, N.W., Douglas, S.E., Johnson, S.C., 2006. Development of the digestive capacity of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture 251, 377-401.

Pérez-Casanova, J.C., Afonso, L.O.B., Johnson, S.C., Currie, S., Gamperl, A.K., in press. The stress and metabolic responses of juvenile Atlantic cod (*Gadus morhua* L.) to an acute thermal challenge. J. Fish Biol.

Pepels, P.P.L.M. and Balm, P.H.M., 2004. Ontogeny of corticotropin-releasing factor and of hypothalamic-pituitary-interrenal axis responsiveness to stress in tilapia (*Oreochromis mossambicus*; teleostei). Gen. Comp. Endocrinol. 139, 251-265.

Pickering, A.D. and Pottinger, T.G., 1987. Crowding causes prolonged leucopenia in salmonid fish, despite interrenal acclimation. J. Fish Biol. 30, 701-712.

Pottinger, T.G. and Mosuwe, E., 1994. The corticosteroidogenic response of brown and rainbow trout alevins and fry to environemntal stress during a "critical period". Gen. Comp. Endocrinol. 95, 350-362.

Pottinger, T.G., Moran, T.A., Cranwell, P.A., 1992. The biliary accumulation of corticosteroids in rainbow trout, *Oncorhynchus mykiss*, during acute and chronic stress. Fish Physiol. Biochem. 10, 55-66.

Puvanendran, V., Brown, J.A., 1999. Foraging, growth and survival of Atlantic cod larvae reared in different prey concentrations. Aquaculture 175, 77-92.

Puvanendran, V., Brown, J.A., 2002. Foraging, growth and survival of Atlantic cod larvae reared in different light intensities and photoperiods. Aquaculture 214, 131-151.

Rainuzzo, J.R., Olsen, Y., Rosenlund, G., 1989. The effect of enrichment diets on the fatty acid composition of the rotifer *Branchionus plicatilis*. Aquaculture 79, 157-161.

Rainuzzo, J.R., Reitan, K.I., Jorgensen, L., Olsen, Y., 1994. Lipid composition in turbot larvae fed live feed cultured by emulsion of different lipid classes. Comp. Biochem. Physiol. A 107, 699-710.

Rainuzzo, J.R., Reitan, K.I., Olsen, Y., 1997. The significance of lipids at early stages of marine fish: a review. Aquaculture 155, 103-115.

Rash, J.M., Jerkunica, I., Sgoutas, D., 1979. Mechanisms of interference of nonesterified fatty acids in radioimmunoassays of steroids. Clin. Chim. Acta 93, 283-294.

Rash, J., Jerkunica, I., Sgoutas, D., 1980. Lipid interference in steroid radioimmunoassay. Clin. Chem. 26, 84-88.

Robin, J.H., Peron, A., 2004. Consumption vs. deposition of essential fatty acids in gilthead sea bream (*Sparus aurata*) larvae fed semi-purified diets. Aquaculture 238, 283-294.

Rodgers, B.D., Weber, G.M., Kelley, K.M., Levine, M.A., 2003. Prolonged fasting and cortisol reduce myostatin mRNA levels in tilapia larvae; short term fasting elevates. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 284, 1277-1286.

Sakakura, Y., Tagawa, M., Tsukamoto, K., 1998. Whole-body cortisol concentrations and ontogeny of aggressive behavior in yellowtail (*Seriola quinqueradiata* temminck & schlegel; carangidae). Gen. Comp. Endocrinol. 109, 286-292.

Sampath-Kumar, R., Byers, R.E., Munro, A.D., Lam, T.J., 1995. Profile of cortisol during the ontogeny of the Asian seabass, *Lates calcarifer*. Aquaculture 132, 349-359.

Sampath-Kumar, R., Lee, S.T.L., Tan, C.H., Munro, A.D., Lam, T.J., 1997. Biosynthesis *in vivo* and excretion of cortisol by fish larvae. J. Exp. Biol. 277, 337-344.

Sapolsky, R.M. and Meaney, M.J., 1986. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Res. 396, 64-76.

Sargent, J.R., Henderson, R.J., Tocher, D.R., 1989. The lipids. In: Halver, J.E. (Ed.), Fish Nutrition. Academic Press, San Diego, pp. 191-199.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J., Tocher, D.R., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol. 11, 183-198.

Sargent, J.R., Bell, J.G., McEvoy, L.A., Tocher, D., Estevez, A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177, 191-199.

Sargent, J.R., Tocher, D.R., Bell, G.J., 2002. The lipids. In: Halver, J.E. and Hardy, R. (Eds.), Fish Nutrition. Academic Press, San Diego, CA, pp. 181-257.

Schreck, C.B., Li, H.W., 1991. Performance capacity of fish: stress and water quality. In: Brune, D.E., Tomasso, J.R. (Eds.), Aquaculture and Water Quality. Advances in World Aquaculture, vol. 3. Pub. Wld. Aquacult. Soc., Baton Rouge, LA, pp. 21–29.

Schreck, C.B., Olla, B.L., Davis, M.W., 1997. Behavior responses to stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Cambridge University Press, Cambridge, pp. 145–170.

Selby, C., 1999. Interference in immunoassay. Ann. Clin. Biochem. 36, 704-21.

Shields, R.J., Irwin, S., Smith, P.L., McEvoy, L.A., 2003. Effects of diet transition regimen on survival, growth and lipid composition of intensively reared Atlantic cod, *Gadus morhua*, larvae. Aqua. Int. 11, 119-130.

Sloman, K.A., Motherwell, G., O'Connor, K.I., Taylor, A.C., 2000. The effect of social stress on the Standard Metabolic Rate (SMR) of brown trout, *Salmo trutta*. Fish Physiol. Biochem. 23, 49-53.

Stephens, S.M., Brown, J.A., Frankling, S.C., 1997a. Stress responses of larval turbot, *Scophthalmus maximus* L., exposed to sub-lethal concentrations of petroleum hydrocarbons. Fish Physiol. Biochem. 17, 433-439.

Stephens, S.M., Alkindi, A.Y.A., Waring, C.P., Brown, J.A., 1997b. Corticosteroid and thyroid responses of larval and juvenile turbot exposed to the water-soluble fraction of crude oil. J. Fish Biol. 50, 953-964.

Stoltze, K. and Buchmann, K., 2001. Effect of *Gyrodactylus derjavini* infections on cortisol production in rainbow trout fry. J. Helminthol. 75, 291-294.

Stoss, J., Hamre, K., Ottera, H., 2004. Weaning and nursery. In: Moksness, E., Kjorsvik, E., Olsen, Y. (Eds.), Culture of Cold-Water Marine Fish. Blackwell Publishing Ltd., Oxford, pp. 337-362.

Sumida, C., 1995. Fatty acids: Ancestral ligands and modern co-regulators of the steroid hormone receptor cell signalling pathway. Pros. Leuk. Ess. Fatty Acids 52, 137-144.

Sutherland, A.B., 2003. Effects of excessive sedimentation on the growth and stress response of whitetail shiner (*Cyprinella galactura*) juveniles. In: Hatcher, K.J. (Ed.), Proceedings of the 2003 Georgia Water Resources Conference, April 23-24, 2003, Athens, Georgia, USA.

Svåsand, T. and Moksness, E., 2004. Marine stock enhancement and sea-ranching. In: Moksness, E., Kjorsvik, E., Olsen, Y. (Eds.), Culture of Cold Water Marine Fish. Blackwell Publishing Ltd., Oxford, pp. 475-485.

Svåsand, T., Ottera, H.M., Taranger, G.L., 2004. The status and perspectives for the species: Atlantic cod. In: Moksness, E., Kjorsvik, E., Olsen, Y. (Eds.), Culture of Cold Water Marine Fish. Blackwell Publishing Ltd., Oxford, pp. 433-443.

Szisch, V., Papandroulakis, N., Fanouraki, E., Pavlidis, M., 2005. Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. Gen. Comp. Endocrinol. 142, 186-192.

Tago, A., Yamamoto, Y.T.,S-I, Kanazawa, A., 1999. Effects of 1,2-di-20:5-phosphatidylcholine PC and 1,2-di-22:6-PC on growth and stress tolerance of Japanese flounder *Paralichthys olivaceus* larvae. Aquaculture 179, 231-239.

Takahashi, A., Kubota, J., Kawauchi, H., Hirano, T., 1985. Effects of N-terminal peptide of salmon proopiocortin on interrenal function of the rainbow trout. Gen. Comp. Endocrinol. 58, 328-335.

Takeuchi, T., Arakawa, T., Satoh, S. and Watanabe, T., 1992. Supplemental effect of phospholipids and requirement of eicosapentaenoic acid and docosahexaenoic acid for juvenile striped jack. Nippon Suisan Gakkaishi 58, 707-713.

Tanaka, M., Tanangonan, J.B., Tagawa, M., de Jesus, E.G., Nishida, H., Isaka, M., Kimura, R., Hirano, T., 1995. Development of the pituitary, thyroid and interrenal

glands and applications of endocrinology to the improved rearing of marine fish larvae. Aquaculture 135, 111-126.

Truscott, B., 1979. Steroid metabolism in fish: identification of steroid moieties of hydrolyzable conjugates of cortisol in the bile of trout *Salmo gairdnerii*. Gen. Comp. Endocrinol. 38, 196-206.

Van Anholt, R.D., Spanings, T., Koven, W.M., Wendelaar Bonga, S.E., 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, and the stress response of tilapia (*Oreochromis mossambicus*). Am. J. Physiol. Regul. Integr. Comp. Physiology 285, 1098-1106.

Van Anholt, R.D., Koven, W.M., Lutzky, S., Wendelaar Bonga, S.E., 2004. Dietary supplementation with arachidonic acid alters the stress response of gilthead seabream (*Sparus aurata*) larvae. Aquaculture 238, 369-383.

Villalta, M., Estevez, A., Bransden, M.P., 2005. Arachidonic acid enriched live prey indices albinism in Senegal sole (*Solea senegalensis*) larvae. Aquaculture 245, 193-209.

Wendelaar Bonga, S. E., 1997. The stress response in fish. Physiol. Rev. 77, 591-625.

Willey, S., Bengtson, D.A., Harel, M., 2003. Arachidonic acid requirements in larval summer flounder, *Paralichthyus dentatus*. Aquacult. Int. 11, 131-149.

Wu, S., Deng, A., Chou, Y., Wu, L., 2005. Exogenous steroids enhance the copper resistance of tilapia larvae (*Oreochromis mossambicus*). Zoological Studies 44, 373-381.

Young, G., 1986. Cortisol secretion *in vitro* by the interrenal of coho salmon (*Oncorhynchus kisutch*) during smoltification: relationship with plasma thyroxine and plasma cortisol. Gen. Comp. Endocrinol. 63, 191-200.

Youson, J.H., 1988. First metamorphosis. In: Hoar, W.S. and Randall, D.J. (Eds.), Fish Physiology. Academic Press, San Diego, pp. 135-196.

Zheng, F., Takeuchi, T., Yoseda, K., Kobayashi, M., Hirokawa, J., Watanabe, T., 1996. Requirements of larval cod for arachidonic acid, eicosapentaenoic acid and

docosahexaenoic acid by using their enriched *Artemia* nauplii. Nippon Suisan Gakkaishi 62, 669-676.