LIPID NUTRITION DURING EARLY DEVELOPMENT OF YELLOWTAIL FLOUNDER (Limanda ferruginea)

LOUISE AUDREY COPEMAN

LIPID NUTRITION DURING EARLY DEVELOPMENT OF YELLOWTAIL FLOUNDER (*Limanda ferruginea*)

by

Louise Audrey Copeman

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Masters of Science

Aquaculture Programme
Memorial University of Newfoundland
May 2001

St. John's Newfoundland Canada

Abstract

Yellowtail flounder (*Limanda ferruginea*) is a candidate species for cold-water aquaculture development in Atlantic Canada. However, mal-pigmentation and high larval mortality are still major obstacles to the successful culture of this species. Starvation due to inadequate nutrition is believed to be a major cause of this mortality. In particular, lipid nutrition has shown significant effects on the early development in a number of marine species. This study is the first examination of the dietary lipid requirements of yellowtail flounder larvae.

Specifically, marine fish require the dietary polyunsaturated fatty acids docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) for normal growth and development. Consequently, in Chapter Two (Part A) an experiment was designed to study the role of dietary ratios of these fatty acids on the early growth, survival, lipid composition, and pigmentation of yellowtail flounder. Rotifers were enriched with experimental emulsions with high concentrations of DHA, DHA+EPA, or DHA+AA, or with a control (no DHA, EPA, or AA) emulsion. After four weeks, larvae fed the high DHA diet were significantly larger (9.7 ± 0.2 mm, $p<0.05$) and had higher survival ($22.1 \pm 0.4\%$), while larvae fed the control diet were significantly smaller (7.3 ± 0.2 mm, $p<0.05$) and showed lower survival ($5.2 \pm 1.9\%$). Larval lipid class and fatty acid profiles showed significant differences ($p<0.05$), with fatty acids reflecting dietary levels in the high PUFA diets. The incidence of mal-pigmentation was higher in the high DHA+AA diet (92%) than in all other

treatments (<64%). It was concluded that yellowtail larvae require diets that are highly enriched with DHA while elevated dietary AA exerts negative effects on larval pigmentation.

In Chapter Two (Part B) I examined the changes in growth and lipid composition that occurred when larvae of differing nutritional status were fed one diet of enriched *Artemia*. Significant changes in larval lipid class and fatty acid composition were observed after just two weeks of feeding on enriched *Artemia*. Control larvae showed a period of 'lipid recovery' while animals fed all other treatments showed a period of decreased lipid unsaturation. However, all larvae demonstrated a dramatic increase in size despite decreased dietary highly unsaturated fatty acids (HUFA). Therefore, it was concluded that high levels of HUFA may not be as essential during later larval development as during initial stages of first-feeding.

In Chapter Three the lipid composition of mal-pigmented (MP) and normally pigmented (NP) newly settled yellowtail flounder were compared in order to elucidate a possible connection between lipids and pigmentation development. NP fish were found to be significantly larger than MP fish ($p=0.04$) at the time of 100% settlement. Higher relative amounts of triacylglycerols were found in NP fish ($p=0.02$) while MP fish had higher relative amounts of phospholipids ($p=0.008$). NP fish had higher relative amounts of DHA in the polar lipids of the body ($p=0.03$) and in the total lipids of the eye ($p=0.04$) than did MP juveniles. These data support previously proposed theories for the importance of DHA in pigmentation development.

Acknowledgements

Thanks to my supervisor, Dr. Christopher Parrish, for encouragement and guidance during the completion of this thesis. I would also like to thank Dr. Joe Brown for the use of his lab space during larval rearing experiments and for positive comments on earlier drafts of this thesis. Dr. Moti Harel kindly formulated the lipid emulsions using in feeding experiments.

I am also grateful to Olav Lingstad and Danny Boyce for helping with the design of both larval fish and live-food rearing protocols. Dr. Suzanne Budge and Jeanette Wells provided valuable technical assistance with lipid analysis. I thank the live food technicians Kim Fifield, Dena Wisemen and Tracy Granter for culturing unenriched rotifers and algae. Finally, I thank the OSC workshop staff for help with the construction of larval rearing tanks.

Both NSERC and a Special Resource Scholarship from the provincial government of Newfoundland provided financial support for this study.

I would like to acknowledge my family, Ben, Doug, Margaret and Laura for their support and patience during the completion of this thesis.

Table on Contents

Abstract ii
Acknowledgements iv
Table of contents v
List of Tables vii
List of Figures ix
List of Abbreviations xi
List of Appendices xiii

Chapter 1 – General Introduction 1
1.1 Yellowtail flounder biology and aquaculture 2
1.2 The importance of lipid nutrition in marine larviculture 3
1.3 Objectives 13

Chapter 2- Effect of dietary ratios of DHA, EPA and AA on early growth, survival, lipid composition, and pigmentation of yellowtail flounder (*Limanda ferruginea*)

2.1 Introduction 14
2.2 Materials and Methods
- 2.2.1 Emulsions 16
- 2.2.2 Rotifers 16
- 2.2.3 *Artemia* 17
- 2.2.4 Larviculture 17
- 2.2.5 Growth and Survival 18
- 2.2.6 Lipid samples 19
- 2.2.7 Lipid analysis 20
- 2.2.8 Pigmentation and eye migration 22
- 2.2.9 Statistical analysis 25

2.3 Results Part A: Effects of feeding differently enriched rotifers during the first four weeks post-hatch on growth, survival, lipid composition and pigmentation of yellowtail flounder.
- 2.3.1 Lipid composition of emulsions 26
- 2.3.2 Lipid composition of rotifers 29
- 2.3.2 Growth and survival 32
- 2.3.4 Lipid composition of eggs and larvae at 100 % hatch 36
- 2.3.5 Lipid class composition of larvae 39
- 2.3.6 Larval fatty acid composition 43
- 2.3.7 Relationship of dietary DHA:EPA ratio to growth and survival 47
- 2.3.8 Pigmentation, eye migration and orientation 52

2.4. Results Part B: Effects of feeding enriched *Artemia* on the growth, survival, and lipid composition of yellowtail larvae with differing nutritional status
2.4.1 Lipid composition of *Artemia* 54
2.4.2 Growth and survival 57
2.4.3 Lipid class composition of larvae 63
2.4.4 Fatty acid composition of larvae 67
2.5 Discussion Part A
2.5.1 Rotifer lipid composition 74
2.5.2 Larval growth and survival 75
2.5.3 Pigmentation 79
2.5.4 Larval lipid composition 81
2.5.5 Conclusions 84
2.6 Discussion Part B
2.6.1 *Artemia* lipid composition 85
2.6.2 Larval growth and survival 86
2.6.3 Larval lipid composition 88
2.6.4 Conclusions 91

Chapter 3 - Comparison of the lipid composition of mal-pigmented and normally pigmented newly settled yellowtail flounder (*Limanda ferruginea*).

3.1 Introduction 92
3.2 Materials and Methods
3.2.1 Live-food 94
3.2.2 Larviculture 94
3.2.3 Lipids and morphometrics 95
3.2.4 Statistical analysis 97
3.3 Results
3.3.1 Live-food 98
3.3.2 Size of juveniles 98
3.3.3 Lipid class composition of juveniles 104
3.3.4 Fatty acid composition of juveniles 108
3.3.5 Principle Component Analysis 112
3.4 Discussion
3.4.1 Size of juveniles 116
3.4.2 Lipid class composition of juveniles 117
3.4.3 Fatty acid composition of juveniles 119
3.4.4 Conclusions 122

Chapter 4- Summary of experiments and suggestions for future research 124

References 126
Appendices 137

List of Tables

		Page
Table 1.1	Lipid composition of the roe from two species of marine fish and live-foods commonly used for first-feeding of marine larvae.	11
Table 2.1	Categories of pigmentation used in evaluation of yellowtail flounder (Simplified from Gara et al., 1998).	23
Table 2.2	Categories of eye migration used in evaluation of yellowtail flounder (Simplified from Gara et al., 1998).	24
Table 2.3	Lipid class composition of the four experimental emulsions (mean ± SEM, n=3).	27
Table 2.4	Fatty acid composition of the four experimental emulsions (mean ± SEM, n=3).	28
Table 2.5	Lipid class composition of rotifers enriched for 12 hours using four different oil emulsions (mean ± SEM, n=4).	30
Table 2.6	Fatty acid composition of rotifers enriched for 12 hours using four different oil emulsions (mean ± SEM, n=4).	31
Table 2.7	Lipid composition of yellowtail flounder eggs and larvae at 100% hatch (mean ± SEM, n=3).	37
Table 2.8	Fatty acid composition of yellowtail flounder eggs and larvae at 100% hatch (mean ± SEM, n=3).	38
Table 2.9	Lipid class composition of larvae after four weeks of feeding on differently enriched rotifers (mean ± SEM, n=6).	42
Table 2.10	Fatty acid composition of week-four larvae fed differently enriched rotifers (mean ± SEM, n=6).	44
Table 2.11	Fatty acid composition of neutral and polar lipids of yellowtail flounder larvae after feeding on differently enriched rotifers for four weeks post-hatch (mean ± SEM, n=6).	49

		Page
Table 2.12	Pigmentation, eye migration, and orientation of yellowtail flounder fed differently enriched rotifers for the first four weeks post-hatch.	53
Table 2.13	Lipid class composition of *Artemia* enriched for 24 hours with Algamac or DHA Selco. Data are mean ± SD (n=2).	55
Table 2.14	Fatty acid composition of *Artemia* after enrichment with Algamac or DHA Selco for 24 hours. Data are mean ± SD (n=2).	56
Table 2.15	Lipid class composition of week-six larvae fed differentially enriched rotifers for the first two weeks post-hatch followed by identically enriched *Artemia*. Values are mean ± SD (n=2).	66
Table 2.16	Fatty acid composition of week-six larvae fed differentially enriched rotifers for four weeks post-hatch followed by identically enriched *Artemia*. Data are mean ± SD (n=2).	68
Table 2.17	Neutral and polar fatty acid composition of six-week-old yellowtail larvae that were fed differently enriched rotifers for 4 weeks post-hatch followed by identically enriched *Artemia*. Data are mean ± SD (n=2).	72
Table 3.1	Summary of data on the lipid composition of rotifers and *Artemia* enriched with Algamac for 18 hours.	99
Table 3.2	Comparison of the body lipid class composition of normally and mal-pigmented newly settled yellowtail flounder.	105
Table 3.3	Comparison of the head lipid class composition of normally and mal-pigmented newly settled yellowtail flounder.	106
Table 3.4	Comparison of the eye lipid class composition of normally and mal-pigmented newly settled yellowtail flounder.	107
Table 3.5	Neutral and polar fatty acid composition of mal-pigmented and normally pigmented newly settled yellowtail flounder larvae.	109

List of Figures

		Page
Figure 1.1	Examples of fatty acid structure and nomenclature.	6
Figure 1.2	Lipid classes commonly discussed in aquaculture.	7
Figure 1.3	Desaturation and elongation pathways for polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series.	9
Figure 2.1	Morphometric measurements and mass of yellowtail flounder larvae fed four types of differently enriched rotifers for the first four weeks post-hatch. Data are mean ± SEM.	34
Figure 2.2	Percent survival of yellowtail flounder larvae after four weeks of feeding on differently enriched rotifers. Data are mean + SD, n=2.	35
Figure 2.3	Lipids in yellowtail flounder fed four types of differently enriched rotifers for the first four weeks post-hatch. Data are mean ± SEM (n=6).	41
Figure 2.4	Fatty acid composition (μg/mg dry weight) of larvae after four weeks of feeding on differently enriched rotifers.	45
Figure 2.5	Relative proportions of specific fatty acids in larvae compared to dietary levels after four weeks of feeding on differently enriched rotifers.	46
Figure 2.6	The highly unsaturated fatty acid composition of the neutral and polar lipids in yellowtail flounder larvae after four weeks of feeding on differently enriched rotifers.	48
Figure 2.7	The relationship between (a) survival and (b) body depth at week four and dietary DHA:EPA for yellowtail flounder fed four types of differently enriched rotifers. Symbols are tank means.	51
Figure 2.8	Morphometric measurements of yellowtail flounder larvae fed four types of differently enriched rotifers for the first four weeks post-hatch followed by identically enriched *Artemia*.	59

		Page
Figure 2.9	The relationship between stocking density and (a) standard length, (b) body depth and (c) dry weight at the end of week 13. Data are tank mean ± SEM.	61
Figure 2.10	Percent survival of larvae from week 4 to week 13.	62
Figure 2.11	Lipids in yellowtail flounder fed four types of differently enriched rotifers four the first four weeks post-hatch followed by two weeks of identically enriched *Artemia*.	65
Figure 2.12	Ratios of fatty acid proportions in yellowtail flounder fed differently enriched rotifers for four weeks post-hatch followed by identically enriched *Artemia* for two weeks.	70
Figure 2.13	Comparison of the neutral and polar highly unsaturated fatty acid composition of yellowtail flounder larvae after four and six weeks of feeding on live-food.	73
Figure 3.1	The (a) wet weight, (b) body depth and (c) standard length of normally pigmented and mal-pigmented fish at the end of week 12 (1999). Data are mean ± SEM.	101
Figure 3.2	The (a) wet weight, (b) body depth, and (c) standard length of normally pigmented fish compared to mal-pigmented fish at the end of week 13 (1998). Data are mean ± SEM.	103
Figure 3.3	Summary of the fatty acid composition in the different body zones of newly settled mal-pigmented and normally pigmented yellowtail flounder: (a) Eye total lipid, (b) Head polar lipid, and (c) Body polar lipid.	111
Figure 3.4	Principle components analysis (PCA) of lipid data from eyes, heads, and bodies of MP and NP juvenile yellowtail flounder. Five samples of MP and NP fish tissue were analyzed from each body zone. The fatty acid parameters used were: 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 20:1n-9, 20:4n-6 (AA), 20:5n-3 (EPA), 22:5n-3, 22:6n-3 (DHA), DHA:EPA, ΣSFA, ΣMUFA, ΣPUFA, TAG, PPL, LIPID per dry weight ($\mu g.mg^{-1}$). (a) Lipid parameter coefficients for PC1 and PC2 (b) Eyes, heads and bodies scores for PC1 and PC2	114

List of Abbreviations

AA	Arachidonic acid (20 4n-6)
ai	anteiso-
ALC	Alcohols
AMPL	Acetone-Mobile Polar Lipids
ANOVA	Analysis of Variance
DHA	Docosahexaenoic acid (22:6n-3)
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic acid (20:5n-3)
FAME	Fatty acid methyl esters
FFA	Free Fatty Acids
GC	Gas Chromatography
HUFA	Highly Unsaturated Fatty Acids (4-6 double bonds)
i	Iso-
MP	Mal-Pigmented
MS-222	3-aminobenzoate methane sulphonate
MT	Million Tonnes
MUFA	Monounsaturated Fatty Acids
NL	Neutral Lipids
NP	Normally Pigmented
PC	Phosphatidylcholine

PCA	Principle Components Analysis
PL	Polar lipids
PPL	Phospholipids
PUFA	Polyunsaturated Fatty Acids (≥ 2 double bonds)
SFA	Saturated Fatty Acids
ST	Sterols
TAG	Triacylglycerols
TLC/FID	Thin Layer Chromatography with Flame Ionization Detection
WE	Wax Esters

List of Appendices

		Page
Appendix A1	Lipid class composition of week-three larvae fed differentially enriched rotifers (mean ± SEM, n=6)	136
Appendix A2	Fatty acid composition of week-three larvae fed differentially enriched rotifers (mean ± SEM, n=6)	137

Chapter 1 - General Introduction

Human consumption of seafood is increasing in part due to its positive effects on human health. Beneficial effects of dietary seafood, relating especially to omega-3 fatty acids, are now known for human cardiovascular disease, hypertension, auto-immune disorders, and infant neural development (Leaf & Weber, 1988; Newton, 2000; Arts et al., 2001). The United Nations has predicted that in 2010, global demand for seafood products will be 105 to 110 million tonnes (MT). However, this demand cannot be met from the wild fishery, whose production has plateaued at 90 MT. In contrast, aquaculture has shown dramatic growth during the past decade from 13 MT to a projected 35 MT in the year 2010 (Report on the State of World Fisheries and Aquaculture, United Nations Publication, FOA 1998).

Despite its vast aquatic environment, Canada is still a minor contributor to world aquaculture production. During the past 15 years, the increase in aquaculture production has been significant with reported production levels of 0.003 MT and 0.1 MT respectively in 1986 and 1999 (Canadian Department of Fisheries and Oceans statistics). As the 1999 Canadian aquaculture production represents less than 0.3% of the estimated 2010 world demand (35MT), there is a tremendous opportunity to be exploited by the expansion of this industry.

Atlantic Canada's fishing industry has been economically devastated due to the collapse of many ground fish stocks (Hutchings et al., 1997; O'Rielly, 2000). This region has many promising qualities relating to future aquaculture development such as: traditional fisheries knowledge, a large skilled employment base, a vast coastal environment, and pre-established seafood markets. Consequently, there is a pressing

need for research into the development of new aquaculture industries. The Canadian Department of Fisheries and Oceans reported that in 1999 over 98% of the total national aquaculture production was composed of salmonids, blue mussels, and American oysters. Clearly, diversification and increased research into candidate species suited to our cold-water marine environment is necessary.

1.1 Yellowtail flounder biology and aquaculture

Yellowtail flounder is a right-eyed small-mouthed flatfish of the order Pleuronectiformes (Scott & Scott, 1988). This species is believed to have potential in cold-water aquaculture due to its established foreign market, high filet-to-body ratio, low commercial supply, and relatively high growth rates at low temperatures (Brown et al., 1995; Brown, 2000). Since 1994, research into yellowtail flounder culture has been ongoing at the Ocean Science Centre, Logy Bay, Newfoundland. Most of these studies have focused on aspects of larval rearing (French, 1995; Puvanendran & Brown, 1995; Copeman, 1996; Morris, 1997; Rabe & Brown, 2000), juvenile husbandry and nutrition (Whalen, 1999; Purchase et al., 2000), and control of broodstock reproduction (Manning & Crim, 1998).

Yellowtail flounder inhabit the continental shelf of the Northwestern Atlantic Ocean from Labrador to Chesapeake Bay and are normally found at depths between 10 and 100 m (Brodie & Walsh, 1994). They have benthic adult and juvenile stages and a pelagic larval stage. Adults and juveniles co-occur on sandy substrates mainly on the Southeastern Shoal of the Grand Banks. Larvae are also found in high numbers on the Southeastern Shoal, which is now believed to serve as a nursery area (Walsh, 1992).

Historically, the highest concentrations of adult yellowtail were found in NAFO regulatory divisions 3L, 3N, and 3O, with 3N being the most important in terms of fishing effort. From 1975 to 1995 yellowtail flounder showed a dramatic decline in abundance which strongly correlated with increased commercial catches, particularly during 1985 and 1986 (Brodie et al., 1998). Reductions in abundance resulted in a moratorium on commercial fishing from 1994 to1997 prompting an interest in aquaculture development.

Yellowtail flounder are batch spawners and in captivity produce on average 14-22 batches per season with a typical total production of 550 000 eggs (Manning & Crim, 1998). Eggs are small, ranging between 0.7 and 1.0 mm in diameter (Laurence & Howell, 1981; Tilseth, 1990) with larvae hatching at approximately 65-degree days. Yellowtail larvae are underdeveloped, small (2.0-3.5 mm), and have limited yolk reserves at hatch. Therefore, providing the correct nutrition to these larvae is both important for larval survival and a challenge to the aquaculture industry.

1.2 The importance of lipid nutrition in marine larviculture

A major obstacle to the successful mass culture of marine finfish species is extremely high mortality during the larval phase. Starvation is thought to be a primary cause of this mortality both in the wild and under culture conditions (Hunter, 1981; Lavens et al., 1995). Due to the limited amount of yolk present at the time of first-feeding, both the quantity and quality of food available are of vital importance. The switch from endogenous to exogenous feeding is termed a 'critical period' due to high levels of mortality which are concentrated over this short period of time (May, 1973). If

larvae do not receive proper nutrition during this period they pass a 'point of no return' and undergo irreversible starvation (Hunter, 1981).

Factors such as total protein, total lipid, and various vitamins and minerals are important in defining dietary quality for first-feeding marine larvae (Watanabe & Kiron, 1994; Ronnestad et al., 1999). However, during the past three decades, certain polyunsaturated fatty acids (PUFA) have been shown to play a crucial role in the early development of marine fish larvae (Bell et al., 1986; Watanabe, 1993; Sargent et al., 1995; Takeuchi, 1997; Sargent et al., 1999) Also, providing correct relative and absolute amounts of dietary PUFA affects the early survival and development in a number of marine species (Rainuzzo et al., 1997).

Lipids are generally defined as a group of compounds that are soluble in organic solvents but are typically insoluble in water. They are often classified by the presence or absence of fatty acids and also by differences in polarity. Fatty acids are the simplest lipids and are found as constituents of other lipid classes such as wax esters, triacylglycerols and phospholipids. Fatty acids are characterised by their hydrophilic carboxyl head group and hydrophobic hydrocarbon tail. Saturated fatty acids (SFA) have a hydrocarbon tail without double bonds while monounsaturated fatty acids (MUFA) have one double bond and polyunsaturated fatty acids have two or more double bonds (PUFA). In aquaculture, highly unsaturated fatty acids (HUFA) usually refers to those with four or more double bonds. The simple nomenclature A:Bn-C is often used to describe the structure of fatty acids where A refers to the number of carbon atoms, B refers to the number of double bonds and C refers to the position of the first double bond

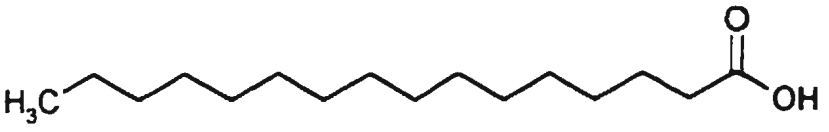
with reference to the methyl end of the molecule. Figure 1.1 shows examples of these different types of fatty acids and demonstrates this simple nomenclature.

Lipids play a vital role in both cell membrane function, energy storage and as substrates for the formation of biologically active molecules. Parrish (1988) described the lipid classes commonly found in the marine environment both in dissolved and particulate matter. Figure 1.2 illustrates examples of the lipid classes which are most commonly discussed in aquaculture; in order of increasing polarity these are wax esters (WE), triacylglycerols (TAG), free fatty acids (FFA), sterols (ST), and phospholipids (PPL).

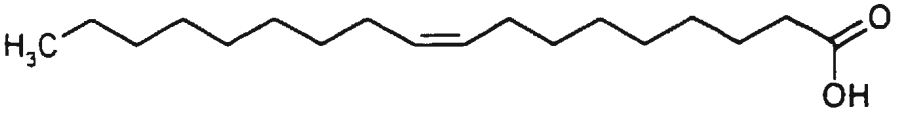
Wax esters (WE) are an important energy reserve for copepods, which are the dominant prey for many species of marine larvae in the wild. Copepods in high latitudes can contain greater than 80% of their lipids as WE (Kattner & Krause, 1987). TAG are the principal form of endogenous energy storage in fish and in enriched live-foods, while FFA are normally found in small amounts (<10% of total lipid) in both live-food and marine larvae. Generally, FFA have been viewed as products of tissue breakdown due to poor storage of samples (Parrish, 1988, Arts et al., 2001). ST play a major structural role as components of cell membranes and are also used as precursors for steroid hormones (Nes, 1974). PPL are also important in maintaining cell membrane structure, however, they also provide vital energy reserves for the early development of certain marine species (Fraser, 1988).

PUFA of the n-3 and n-6 series, docosahexaenioc acid (22:6 n-3, DHA), eicosapentaenoic acid (20:5 n-3, EPA), and arachidonic acid (20:4 n-6, AA) are essential for marine fish because they require them for normal growth and development but

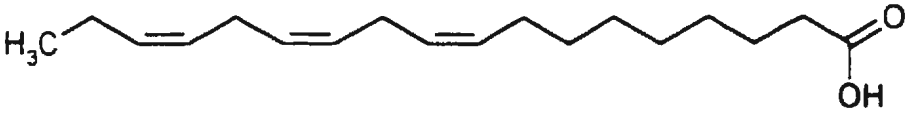
A:Bn-C
A=number of carbon
B=number of double bonds
C=position of double bond relative to methyl end



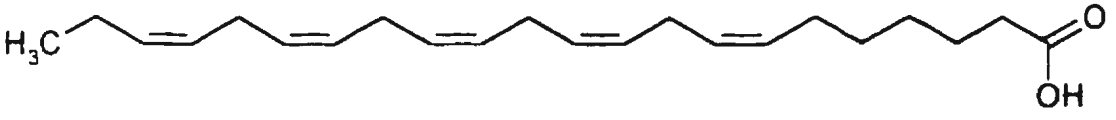
Saturated fatty acid, 16:0



Monounsaturated fatty acid, 18:1n-9

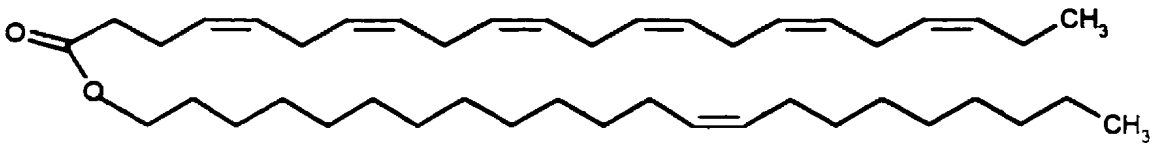


Polyunsaturated fatty acid, 18:3n-3

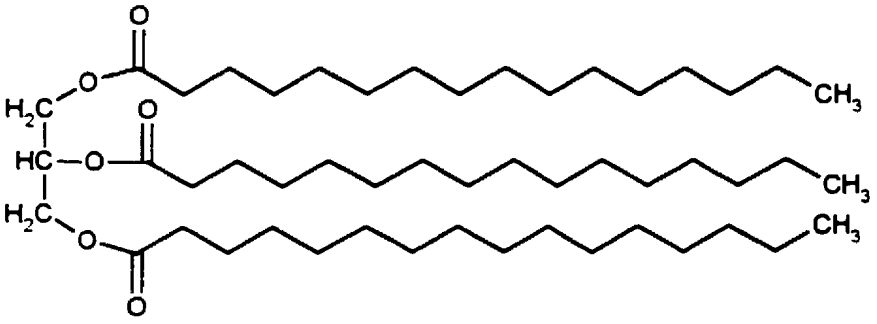


Highly unsaturated fatty acid, 22:6n-3

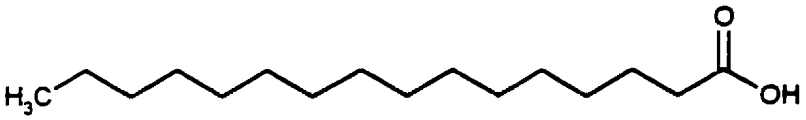
Figure 1.1: Examples of fatty acid structure and nomenclature



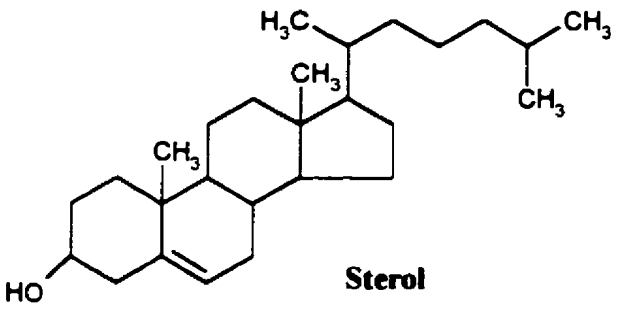
Wax Ester



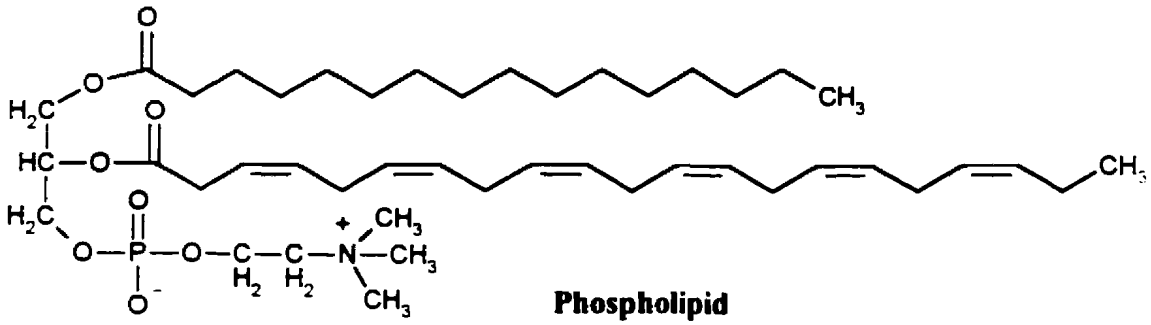
Triacylglycerol



Free Fatty Acid



Sterol



Phospholipid

Figure 1.2: Lipid classes commonly discussed in aquaculture

cannot synthesise them '*de novo*' from shorter chain precursors. It was previously thought that Δ4 desaturase was used to convert 22:5n-3 and 22:4n-6 into 22:6n-3 and 22:5n-6, respectively (Sargent, 1995). However, Figure 1.3 shows the current views on the pathways for the elongation and desaturation of PUFA of the n-3 and n-6 series. It is now believed that Δ6 and Δ5 are used to produce 24:6n-3 and 24:5n-6 which are then shortened to 22:6n-3 and 22:5n-6 respectively by β-oxidation (Voss et al., 1991; Buzzi et al., 1996).

The differing abilities of marine and freshwater species to chain elongate and desaturate shorter chain dietary precursors into longer chain HUFA can be predicted by examining their natural diet. The marine environment is characterised by elevated levels of long chain fatty acids (with 20 and 22 carbons) of the n-3 series, which are produced in large amounts by marine algae. Marine phytoplankton may contain approximately 20% of their dry weight as lipid, of which up to 50% of the fatty acids may be n-3 HUFA (Sargent et al., 1985). Zooplankton, which feed heavily on phytoplankton, maintain high levels of these n-3 HUFA in their energy reserves (Evjemo & Olsen, 1997). In contrast, freshwater phytoplankton have high levels of 18:3n-3 and 18:2n-6 while having low levels of C20 and C22 fatty acids. The terrestrial contribution of lipids to the freshwater environment is significant and is also characterised by high levels of 18:3n-3 and 18:2n-6. Therefore, most marine species show little ability to chain elongate and desaturate C18 PUFA because C20 and C22 are present in high amounts in their diet. However, freshwater fish generally do not have C20 and C22 fatty acids in their diets and therefore must synthesise their required amounts from shorter chain dietary precursors.

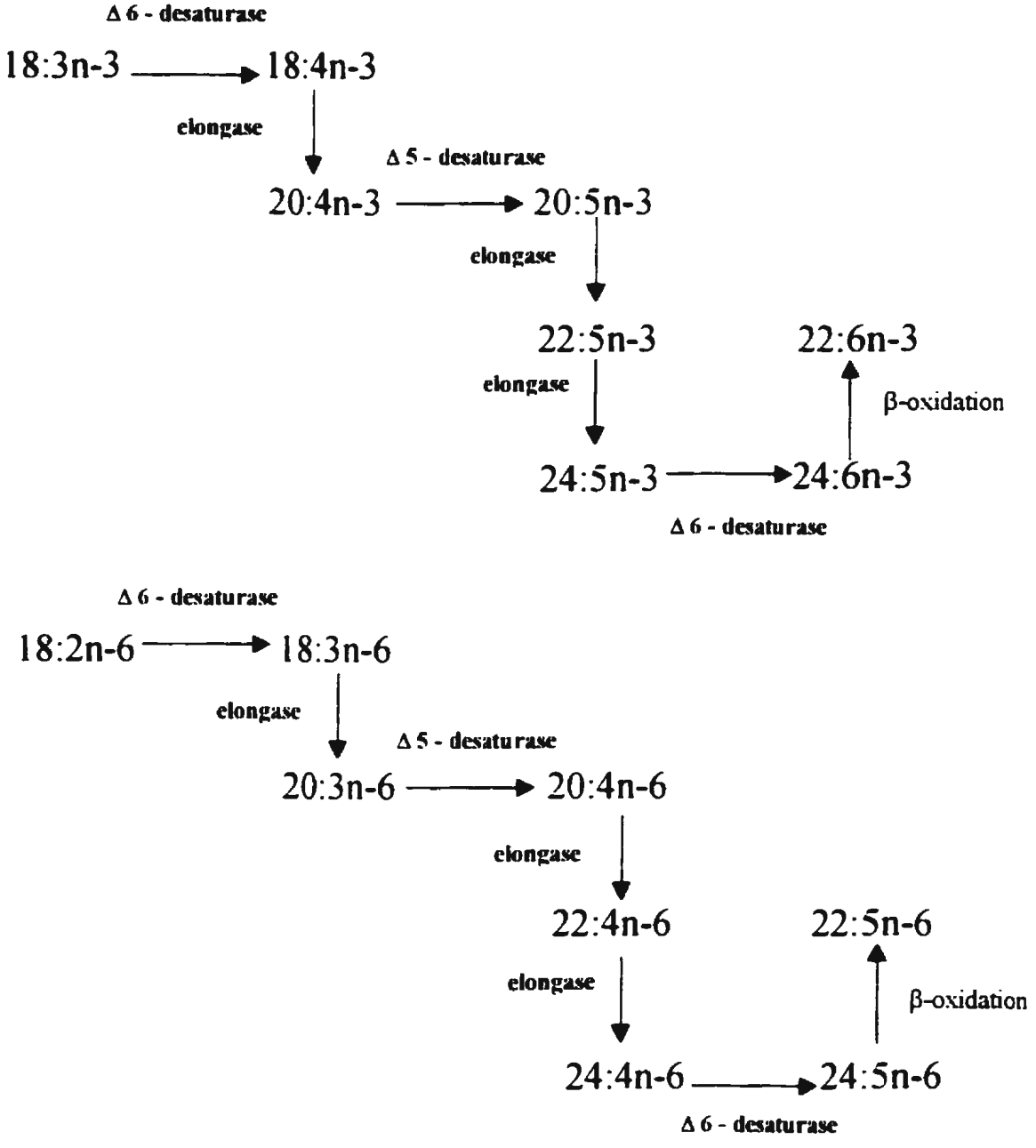


Figure 1.3: Desaturation and elongation pathways for polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series (after Voss et al., 1991; Buzzi et al., 1996)

Currently, a major challenge to successful marine larviculture is the production of live-feed organisms which is costly, labour intensive, unpredictable, and often provides sub-optimal nutrition. However, live-feeds are necessary due to both the small size of larvae during first-feeding and their strong reliance on visual cues. Both of these issues make the formulation of dry diets particularly difficult, although, considerable progress has been made in this area (Le Ruyet et al., 1993; Barrows & Rust, 2000). The two most common live-feeds used in marine larviculture are rotifers (*Brachionus plicatilis*) and brine shrimp (*Artemia franciscana*). Wild zooplankton have also been used successfully for the production of marine larvae, particularly in Norway (Olsen et al., 1999). However, wild zooplankton are often unavailable in sufficient quantities and with optimal species composition during peak larval production. Also, availability is variable due to dependence on oceanic conditions.

However, the development of intensive culture techniques for marine copepods such as *Tisbe* sp. and *Amonardia* sp. shows promise (Stottrup & Norsker, 1997; Stottrup, 2000). Cultured copepods are able to conserve DHA and EPA when maintained on a diet containing low levels of these fatty acids. They also have a much higher level of n-3 HUFA present in their polar lipid portion when compared to rotifers and *Artemia* (Nanton & Castell, 1998; Nanton & Castell, 1999). A prevalent postulate in marine larviculture is that larval foods should have a similar composition to either fish roe or wild marine zooplankton (Sargent, 1995; Sargent et al., 1999).

Table 1.1 compares the lipid composition of fish roe from two marine species to live-food organisms commonly used in aquaculture. Generally, both fish roe and wild marine copepods have higher levels of phospholipids and lower overall levels of lipid per

Table 1.1: Lipid composition of the roe from two species of marine fish and live-foods commonly used for first-feeding of marine larvae.

Lipid Components	Atlantic cod[1] Roe	Haddock[2] Roe	Copepods[3]	Copepods[4]	Enriched Rotifers[5,6]	*Artemia* Nauplii[7]	Enriched Artemia[8]
Total lipid (% dry weight)	13.2	10.7	9-11	-	21.2	9.2	19.2
Lipid classes (% total lipid)							
Triacylglycerols	12.5	8.3	-	19.6	55.5	23.4	76.2
Sterols	6.1	9.5	-	9.3	2.7	12.5	3.3
Phospholipids	71.5	70.9	-	59.6	24.9	12.3	5.7
Fatty acids (% fatty acids)							
16:0	23.7	27.4	8-12	13.7	6.7	20.6	12.0
18:1n-9	11.0	4.8	-	1.0	11.1	22.8	24.1
18:3n-3	0.3	n.d.	-	1.1	0.6	11.9	14.4
20:4n-6 (AA)	1.9	1.0	-	0.6	0.7	n.d.	1.6
20:5n-3 (EPA)	15.3	13.7	21-24	13.2	18.6	n.d	8.7
22:6n-3 (DHA)	28.6	31.4	40-45	44.4	35.9	n.d.	9.6
Total SFA	28.1	32.7	-	22.3	10.0	31.3	20.4
Total MUFA	20.3	14.5	-	6.2	23.6	36.1	30.7
Total PUFA	46.1	47.1	-	67.4	65.8	21.9	48.9
DHA:EPA	1.9	2.3	1.8-1.9	3.4	1.9	-	1.1
EPA:AA	8.1	13.7	-	22	26.6	-	5.4

[1,2] Tocher et al., 1984 (Fish caught near Gourdon, Scotland)
[3] Evjemo & Olsen, 1997 (*Temora longicornis*, collected from halibut hatcheries in Norway, 93% copepodid V and adult stages)
[4] McEvoy et al., 1998 (63% Calanoid nauplii, 21.3% *Eurytemora affinis* adults, collected from Svartatjohn marine lagoon, Norway)
[5] Oie et al., 1997 (slow growing rotifers cultures maintained on bakers yeast and Super Selco then short term enriched with DHA-Selco, fatty acid and total lipid data only)
[6] This study (Rotifers enriched with DHA emulsion for 12 hours, lipid class % data)
[7] Navarro et al., 1993 (Great Salt Lake nauplii)
[8] This study (Artemia enriched with DHA-Selco for 24 hrs)

dry weight than enriched rotifers and *Artemia*. The fatty acids profiles of both zooplankton and fish roe are characterised by higher levels of DHA, EPA, and moderate levels of AA, while nauplii from Great Salt Lake *Artemia* have undetectable amounts of these fatty acids and elevated levels of 18:1n-9 and 18:3n-3 (Table 1.1). Following enrichment, *Artemia* have elevated levels of both DHA and EPA, however, these values are still considerably lower (50%) than those found in marine copepods and fish roe. Growth, survival, and pigmentation often improve when larvae are fed wild zooplankton compared to enriched rotifers or *Artemia* (Seikai et. al., 1987; Naess & Lie, 1998). It is now believed that these differences in culture success are to some extent due to differences in lipid composition between the two food sources.

Much attention has been focused on lipid enrichment of both rotifers and *Artemia*. This is made challenging, particularly in *Artemia*, by physiological processes that occur within live-food such as: retroconversion of DHA to EPA in first-feeding tanks prior to consumption, autoxidation of PUFA, and lipid class conversions (McEvoy et al., 1998). However, significant advances have been made using speciality oils, which are particularly high in DHA such as tuna orbital oil and purified algae lipid extracts. The lipids from *Crypthecodinium cohnii* are high in DHA and purified extracts from this algal species were used in the rotifer enrichments described in Chapter Two. Also, vitamin C, vitamin E and lecithin were added to enrichment oils in order to minimise oxidation. In this study, husbandry protocols were adapted in order to reduce enrichment loss in live-feeds. This was accomplished by reducing the time that live-food remained in first-feeding tanks prior to larval consumption.

1.3 Objectives

The objective of Chapter Two was to use dietary manipulation in yellowtail flounder larvae, in order to determine both the importance of essential fatty acids in early growth, survival and pigmentation, as well as the time required for nutritionally deficient larvae to recover. The objective of Chapter Three was to investigate a possible role for lipids in pigmentation development by comparing the lipid composition of normally and mal-pigmented fish.

Chapter 2 - Effect of dietary ratios of DHA, EPA and AA on early growth, survival, lipid composition, and pigmentation of yellowtail flounder (*Limanda ferruginea*)

2.1 Introduction

The importance of polyunsaturated fatty acids (PUFA) in larval fish nutrition has been extensively investigated during the past 20 years (Wantanabe, 1993; Wantanabe & Kiron, 1994; Sargent et al., 1999). Docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (AA, 20:4n-6) are essential fatty acids (EFA) for many marine species. However, live-foods that are commonly used for first-feeding of marine larvae, such as rotifers and *Artemia*, are naturally low in these PUFA. Therefore, enrichment of live foods with lipids rich in PUFA prior to feeding is usually necessary.

Recently the importance of considering the relative amounts of DHA, EPA, and AA simultaneously has been demonstrated (McEvoy et al., 1998; Estevez et al., 1999; Sargent et al., 1999). This is due to competitive interactions between not only DHA and EPA, but also between EPA and AA. The DHA and EPA competition results from both molecules using the same enzyme system to esterify fatty acids into phospholipid backbones. Given that DHA is naturally found at very high levels in neural tissue, it is believed to play a specialised role in neural membrane structure and function. Therefore, elevated dietary EPA relative to DHA is postulated to have a negative impact on larval neural function and thus growth and survival (Bell et al., 1995; Rodriguez et al., 1997).

The competitive interactions between EPA and AA are important in the formation of eicosanoids. Eicosanoids are a group of biologically active molecules, once known as

local hormones, which include prostaglandins, thromboxanes, and leukotrienes. Eicosanoids play a wide variety of localised physiological roles in fish which can range from ionic regulation to the induction of egg shedding in ripe females (Sargent, 1995). EPA and AA are both substrates for the formation of eicosanoids, with AA being the preferred substrate and producing eicosanoids of higher biological activity (Bell et al., 1994). EPA produces eicosanoids of lower biological activity and therefore modulates the efficiency of AA. Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*) larvae when fed high levels of AA relative to EPA developed high levels of mal-pigmentation (McEvoy et al., 1998; Estevez et al., 1999). These results are hypothesised to be a result of stress induced by elevated eicosanoid activity (Estevez et al., 1999, Sargent et al., 1999)

The yellowtail flounder is a candidate species for cold-water aquaculture in Atlantic Canada. However, high mortality and mal-pigmentation are still substantial challenges to the successful early culture of yellowtail. Both of these issues have been associated with the PUFA content of live food used in first-feeding in a number of marine flatfish species (Rainuzzo et al., 1997; Sargent et al., 1999). Currently, there are no studies that have addressed the optimal lipid requirements of yellowtail flounder larvae. Therefore, this experiment was designed to investigate the role of dietary ratios of DHA:EPA and EPA:AA on their early growth, survival, and pigmentation.

2.2 Materials and Methods

2.2.1 Emulsions

Dr. Moti Harel of the University of Maryland prepared the four experimental emulsions tested in this study. Three emulsions were formulated by blending different ratios of DHA and AA rich triacylglycerols (TAG) from heterotrophic algae production (DHASCO and ARASCO, Martek BioSci. Columbia, MD. USA) and a marine oil (TG 22/33, Marine Lipids Leknes, Norway). The fatty acid composition of DHASCO and ARASCO were 49 % DHA and 54 % AA respectively and both had less than 0.5 % EPA. The marine oil contained approximately 20 % DHA and 30 % EPA. A fourth emulsion was prepared as a control using olive oil, which was low in PUFA. A mixture of 2 % alginic acid, 2 % polyoxyethylene sorbitan monooleate (Tween 80), 1 % ascorbic acid, 1 % vitamin E, 1 % silicon based anti-foaming agent (Sigma Co, MO, USA) and 5 % soy lecithin (80 % PC: Archer Daniels Midland Co. Decatur, IL, USA) were added to the oils. Oil mixtures were emulsified with equal amounts of distilled water by first homogenising at low speed (Ultra-turrax T8, IKA Labortechnik, Staufen, Germany) for 15 seconds and then sonicating for an additional 15 seconds at one third of the maximum sonication energy level (Sonifier 450: Branson Sonic Power Company, Danbury, CT. USA). Emulsions were stored under nitrogen at 4°C for daily use.

2.2.2 Rotifers

Rotifers were cultured on baker's yeast, *Saccharomyces cerevisiae*, and culture Selco (INVE, Dendermond, Belgium) for five days prior to enrichment. They were then taken from stock cultures and placed into smaller 10-L enrichment vessels at a density of 5 X

10^5 rotifers L^{-1}. Two batches of enriched rotifers were produced every 24 hours. Rotifer batches were enriched for approximately 12 hours at 0.1 g of enrichment oil L^{-1} of rotifer culture. Enrichment was added at time zero and after six hours of enrichment. Emulsions were blended in 1 L of distilled water for two minutes prior to addition to enrichment vessels. Rotifers were sampled from each enrichment vessel in triplicate for lipid analysis four times during the experiment.

2.2.3 *Artemia*

All larval groups were fed identically enriched *Artemia* (Great Salt Lake, UT, USA). Enrichment alternated daily between 24 hrs of DHA Selco (INVE, Dendermond, Belgium) or 24 hrs of Algamac (Bio-Marine, Hawthorne, CA, USA). Second instar stage *Artemia* were stocked in 300-L tanks at a density of 2 X 10^5 animals L^{-1}. During enrichment, the temperature was maintained at 26°C and vigorous bottom aeration was applied. Enrichment was added at a concentration of 2 g per 10^6 animals. After 12 hours of enrichment, *Artemia* were transferred to a new enrichment vessel to receive a second 12-hour enrichment. *Artemia* were sampled in triplicate four times during the experimental period, with two sampling days, respectively, for each Algamac and DHA Selco.

2.2.4 Larviculture

Yellowtail flounder broodstock were collected from Witless Bay, Newfoundland and brought to the Ocean Sciences Centre in late June of 1998. Eggs for this experiment were obtained from these fish between July 6 to 7, 1998 and were pooled to obtain the required

quantity. They were then incubated in a 300-L cylindro-conical upwelling tank and hatched at approximately 60-degree-days.

At 100 % hatch, larvae were transferred to eight 230 L round, flat-bottomed tanks at a density of 60 larvae L^{-1}. Water flow was set at 2-L min^{-1} and aeration was provided by one airstone placed in the centre of each tank. Fish were reared at ambient temperature ~13°C (10.5°-16.5°C) on a 24-hour photoperiod of ~1000 lux.

Differentially enriched rotifers were added to tanks twice per day at a density of 7000 prey L^{-1}, from day 2 to 28 post-hatch (Puvandndran & Brown, 1995; Rabe & Brown, 2000). Tanks were 'greened' daily with a 50:50 mix of 10 L of *Isochrysis galbana* and *Nannochloropsis spp*. This combined algae mixture had a concentration of 6.1 X 10^{10} cells L^{-1}. After day 30, identically enriched *Artemia* were added to all tanks twice per day at a density of 2000 prey L^{-1}.

2.2.5 Growth and Survival

Standard length, body depth, dry weight, and ash free dry weight were measured at 100 % hatch and at weeks 2, 3, 4, 6 and 13. Standard length was defined as the length in mm from the tip of the snout to the end of the notochord. Body depth was defined as the width of the larvae just posterior to the anus not including the fin fold. Larvae were sampled and placed in beakers that were kept on ice and were then sacrificed using an overdose of 3-aminobenzoate methane sulphonate (MS 222). Measurements were completed within 30 minutes of death to minimise shrinkage due to osmotic loss. These measurements were completed on 15 larvae $tank^{-1}$ $week^{-1}$ using a dissecting microscope and a depression slide.

After morphometric measurements, larvae were washed with 3 % ammonium formate to remove salt, and five larvae were placed on a 1.5 cm^2 pre-weighed foil. This method resulted in three samples of five larvae being analysed for dry weight $tank^{-1}$ $week^{-1}$. At week 13 only one larva was place on each foil due to their increased size. The foils were dried at 60^0C for 24 hours. Foils were then stored in a desiccator and reweighed. Ash weights were also taken on the same samples by placing them in a muffle furnace (450^0C) for a further 12 hours and then re-weighing.

Yellowtail flounder larvae are too small and fragile to easily determine survival in large tanks during the first few weeks post-hatch. Therefore, after the rotifer-feeding period at four weeks, survival was determined by counting larvae in subsamples of water. Aeration was increased prior to sampling to provide enhanced mixing of the tank water and then each tank was thoroughly stirred with a glass rod. Five sub-samples of four L of water were counted per tank to calculate an average number of larvae per tank. At week 13 all remaining larvae were counted.

2.2.6 Lipid samples:

Lipid samples were taken from eggs and larvae throughout the experiment. Three samples of 500 individuals were taken on both day two of incubation and at 100 % hatch. Triplicate samples from each tank were also collected after weeks three, four and six for a total of six samples taken per treatment. The number of individuals per sample was based on the dry weight from the previous week. Samples ranged between 10 and100 individuals and 10 to 30 mg dry weight.

2.2.7 Lipid Analysis

Lipid samples were placed directly in chloroform and stored under nitrogen at -20°C until analysis. Lipids were extracted in chloroform and methanol according to Parrish (1998) using a modified Folch procedure (Folch et al. 1957). Samples were homogenised and then sonicated and centrifuged four times in an 8 4:3 (v/v/v) chloroform:methanol:water solution. The sonication and centrifugation were carried out four times and the chloroform layers were collected and pooled.

Lipid classes were determined using thin layer chromatography with flame ionisation detection (TLC/FID) with a MARK V Iatroscan, as described by Parrish (1987). Lipid extracts were spotted on silica gel coated Chromarods and a three stage development system was used to separate lipid classes. The first separation consisted of two developments in 99:1:0.05 (v/v/v) hexane:diethyl ether:formic acid. The first development was for 25 minutes followed by a second development for 20 minutes. The second separation consisted of a 40-minute development in 80:20:1 (v/v/v) hexane:diethyl ether:formic acid. The last separation consisted of two 15-minute developments in 100 % acetone followed by two 10-minute developments in 5:4:1 (v/v/v) chloroform:methanol:water. After each separation the rods were scanned and the three chromatograms were combined to form one complete chromatogram using T-data scan software (RSS Inc., Bemis, TN, USA). A calibration curve was used to convert given peak areas into lipid mass values. The standards used in the calibration curve were: n-nonadecane (hydrocarbon), cholesteryl palmitate (steryl ester), hexadecan-3-one (ketone), tripalmitin (triacylglycerol), palmitic acid (free fatty acid), hexadecan-1-ol (alcohol),

cholesterol (sterol), glyceryl-1-monohexadecanoate (acetone mobile polar lipid), DL-α-phosphatidylcholine (phospholipid). All standards were obtained from Sigma Chemical Inc. (St. Louis, MO, USA).

Total lipid as well as neutral and polar lipids were analysed for fatty acid composition. Prior to separation of total lipids, 23:0 tricosanoic acid methyl ester was added to larval fish extracts at an amount that was approximately 10 % of the total fatty acids. Preliminary analysis of samples without 23:0 revealed that it would be a suitable internal standard. Total lipids were separated into neutral and polar lipids using column chromatography (Yang, 1995; Budge, 1999). Glass wool was placed in the tip of a Pasteur pipette so that it just filled the tip, and pipettes were then placed in a muffle furnace (450°C) for 12 hours to remove any organic contamination. Approximately 0.8 g of activated silica gel was packed into each pipette. Prior to use, silica gel was activated in an oven at 110°C for one hour. The silica was washed with four ml of methanol followed by four ml of chloroform. Lipid extracts were applied to the top of the column and the neutral lipids were eluted with three ml of 99:1:0.5 (v/v/v) chloroform:methanol:formic acid. The remaining polar lipids were removed using six ml of methanol

Fatty acid methyl esters (FAMEs) were prepared by transesterification with 10% BF_3 in methanol (Morrison & Smith, 1964). Lipid extracts were evaporated to near dryness under nitrogen and 0.5 ml of hexane and one ml of BF_3/methanol were added. Vials were shaken and sealed with Teflon tape before placing in an oven at 85°C for one hour. Samples were removed from the oven and allowed to cool for five minutes. Then

0.5 ml of choloroform extracted water and two ml of hexane were added and the mixture was shaken, centrifuged and the upper layer containing the FAME was removed.

A Varian model 3400 GC equipped with a Varian 8100 autosampler was used for fatty acid analysis. The column was an Omegawax 320 column, 30 m, 0.32 mm i.d., 0.25 μm film thickness (Supelco, Ind., Bellefonte, PA, USA). Hydrogen was used as the carrier gas and the flow rate was set at 2 ml $minute^{-1}$. The column temperature profile was as follows: 65°C for 0.5 minute, hold at 195°C for 15 minute after ramping at 40°C $minute^{-1}$, and hold at 220°C for 0.75 minute after ramping at 2°C $minute^{-1}$. The injector temperature increased from 150 to 250°C at 200°C $minute^{-1}$. Peaks were detected by flame ionisation and the detector was held at 260°C. Fatty acid peaks were integrated using Varian Star Chromatography Software (version 4 02) and identification was made with reference to known standards (PUFA 1 and 37 Component FAME Mix, Supelco Inc).

2.2.8 Pigmentation and Eye Migration

Pigmentation, eye migration, and orientation of the fish were classified at week 13. Scales were used to summarise the range in both pigmentation (1-6) and eye migration (0-3) that were based on categories previously defined for Atlantic halibut (Simplified from Gara et al, 1998; Table 1 & 2). These two classifications were made while viewing the fish on a flat petri dish from directly above. Also, the side on which the fish were lying was recorded. These parameters along with wet weight were recorded for 50 fish per tank.

Table 2.1: Categories of pigmentation used in evaluation of yellowtail flounder (Simplified from Gara et al., 1998)

Categories of Pigmentation	Definition
1	No pigmentation visible
2	Pigmentation only visible on the head
3	Pigmentation visible on the head and tail
4	Pigmentation visible on the head and abdomen
5	Completely pigmented on the ocular side
6	Completely pigmented on both the ocular and blind side

Table 2.2: Categories of eye migration used in evaluation of yellowtail flounder (Simplified from Gara et al., 1998)

Categories of Eye Migration	Definition
0	Blind side eye not yet visible
1	Blind side eye only partially visible but not the full diameter
2	Blind side eye diameter fully visible but not past the dorsal margin
3	Blind side eye visible and fully past the dorsal margin

2.2.9 Statistical Analysis

Differences in growth and fatty acid parameters amoung treatments were analysed using an ANOVA with tanks nested into treatments. In all cases, except for week six lipid data, the effect of tanks was not found to be significant and was dropped from the model. Week six lipid data were analysed based on tank means due to differences between tanks in the same treatment. The interaction term between treatments and week was significant. Therefore, one way ANOVAs with Tukey's multiple comparison test were used to compare weekly differences in these parameters between treatments. Significance was set at alpha = 0.05. Residuals versus fitted values were examined to check for normality and heteroscedasticity. Body depth and dry weight data were log transformed and certain percentage data was arcsine-square root transformed in order to meet the assumption of the model.

Pigmentation and eye migration and side of settlement data were analysed for difference between treatments using a G-test based on the chi-square distribution. Where differences were detected, three *a priori* multiple comparison test where carried out to detect differences between treatments with an alpha of 0.05.

2.3 Results Part A:

Effects of feeding differently enriched rotifers during the first four weeks post-hatch on growth, survival, lipid composition, and pigmentation of yellowtail flounder

2.3.1 Lipid composition of emulsions

The four experimental emulsions were similar in lipid class composition with triacylglycerol (TAG) as their main component. The DHA, DHA+AA, and control emulsions were ~86% TAG with alcohol (ALC), sterol (ST), acetone mobile polar lipid (AMPL) and phospholipid (PPL) making up the remainder of the emulsions. However, the DHA+EPA emulsion had less TAG, 64%, and more ALC, 12%, than the other three emulsions (Table 2.3, $p<0.05$). The DHA+EPA emulsion was the only one that contained the marine oil.

Table 2.4 shows the fatty acid composition of the four emulsions. The emulsions that were high in polyunsaturated fatty acids (PUFA) contained high levels of DHA (36.0-43.3%), while the control emulsion had only 0.5% DHA. The DHA+EPA treatment had a high level of EPA (14.2%) and the DHA+AA emulsion had a high level of AA (8.9%). The major monounsaturated fatty acid (MUFA) in all emulsions was 18:1n-9, however, the control diet contained higher levels of this fatty acid (70%) than the other emulsions (~22%, Table 2.4).

Table 2.3: Lipid class composition of the four experimental emulsions (mean ± SEM, n=3)

Lipid class (% of total lipid)*	DHA	DHA+EPA	DHA+AA	CONTROL
Triacylglycerols	86.3 ± 1.3[a]	64.1 ± 0.5[b]	83.6 ± 1.3[a]	87.0 ± 1.5[a]
Free Fatty Acids	1.2 ± 0.5	0.5 ± 0.1	1.3 ± 0.1	1.9 ± 1.1
Alcohols	2.1 ± 1.0[a]	12.3 ± 0.5[b]	2.8 ± 0.9[a]	2.4 ± 0.3[a]
Sterols	2.7 ± 0.8	7.9 ± 2.6	3.0 ± 0.4	1.4 ± 0.2
Acetone-Mobile Polar Lipids	2.5 ± 0.1	5.8 ± 1.3	2.3 ± 0.7	0.8 ± 0.1
Phospholipids	3.3 ± 0.2	6.1 ± 1.0	5.4 ± 1.5	6.0 ± 0.5

*Also contained < 1% hydrocarbons, ketones, and diacylglycerols.
[ab] Different letters represent a significant difference between emulsions (One Way ANOVA with Tukey's Multiple Comparison Test, $p < 0.05$, $F_{3,8}$)

Table 2.4: Fatty acid composition of the four experimental emulsions (mean ± SEM, n=3)

Fatty acids (% total)	DHA	DHA+EPA	DHA+AA	CONTROL
14:0	14.0 ± 0.1	8.4 ± 0.0	11.5 ± 0.3	0.6 ± 0.0
16:0	10.7 ± 0.0	6.9 ± 0.0	10.3 ± 0.1	12.6 ± 0.2
18:0	1.1 ± 0.1	1.9 ± 0.0	2.9 ± 0.1	2.2 ± 0.2
ΣSFA[a]	**26.1 ± 0.2**	**17.8 ± 0.1**	**25.1 ± 0.6**	**16.8 ± 1.4**
16:1n-7	1.1 ± 0.0	1.2 ± 0.0	1.0 ± 0.0	1.2 ± 0.9
18:1n-9	24.7 ± 0.1	18.6 ± 0.2	23.1 ± 0.6	69.5 ± 1.0
ΣMUFA[b]	**26.2 ± 0.2**	**21.5 ± 0.6**	**24.6 ± 0.7**	**71.3 ± 2.0**
18:2n-6	2.6 ± 0.0	2.5 ± 0.0	3.9 ± 0.1	10.1 ± 0.1
20:4n-6 (AA)	0.7 ± 0.0	1.6 ± 0.0	8.9 ± 0.2	0.0 ± 0.0
20:5n-3 (EPA)	0.1 ± 0.1	14.2 ± 0.2	0.1 ± 0.0	0.0 ± 0.0
22:5n-3	0.5 ± 0.0	2.6 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
22:6n-3 (DHA)	43.3 ± 0.4	37.4 ± 0.3	36.0 ± 0.4	0.5 ± 0.1
ΣPUFA[c]	**47.7 ± 0.5**	**60.7 ± 0.6**	**50.3 ± 0.7**	**11.9 ± 0.3**

[a] includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, 20:0
[b] includes 18:1n-11, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 22:1n-11, 22:1n-9, and 24:1
[c] includes 16:2n-6, 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, 22:4n-6 and 22:5n-6

2.3.2 Lipid Composition of Rotifers

Following enrichment, all rotifer groups contained approximately 16% (160 $\mu g.mg^{-1}$) of their dry weight as lipid (Table 2.5). TAG was the major lipid class in all rotifer groups reflecting high levels of TAG in enrichment emulsions. The PUFA enriched rotifers had significantly higher levels of TAG (~54%) than the control group (38%), while levels of free fatty acids were significantly higher in the control treatment (20.5%) than in the PUFA enriched groups (~3.9%). PPL levels were similar in all treatments and accounted for ~26% of the total lipid.

The fatty acid composition of the enriched rotifers is given in Table 2.6. All rotifer groups contained ~19% saturated fatty acids (SFA) while the control diet had higher levels of MUFA (64%, $p<0.05$) than seen in the three PUFA treatments (~35%). Control rotifers had lower levels of PUFA (~20%, $p<0.05$) than all other groups, while the DHA+EPA treatment had the highest levels of PUFA (49%, $p<0.05$). Levels of PUFA in the rotifers reflected that of enrichment emulsions. The three PUFA treatments resulted in high levels of DHA (21.5-28.2%) with various levels of EPA (3.5-11.0%) and AA (1.2-7.1%). The control treatment had low concentrations of all these PUFA (<2.5%). The ratio of DHA:EPA in the rotifers varied significantly among groups from a high of 8:1 in the DHA treatment to a low of 0.7:1 in the control diet. Similarly, there was a wide range in the DHA:AA ratio from a high of 24:1 in the DHA treatment to a low of 2.5:1 in the control treatment. The EPA:AA ratio was highest in the DHA+EPA treatment (5:1) and significantly lower in the DHA+AA enriched group (0.5:1).

Table 2.5: Lipid class composition of rotifers enriched for 12 hours using four different oil emulsions (mean ± SEM, n = 4).

	DHA	DHA+EPA	DHA+AA	CONTROL
Total lipid* ($\mu g.mg^{-1}$)	169.7 ± 3.4	168.2 ± 15.3	156.5 ± 22.4	165.2 ± 9.6
Lipid class (% total lipid)				
Steryl/Wax Esters	4.1 ± 1.4	4.1 ± 1.1	4.4 ± 0.9	3.9 ± 1.4
Methyl ketones	2.1 ± 0.6	1.9 ± 0.7	2.7 ± 0.5	2.0 ± 0.5
Triacylglycerols	55.5 ± 1.5[a]	52.3 ± 2.3[a]	53.9 ± 3.1[a]	38.0 ± 4.3[b]
Free Fatty Acids	4.3 ± 0.9[a]	4.5 ± 0.9[a]	2.8 ± 0.8[a]	20.5 ± 3.0[b]
Sterols	2.7 ± 0.6	2.3 ± 0.8	2.8 ± 0.3	2.7 ± 0.2
Acetone Mobile Polar Lipids	3.1 ± 0.8	5.3 ± 0.8	3.9 ± 1.1	2.6 ± 0.9
Phospholipids	24.9 ± 0.5	26.1 ± 1.8	24.8 ± 0.9	26.7 ± 2.0

*Also contained < 2.5 % ethyl ketones, glyceryl esters, alcohols, and diacylglycerols

[ab] Different letters represent a significant difference among groups $p < 0.05$, $F_{3,12}$, One-Way ANOVA with Tukey's Multiple Comparison Test

Table 2.6: Fatty acid composition of rotifers enriched for 12 hours using four different oil emulsions (mean ± SEM, n = 4).

Fatty acid (% total)	DHA	DHA+EPA	DHA+AA	CONTROL
14:0	6.9 ± 0.3[a]	3.7 ± 0.4[b]	5.4 ± 0.2[ab]	1.8 ± 0.5[c]
16:0	10.3 ± 0.9	9.3 ± 0.9	9.7 ± 0.8	10.1 ± 1.4
18:0	2.8 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
Σ SFA[1]	**21.0 ± 1.2**	**17.6 ± 1.3**	**19.6 ± 1.2**	**16.7 ± 1.6**
16:1n-7	4.9 ± 0.4	4.9 ± 0.4	4.7 ± 0.5	5.6 ± 0.5
18:1n-9	26.7 ± 1.0[a]	20.8 ± 0.4[b]	26.4 ± 0.8[a]	52.1 ± 1.0[c]
18:1n-7	0.7 ± 0.4	2.0 ± 0.4	1.1 ± 0.4	1.8 ± 0.9
20:1n-9	2.0 ± 0.1	2.3 ± 0.3	2.0 ± 0.1	2.0 ± 0.2
Σ MUFA[2]	**36.8 ± 1.3[a]**	**33.4 ± 0.4[a]**	**36.3 ± 0.8[a]**	**63.5 ± 1.3[b]**
18:2n-6	4.5 ± 0.3[a]	4.4 ± 0.4[a]	5.5 ± 0.4[a]	10.2 ± 0.5[b]
20:3n-6	0.2 ± 0.0	1.2 ± 0.8	0.7 ± 0.0	0.3 ± 0.0
20:4n-6	1.2 ± 0.0[a]	2.2 ± 0.4[b]	7.1 ± 0.2[c]	0.7 ± 0.0[a]
20:4n-3 (AA)	0.7 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.0
20:5n-3 (EPA)	3.5 ± 0.3[a]	11.0 ± 0.2[c]	3.2 ± 0.2[ab]	2.5 ± 0.2[b]
22:5n-3	2.0 ± 0.3[a]	4.1 ± 0.3[b]	2.0 ± 0.1[a]	1.5 ± 0.1[a]
22:6n-3 (DHA)	28.2 ± 0.5[a]	21.5 ± 0.7[b]	23.4 ± 0.8[b]	1.7 ± 0.1[c]
Σ PUFA[3]	**42.1 ± 0.4[a]**	**49.0 ± 1.0[b]**	**44.2 ± 1.2[a]**	**19.8 ± 0.5[c]**
DHA/EPA	**8.2 ± 0.7[a]**	**1.9 ± 0.1[b]**	**7.5 ± 0.6[a]**	**0.7 ± 0.0[c]**
DHA/AA	**24.5 ± 1.1[a]**	**10.5 ± 1.4[b]**	**3.3 ± 0.0[c]**	**2.5 ± 0.2[c]**
EPA/AA	**3.0 ± 0.3[a]**	**5.4 ± 0.7[a]**	**0.5 ± 0.0[b]**	**3.7 ± 0.4[a]**

[1] includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, and 20:0
[2] includes 18:1n-11, 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, and 24:1
[3] includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 22:4n-6, and 22:5n-6

[a,b] Different letters represent a significant difference among groups $p<0.05$, $F_{3,12}$, One-Way ANOVA with Tukey's Multiple Comparison Test

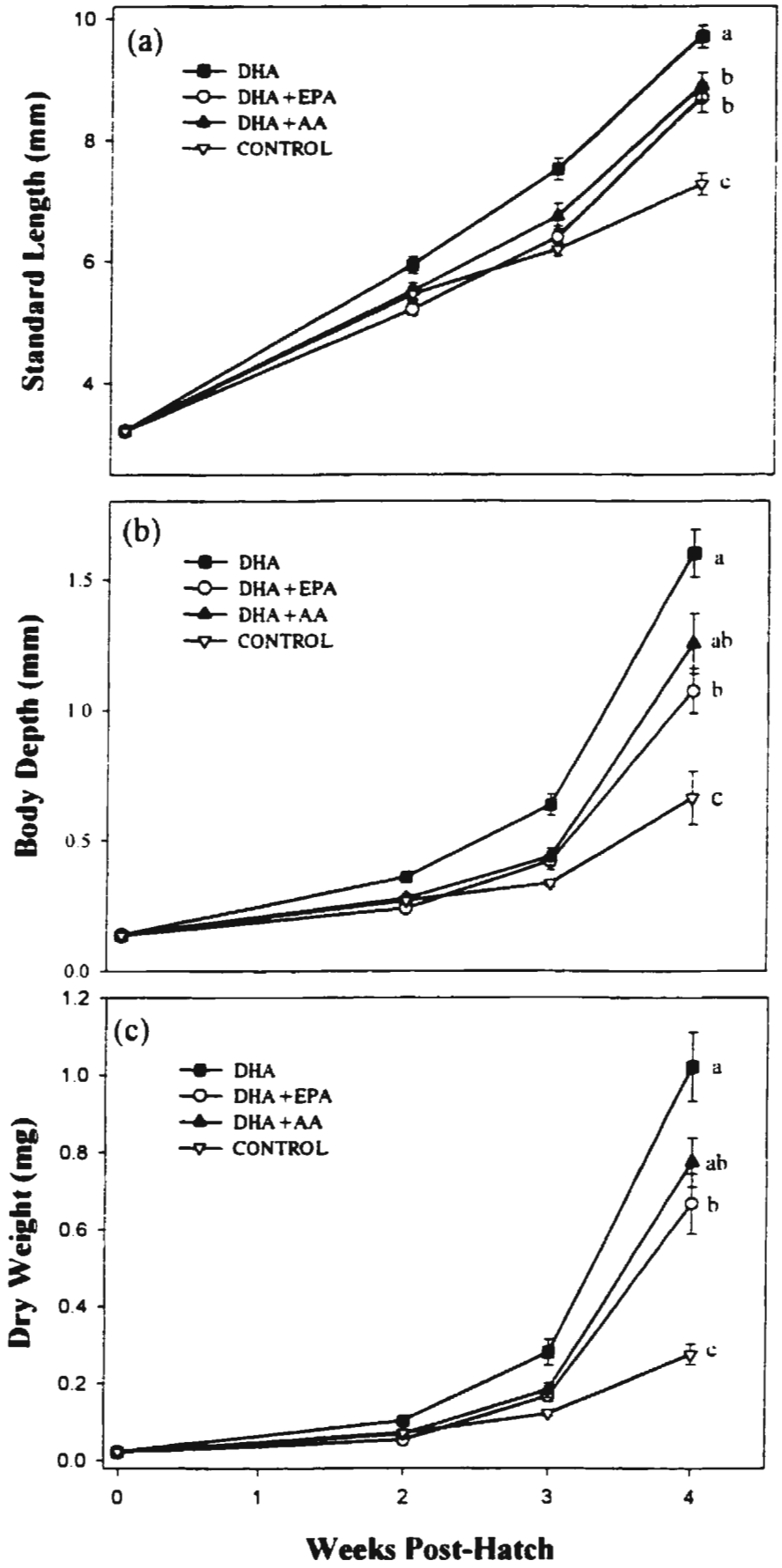
2.3.2 Growth and Survival

Larvae grew and survived in all treatments throughout the first four weeks of rotifer feeding. Feeding differently enriched rotifers over the first four weeks resulted in differences in standard length ($p<0.05$, $F_{3,344}$), body depth ($p<0.05$, $F_{3,344}$), and dry weight ($p<0.05$, $F_{3,56}$). After only two weeks post-hatch, larvae fed the DHA enriched diet were significantly larger in terms of standard length than larvae from the control and the DHA+EPA enriched diets. By week four, larvae in the DHA treatment were significantly larger than all other groups while the control treatment was significantly smaller. There was no significant difference between larvae from the DHA+EPA and DHA+AA groups (Figure 2.1a).

The effect of diet on body depth and dry weight was similar to that on standard length. At week four, larvae fed the control diet were significantly smaller in body depth and dry weight than all other treatments. Conversely, larvae in the DHA treatment were significantly larger than the DHA+EPA and control groups in terms of dry weight and body depth. There were no significant differences between the DHA+EPA or DHA+AA treatments in either body depth or dry weight (Figures 2.1b & 2.1c).

At week four, trends in survival reflected those seen in growth. Larvae in the DHA enriched treatment showed the highest survival (22%) whereas survival in the control group was lowest (5%). Larvae from the DHA+AA group had a survival of 19% while the DHA+EPA group had 12% survival (Figure 2.2).

Figure 2.1: Morphometric measurements and mass of yellowtail flounder larvae fed four types of differently enriched rotifers for the first four weeks post-hatch. Data are mean ± SEM.
(a) Standard length, n=30 ($P<0.05$, $F_{3,116}$)
(b) Body depth, n=30 ($P<0.05$, $F_{3,116}$)
(c) Dry weight, n=6, ($P<0.05$, $F_{3,20}$).
^{abc} Different letters represent significant differences between dietary groups (ANOVA, Tukey's Multiple Comparison).


(a)
DHA
DHA+EPA
DHA+AA
CONTROL
Standard Length (mm)
10
8
6
4
a
b
b
c
(b)
DHA
DHA+EPA
DHA+AA
CONTROL
Body Depth (mm)
1.5
1.0
0.5
0.0
a
ab
b
c
(c)
DHA
DHA+EPA
DHA+AA
CONTROL
Dry Weight (mg)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
ab
b
c
0
1
2
3
4
Weeks Post-Hatch

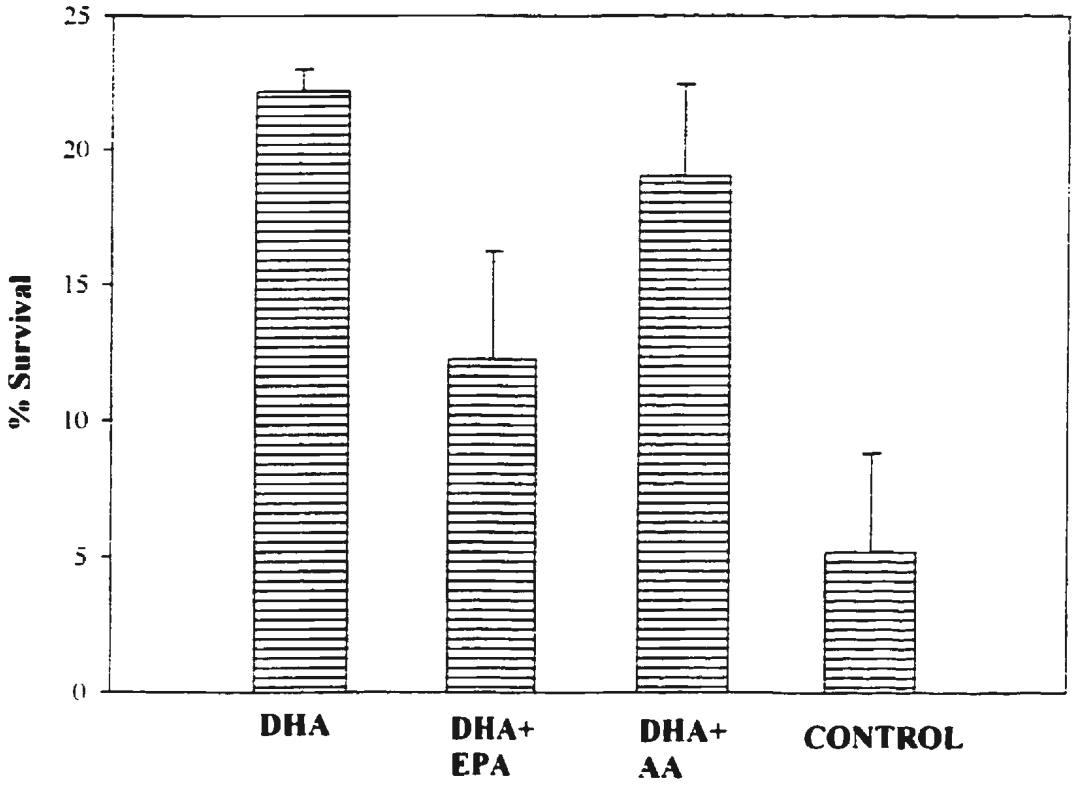


Figure 2.2: Percent survival of yellowtail flounder larvae after four weeks of feeding on differently enriched rotifers. Data are mean + SD, n=2.

2.3.4 Lipid composition of eggs and larvae at 100% hatch

There were no significant differences in lipid class and fatty acid composition between eggs (3 days post-fertilisation) and larvae at 100% hatch (Table 2.7 & 2.8). Both contained ~6 % of their dry weight as lipid. PPL, the major lipid class, accounted for 63% of total lipid. ST was the second largest lipid class at 13% while TAG was present in low levels in both eggs and larvae (~4%). SFA, MUFA, and PUFA accounted for on average 29, 21, and 50% of the total fatty acids respectively. In both groups, the major SFA was 16:0 (21%), while the major MUFA was 18:1n-9 (10%) and the major PUFA was DHA (24%). EPA was also present in significant amounts (16%) and this resulted in a DHA:EPA ratio of approximately 1.5:1 in both eggs and larvae.

Table 2.7: Lipid class composition of yellowtail flounder eggs and larvae at 100% hatch. (mean ± SEM, n=3)

	Egg	100% hatch
Total lipid ($\mu g.mg^{-1}$)	66.3 ± 1.1	62.0 ± 3.1
Lipid Class (% total lipid)		
Hydrocarbons	1.3 ± 0.4	3.4 ± 0.6
Glyceryl esters	0.4 ± 0.4	0.4 ± 0.4
Triacylglycerols	4.7 ± 1.2	2.9 ± 0.1
Free Fatty Acids	6.3 ± 0.8	6.1 ± 0.7
Alcohols	2.7 ± 1.2	1.3 ± 0.2
Sterols	12.8 ± 1.2	12.5 ± 0.6
Diacylglycerols	0.0 ± 0.0	1.3 ± 0.4
Acetone-Mobile Polar Lipids	6.2 ± 1.9	9.2 ± 1.3
Phospholipids	64.1 ± 1.6	62.9 ± 1.8

Table 2.8: Fatty acid composition of yellowtail flounder eggs and larvae at 100% hatch (mean ± SEM, n=3)

% total fatty acids	Eggs	100% hatch
14:0	2.0 ± 0.1	2.1 ± 0.3
16:0	20.7 ± 0.9	21.3 ± 0.6
18:0	3.0 ± 0.1	5.3 ± 2.1
Σ SFA[a]	27.8 ± 0.8	30.8 ± 1.9
16:1n-7	3.2 ± 1.5	3.9 ± 0.8
18:1n-9	10.1 ± 0.8	9.9 ± 0.9
18:1n-7	4.7 ± 0.1	4.7 ± 0.0
20:1n-9	1.0 ± 0.3	0.9 ± 0.4
20:1n-7	0.5 ± 0.2	0.3 ± 0.3
Σ MUFA[b]	20.9 ± 1.2	20.6 ± 0.5
18:2n-6	1.0 ± 0.0	1.2 ± 0.3
20:4n-6 (AA)	2.4 ± 0.3	2.7 ± 0.1
20:5n-3 (EPA)	17.3 ± 0.1	15.6 ± 1.9
22:5n-3	3.8 ± 0.1	3.5 ± 0.4
22:6n-3 (DHA)	24.4 ± 0.9	23.4 ± 0.3
Σ PUFA[c]	51.3 ± 0.5	48.6 ± 2.4
DHA/EPA	1.4 ± 0.1	1.5 ± 0.2
DHA/AA	10.8 ± 2.1	8.8 ± 0.1
EPA/AA	7.6 ± 1.1	5.9 ± 0.6

[a] includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, and 20:0
[b] includes 18:1n-11, 18:1n-5, 22:1n-11, 22:1n-9, and 24:1
[c] includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, 22:4n-6 and 22:5n-6

2.3.5 Lipid Class Composition of Larvae

Figure 2.3a shows the amount of lipid per dry weight from hatch until the end of the rotifer-feeding period. Larvae from the DHA and DHA+EPA diets increased from ~60 $\mu g.mg^{-1}$ at hatch to ~126 $\mu g.mg^{-1}$ at week four, while larvae from the control and DHA+AA diets had only ~87 $\mu g.mg^{-1}$ at week four. Most treatments showed a trend of increasing lipid from hatch until the end of week four, however, larvae from the DHA+AA group decreased in total lipid from week three to week four.

TAG increased significantly in all groups from 100% hatch until the end of the rotifer stage (Table 2.9, Figure 2.3b). At both weeks three and four, larvae fed the high PUFA diets had significantly higher percentages of TAG (~18-22%, $p<0.05$) than larvae in the control group (11%). PPL also increased in all treatments over the first four weeks post-hatch. However, there was no significant difference between dietary groups.

The larval condition index TAG/ ST is shown over the first four weeks post-hatch in Figure 2.3c. This factor increased in all treatments during the first four weeks from a low of 0.4 at 100% hatch. Larvae in the high PUFA treatments showed higher condition factors at weeks three and four than larvae in the control group (Table 2.9, Figure 2.3c, $p<0.05$).

Figure 2.3: Lipids in yellowtail flounder fed four types of differently enriched rotifers for the first four weeks post-hatch. Data are mean ± SEM (n=6).
(a) lipid dry weight^{-1}
(b) TAG (%)
(c) TAG/ST
* represents a significant difference between dietary groups, ANOVA, Tukey's Multiple Comparison test ($P<0.05$, $F_{3,20}$)

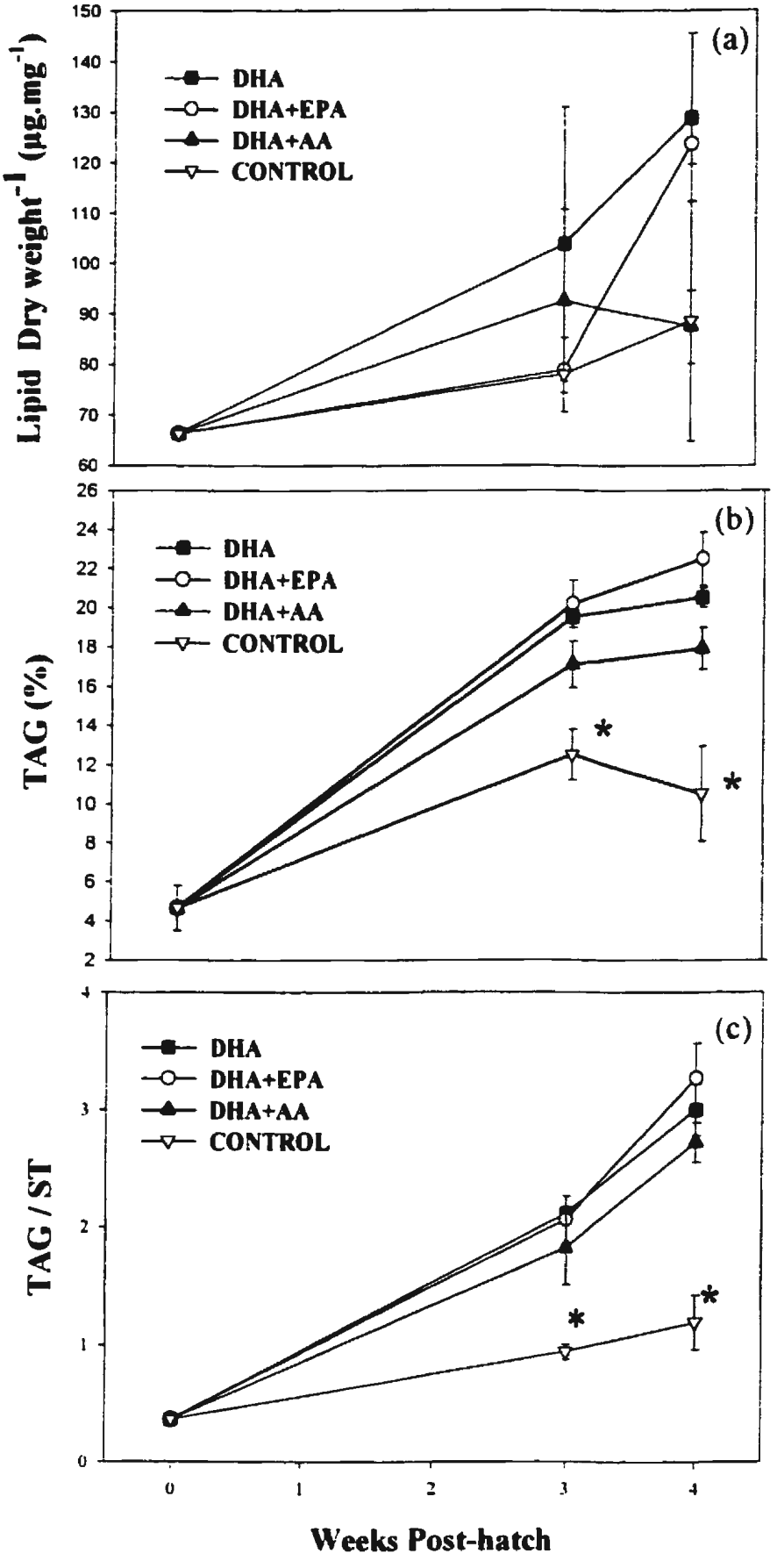

(a)
DHA
DHA+EPA
DHA+AA
CONTROL
Lipid Dry weight^{-1} (µg.mg^{-1})
150
140
130
120
110
100
90
80
70
60
(b)
DHA
DHA+EPA
DHA+AA
CONTROL
TAG (%)
26
24
22
20
18
16
14
12
10
8
6
4
2
*
*
(c)
DHA
DHA+EPA
DHA+AA
CONTROL
TAG / ST
4
3
2
1
0
*
*
0
1
2
3
4
Weeks Post-hatch

Table 2.9: Lipid class composition of larvae after four weeks of feeding on differently enriched rotifers (mean ± SEM, n=6)

	DHA	DHA+EPA	DHA+AA	CONTROL
Total Lipid* ($\mu g.mg^{-1}$)	128.9 ± 9.4	123.7 ± 12.1	87.4 ± 6.6	88.6 ± 12.4
Lipid class (% total lipid)				
Triacylglycerols	20.6 ± 0.8[a]	22.5 ± 0.8[a]	17.9 ± 1.5[a]	10.5 ± 1.3[b]
Free Fatty Acids	1.8 ± 0.1	2.6 ± 0.6	3.0 ± 0.6	3.9 ± 0.5
Sterols	7.1 ± 0.4	6.9 ± 0.4	6.6 ± 0.4	8.8 ± 0.6
Diacylglycerols	3.1 ± 0.7	3.5 ± 0.6	5.0 ± 0.6	6.1 ± 2.2
Acetone-Mobile Polar Lipids	1.6 ± 0.3	2.1 ± 0.4	1.5 ± 0.2	1.3 ± 0.6
Phospholipids	65.1 ± 1.0	61.5 ± 1.1	63.7 ± 3.7	68.3 ± 2.5
TAG/ST	3.0 ± 0.2[a]	3.3 ± 0.3[a]	2.7 ± 0.2[a]	1.2 ± 0.2[b]

*Also contain <1% hydrocarbons, steryl/wax esters, ketones, and glyceryl esters

[ab] Different letters represent a significant difference among groups $p<0.05$, $F_{3,20}$, One Way ANOVA with Tukey's Multiple Comparison Test

2.3.6 Larval fatty acid composition

There was less than 5% difference in the relative fatty acid composition of larvae between week three and four (Appendix a & b, Table 2.10). At either time, the DHA treatment contained significantly higher levels of DHA ($p<0.05$) than all other groups, while larvae in the control group had significantly lower levels (Table 2.10, Figure 2.4). The DHA+EPA treatment had the highest relative and absolute amounts of EPA and larvae in the DHA+AA group had the highest level of AA ($p<0.05$, Figure 2.4). The control diet had the lowest levels of DHA, EPA, and AA with a higher level of 18:2n-6 and 18:1n-9 ($p<0.05$). The DHA:EPA ratio in the total lipid was different amoung all dietary groups with the DHA treatment having the highest and the control treatment the lowest (Table 2.10, $p<0.05$).

Dietary groups showed different trends when the relative incorporation of specific fatty acids was examined (Figure 2.5). Larvae in the high PUFA diets had fatty acid profiles that closely reflected dietary levels, while in the control treatment, larvae showed preferential retention of PUFA and a decrease in 18:1n-9 compared to dietary levels (Figure 2.5). All larvae had lower relative levels of 18:1n-9 in their bodies than in their diet. In all cases, larvae had higher levels of AA than were presented in the diet and this was especially true of the control treatment which had 0.7% AA in the diet and 2.8% AA in the larvae. EPA was present in larval tissue in higher amounts than found in rotifers except for in the DHA+EPA treatment, which had approximately the same amount. The control treatment larvae also showed higher levels of 22:5n-3 in their bodies (3.5%) than was seen in the enriched rotifers (1.7%). DHA was not conserved in the high

Table 2.10: Fatty acid composition of week-four larvae fed differentially enriched rotifers (mean ± SEM, n=6)

	DHA	DHA+EPA	DHA+AA	CONTROL
Total Fatty Acids ($\mu g.mg^{-1}$ dry wt)	64.01 ± 13.00	60.6 ± 3.6	39.1 ± 0.7	48.1 ± 13.4
% total fatty acids				
14:0	4.1 ± 0.2^{a}	3.2 ± 0.3^{b}	3.4 ± 0.0^{b}	1.6 ± 0.4^{c}
16:0	11.0 ± 0.2^{a}	11.3 ± 0.2^{a}	11.9 ± 0.2^{ab}	12.7 ± 0.6^{b}
18:0	4.9 ± 0.1^{a}	5.6 ± 0.1^{b}	5.4 ± 0.0^{ab}	6.0 ± 0.3^{b}
Σ SFA[1]	21.5 ± 0.6	21.9 ± 0.6	22.1 ± 0.3	22.1 ± 1.5
16:1n-7	5.0 ± 0.1^{a}	5.8 ± 0.1^{b}	5.0 ± 0.1^{a}	6.2 ± 0.1^{b}
18:1n-11	1.0 ± 0.2	1.4 ± 0.0	1.2 ± 0.0	1.1 ± 0.1
18:1n-9	21.4 ± 0.5^{a}	18.9 ± 0.9^{b}	18.5 ± 0.6^{b}	26.5 ± 0.7^{c}
18:1n-7	2.5 ± 0.1^{a}	2.9 ± 0.1^{b}	2.3 ± 0.1^{c}	3.3 ± 0.1^{d}
20:1n-9	2.0 ± 0.1^{a}	2.2 ± 0.1^{ab}	2.1 ± 0.0^{a}	2.5 ± 0.0^{b}
Σ MUFA[2]	34.1 ± 1.2^{a}	33.8 ± 1.5^{a}	31.2 ± 1.0^{a}	42.1 ± 1.1^{b}
18:2n-6	5.0 ± 0.2^{a}	4.8 ± 0.1^{a}	5.3 ± 0.2^{b}	10.8 ± 0.0^{c}
20:4n-6 (AA)	2.2 ± 0.0^{a}	2.6 ± 0.2^{b}	8.0 ± 0.2^{c}	2.8 ± 0.1^{d}
20:5n-3 (EPA)	4.8 ± 0.0^{a}	10.1 ± 0.4^{b}	4.6 ± 0.4^{a}	6.7 ± 0.0^{c}
22:5n-3	2.0 ± 0.0^{a}	3.6 ± 0.1^{b}	2.1 ± 0.1^{a}	3.5 ± 0.1^{b}
22:6n-3 (DHA)	27.1 ± 1.1^{a}	18.7 ± 1.0^{b}	22.3 ± 1.0^{a}	7.0 ± 0.2^{c}
Σ PUFA[3]	44.4 ± 1.6^{a}	44.3 ± 1.9^{a}	46.7 ± 2.4^{a}	35.8 ± 0.9^{a}
DHA/EPA	5.6 ± 0.2^{a}	1.9 ± 0.0^{b}	4.9 ± 0.6^{c}	1.1 ± 0.0^{d}
DHA/AA	12.5 ± 0.5^{a}	7.2 ± 0.2^{b}	2.8 ± 0.1^{c}	2.5 ± 0.2^{d}
EPA/AA	2.2 ± 0.0^{a}	3.9 ± 0.2^{b}	0.6 ± 0.1^{c}	2.4 ± 0.1^{d}

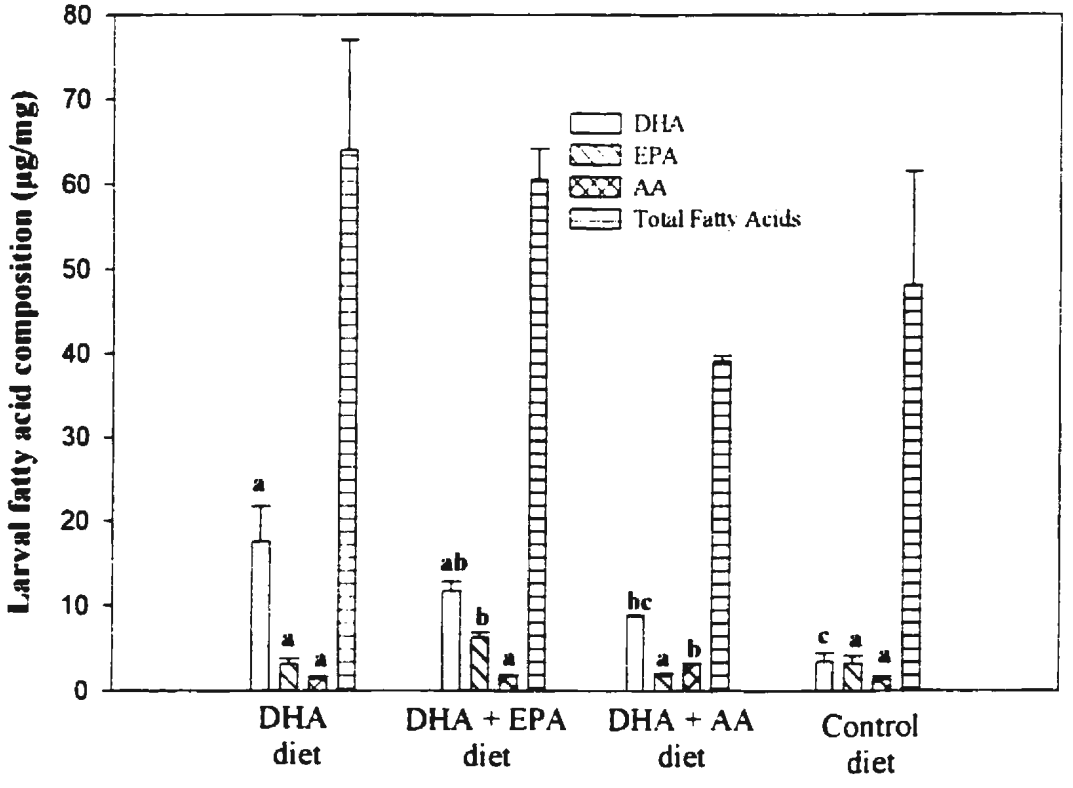
[1] includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, and 20:0

[2] includes 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, and 24:1

[3] includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, 22:4n-6, and 22:5n-6

[abc] Different letters represent a significant difference among groups $p < 0.05$, $F_{3,20}$, One-Way ANOVA with Tukey's Multiple Comparison Test

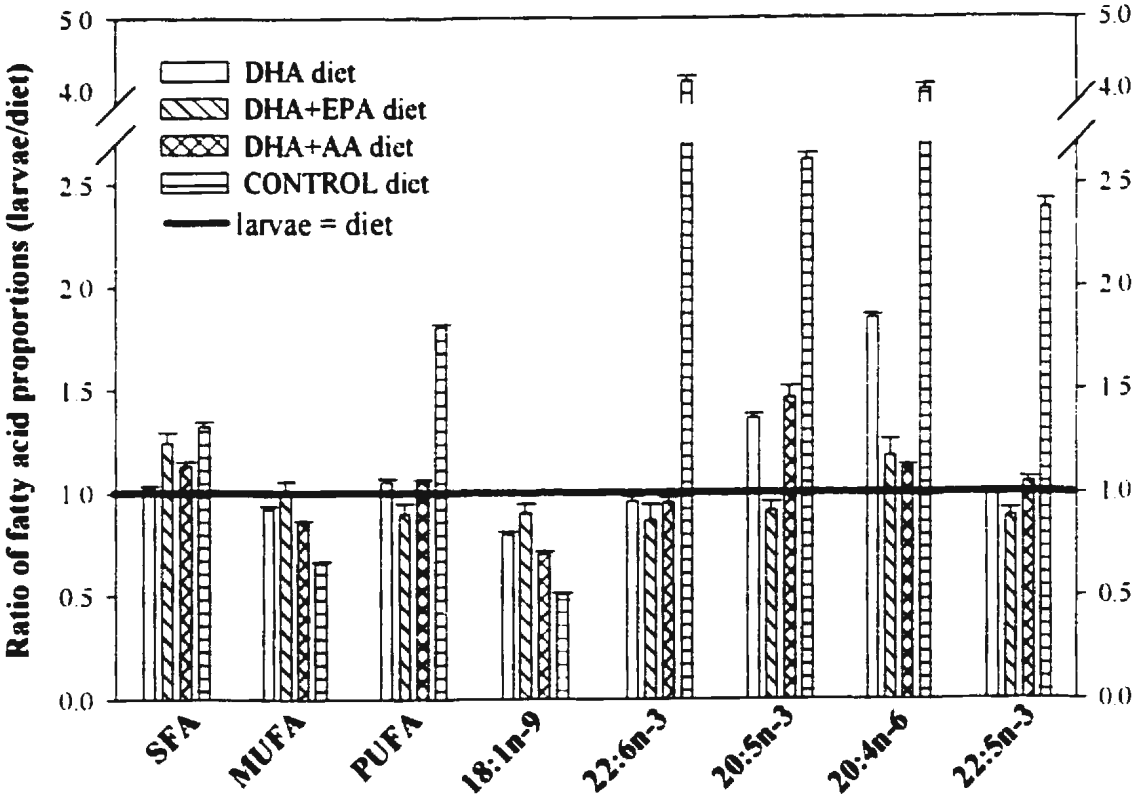
Figure 2.4: Fatty acid composition (µg/mg dry weight) of larvae after four weeks of feeding on differently enriched rotifers.



[abc] Different letters represent significant differences amoung dietary groups (ANOVA, Tukey's Multiple Comparison Test. $P<0.05$ $F_{3,20}$).

Values are mean + SEM, n=6.

Figure 2.5: Relative proportions of specific fatty acids in larvae compared to dietary levels after four weeks of feeding on differently enriched rotifers



Data are means + SEM, n=6.

PUFA treatments, however, the control group had much higher levels (6.9%) than in enriched rotifers (1.7%).

The neutral and polar fatty acid composition of larvae after week four is given in Table 2.11. Larvae in all groups had higher levels of SFA and PUFA in their polar lipids than in their neutral lipids. Conversely, all groups had higher levels of MUFA in their neutral lipid portion than in their polar lipids. In particular, DHA was concentrated in the polar lipid fraction, while in larvae fed the high PUFA diets EPA was present in slightly higher levels in the neutral lipid portion. Only larvae in the control treatment showed higher levels of EPA in the polar lipids than in the neutral. AA was also concentrated in the polar lipid portion and in the DHA+AA diet was present as ~10 % of the fatty acids in the polar lipids (Figure 2.6). The DHA/EPA ratio in all groups was higher in the polar lipid portion than in the neutral lipids. This ratio was highest in the DHA and DHA+AA treatments at ~7:1 and lowest in the control treatment at ~1.5:1.

2.3.7 Relationship of Dietary DHA/EPA and growth and survival

The relationship between dietary DHA/EPA and growth and survival at week four was significant ($p<0.005$). The highest ratio of DHA/EPA was found in the DHA treatment followed by DHA+AA, DHA+EPA, and control treatment. Of all the relationships, the most significant relationship was between larval survival at week four and dietary DHA/EPA (Figure 2.7a, $r^2 = 86.5\%$, $p=0.001$). Standard length and dry weight were also significant related to dietary DHA/EPA, although, body depth showed the most significant relationship (Figure 2.7b, $r^2 = 75.4\%$, $p=0.005$).

Table 2.11: Fatty acid composition of the neutral and polar lipids of yellowtail flounder larvae after feeding on differently enriched rotifers for four weeks post-hatch (mean ± SEM, n=6)

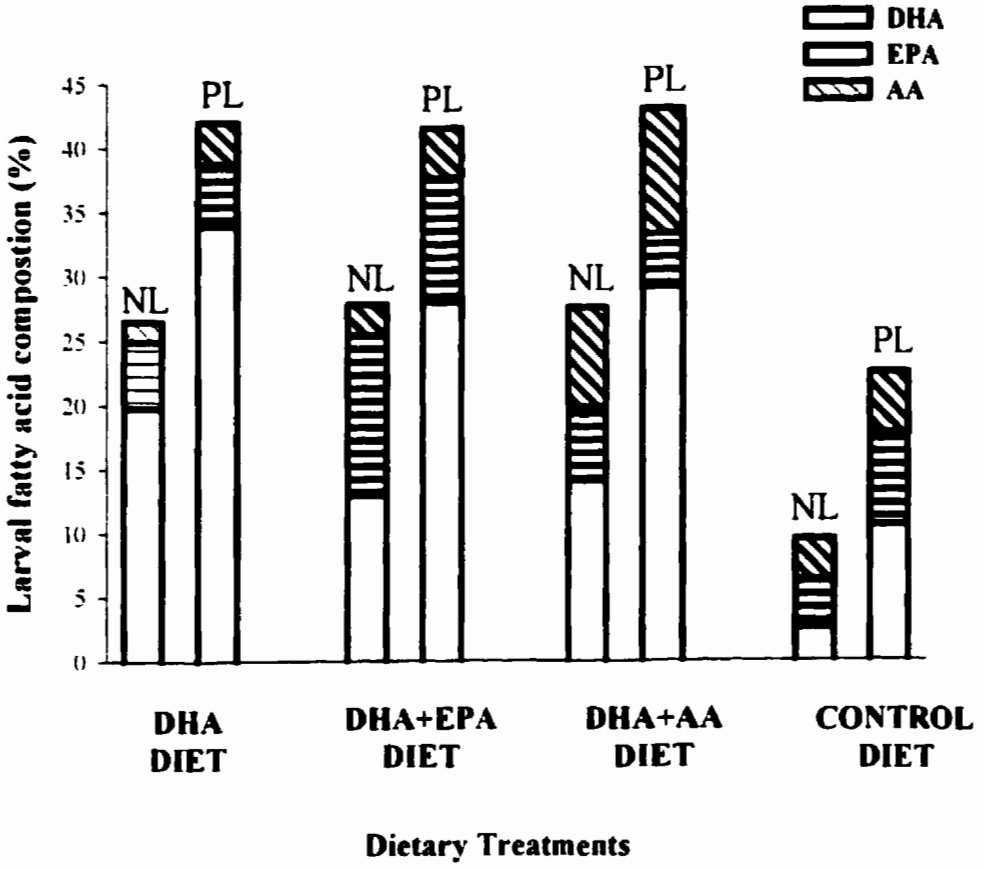
% of total fatty acids	DHA Neutral	DHA Polar	DHA+EPA Neutral	DHA+EPA Polar	DHA+AA Neutral	DHA+AA Polar	Control Neutral	Control Polar
14:0	6.3 ±0.3	2.7 ±0.2	4.8 ±0.2	2.1 ±0.1	6.2 ±0.6	2.2 ±0.1	2.4 ±0.2	1.0 ±0.0
16:0	6.1 ±0.0	12.1 ±0.6	5.9 ±0.1	12.5 ±0.3	6.9 ±0.2	12.6 ±0.2	8.1 ±0.5	12.9 ±2.2
18:0	3.8 ±0.2	7.0 ±0.6	4.5 ±0.20	7.0 ±0.8	3.9 ±0.1	7.3 ±0.2	5.4 ±1.3	7.8 ±1.1
Σ SFA[a]	**17.1 ±0.6**	**23.0 ±1.6**	**16.4 ±0.6**	**22.8 ±1.3**	**19.5 ±2.1**	**23.2 ±0.5**	**17.4 ±2.5**	**23.1 ±3.5**
16:1n-7	7.9 ±0.1	3.2 ±0.3	8.4 ±0.1	3.8 ±0.2	6.5 ±1.2	3.3 ±0.0	9.0 ±0.0	3.9 ±0.5
18:1n-9	27.7 ±0.8	17.4 ±0.4	22.2 ±0.3	15.0 ±0.2	24.0 ±2.3	15.4 ±0.7	35.8 ±1.8	22.2 ±1.0
18:1n-7	2.0 ±0.2	2.8 ±0.2	2.8 ±0.2	3.3 ±0.3	2.0 ±0.3	2.8 ±0.0	3.7 ±1.0	3.7 ±0.3
20:1n-9	2.7 ±0.1	1.7 ±0.3	3.0 ±0.1	1.5 ±0.1	2.9 ±0.1	1.7 ±0.0	3.9 ±0.5	2.1 ±0.2
Σ MUFA[b]	**43.4 ±1.4**	**26.3 ±1.4**	**40.1 ±0.8**	**24.7 ±1.0**	**38.8 ±4.3**	**24.3 ±0.8**	**56.0 ±3.7**	**33.2 ±2.1**
18:2n-6	6.4 ±0.2	4.1 ±0.2	6.3 ±0.0	3.8 ±0.0	6.0 ±1.3	4.3 ±0.2	9.3 ±0.3	11.7 ±0.6
20:4n-6 (AA)	1.6 ±0.0	3.3 ±0.1	2.4 ±0.4	3.9 ±0.2	7.7 ±0.0	9.8 ±0.1	3.2 ±0.1	4.7 ±0.2
20:5n-3 (EPA)	5.1 ±0.1	4.8 ±0.1	12.4 ±0.1	9.7 ±0.4	5.7 ±0.3	4.1 ±0.5	3.7 ±0.2	7.2 ±1.8
22:5n-3	2.3 ±0.1	1.9 ±0.0	3.9 ±0.0	3.9 ±0.1	2.2 ±0.1	2.2 ±0.2	1.4 ±0.2	5.1 ±0.2
22:6n-3 (DHA)	19.8 ±0.3	33.9 ±2.0	12.9 ±0.4	28.0 ±1.3	14.1 ±1.5	29.1 ±0.2	2.5 ±0.1	10.5 ±0.6
Σ PUFA[c]	**39.4 0.8**	**50.7 2.8**	**43.5 1.3**	**52.5 2.1**	**41.7 4.6**	**52.5 1.5**	**26.5 3.2**	**43.7 3.5**
DHA/EPA	3.9 ±0.0	7.0 ±0.2	1.0 ±0.0	2.9 ±0.0	2.5 ±0.4	7.2 ±0.9	0.7 ±0.0	1.5 ±0.3
DHA/AA	12.6 ±0.2	10.3 ±0.9	5.6 ±1.2	7.3 ±0.7	1.8 ±0.2	3.0 ±0.0	0.8 ±0.0	2.3 ±0.2
EPA/AA	3.3 ±0.1	1.5 ±0.1	5.3 ±0.9	2.5 ±0.2	0.8 ±0.0	0.4 ±0.1	1.2 ±0.0	1.5 ±0.4

[a] includes 15:0, ai-15:0, i-17:0, 17:0, and 20:0

[b] includes 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, 24:1

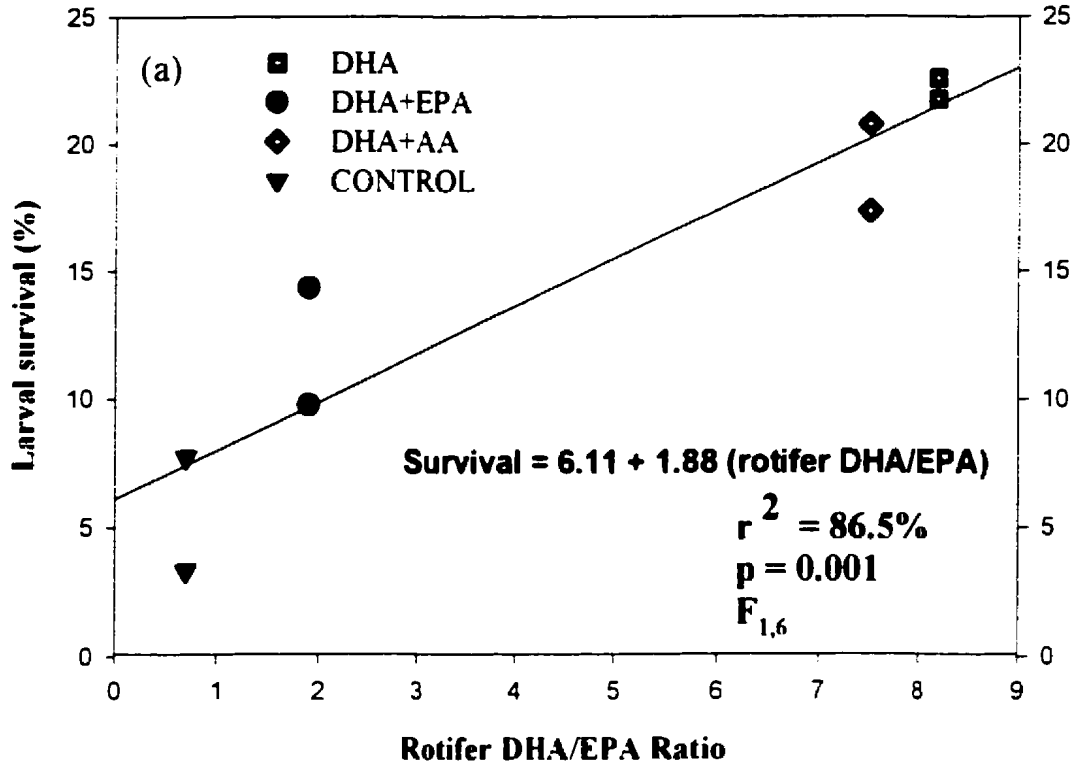
c includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 20:2n-6, 20:3n-6, 20:4n-3, and 22:4n-6

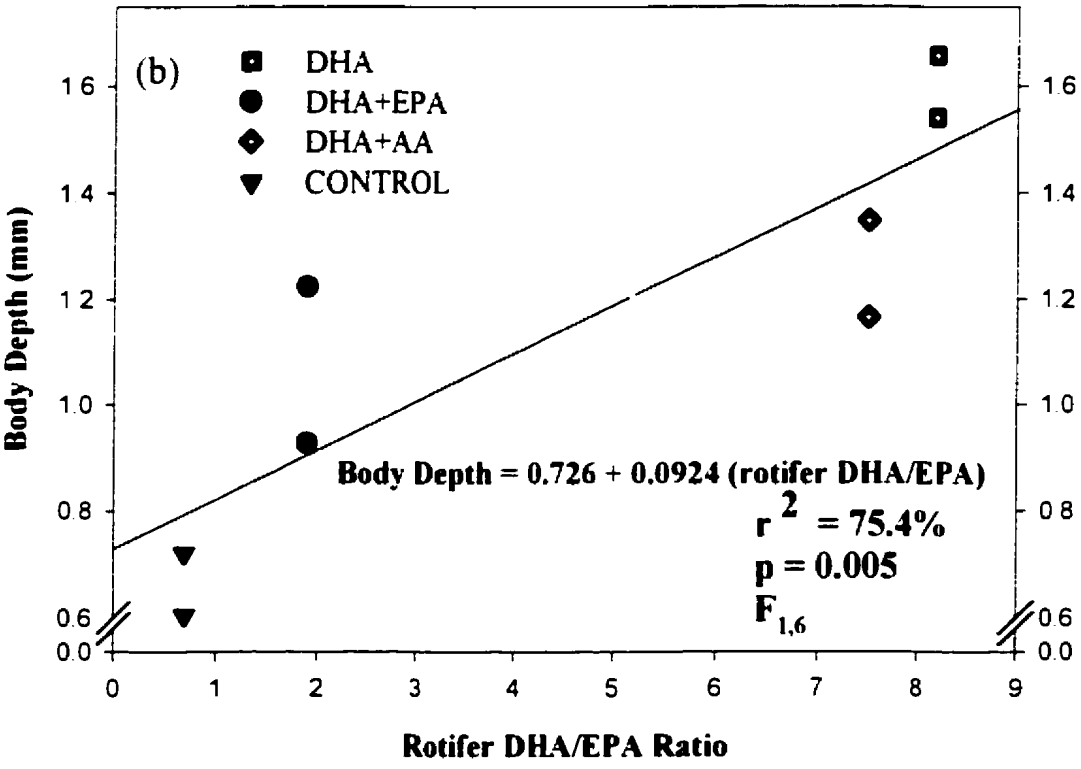
Figure 2.6: The highly unsaturated fatty acid composition of the neutral and polar lipids in yellowtail flounder larvae after four weeks of feeding on differently enriched rotifers



Bars are means (n=6).
NL= neutral lipid and PL= polar lipid

Figure 2.7a: The relationship between (a) survival and (b) body depth (mm) at week four and dietary DHA:EPA for yellowtail flounder fed four types of differently enriched rotifers. Symbols are tank means.


(a)
DHA
DHA+EPA
DHA+AA
CONTROL
Larval survival (%)
Survival = 6.11 + 1.88 (rotifer DHA/EPA)
r 2 = 86.5%
p = 0.001
F1,6
Rotifer DHA/EPA Ratio


(b)
DHA
DHA+EPA
DHA+AA
CONTROL
Body Depth (mm)
Body Depth = 0.726 + 0.0924 (rotifer DHA/EPA)
r 2 = 75.4%
p = 0.005
F1,6
Rotifer DHA/EPA Ratio

2.3.8 Pigmentation, Eye migration, and Orientation

In Table 2.12, pigmentation data are categorised as either normal (stages 5-6) or mal-pigmented on the ocular side (stages 1-4). High proportions of mal-pigmented fish were observed in all dietary groups at the end of the experiment. However, at least 39% of fish were normally pigmented in the DHA, DHA+EPA, and control groups. In the DHA+AA treatment, there was a significantly higher proportion of albinos ($p<0.05$) with only 8% of the fish normally pigmented on their ocular side.

Eye migration is presented as either complete (stage 3) or incomplete (0-2). In all treatments, the percentage of fish with complete eye migration was very low and averaged ~52%. The DHA+EPA treatment had the highest levels of complete eye migration at 75% (Table 2.12, $p<0.05$). The percentage of fish that were settled on their right or left side is also shown in Table 2.12.

Table 2.12: Pigmentation, eye migration, and orientation of yellowtail flounder fed differently enriched rotifers for the first four weeks post-hatch.

Diet	% Normal Pigmentation	% Complete Eye Migration	% Right sided Orientation
DHA	47	47	61
DHA+EPA	39	75*	73
DHA+AA	8 *	46	57
CONTROL	46	42	84

Data are the average of duplicate tanks except for the control treatment where data are from only one tank.

*Represents a significant differences in the odds of having normal pigmentation or complete eye migration. G-test. Chi-square df=3.

2.4 Results Part B

Effects on growth survival and lipid composition of feeding one *Artemia* diet to larvae of differing nutritional status

2.4.1 Lipid composition of *Artemia*

The lipid class and fatty acid composition of *Artemia* after 24 hours of enrichment is given in Tables 2.13 & 2.14. TAG was the major lipid class in enriched *Artemia* and was on average 63% of the total lipid. As in the rotifers, the average percent SFA, MUFA, and PUFA in *Artemia* were ~24%, 31%, and 46% respectively. Also, like rotifers, the major SFA in *Artemia* was 16:0 while the major MUFA was 18:1n-9. However, the PUFA composition of *Artemia* was very different from that of the enriched rotifers. The major PUFA in enriched *Artemia* was 18:3n-3, while this fatty acid made up <1% of enriched rotifers. DHA was on average 7% of the total fatty acids in *Artemia*, which is much lower than the levels in PUFA enriched rotifers (21.5 - 28.2%). EPA and AA on average made up 8% and 2% of the total fatty acids. While, the DHA:EPA, DHA:AA, and EPA:AA ratios were 0.8:1, 4:1, and 4:1 respectively in enriched *Artemia*.

Table 2.13: Lipid class composition of *Artemia* enriched for 24 hours with Algamac or DHA Selco. Data are means ± SD (n = 2).

Lipid class (% total lipid)	Algamac	DHA Selco	Average
Lipid per dry weight ($\mu g.mg^{-1}$)	151.8 ± 33.2	192.7 ± 45.6	172.3 ± 20.5
Ketones	1.4 ± 0.1	0.6 ± 0.3	1.0 ± 0.3
Triacylglycerols	50.0 ± 1.2	76.2 ± 4.5	63.1 ± 13.1
Free Fatty Acids	12.0 ± 1.8	8.6 ± 1.0	10.3 ± 1.7
Sterols	5.4 ± 0.1	3.3 ± 0.5	4.3 ± 1.1
Diacylglycerols	0.4 ± 0.4	0.7 ± 0.0	0.5 ± 0.2
Acetone-Mobile Polar Lipids	6.0 ± 2.7	4.5 ± 0.7	5.2 ± 0.8
Phospholipids	23.1 ± 3.2	5.7 ± 2.1	14.4 ± 8.7

Table 2.14: Fatty acid composition of *Artemia* after enrichment with Algamac or DHA Selco for 24 hours. Data are means ± SD (n = 2).

% total fatty acids	Algamac	DHA Selco	Average
14:0	2.4 ± 0.1	2.1 ± 0.1	2.3 ± 0.1
16:0	15.3 ± 1.0	12.0 ± 0.1	13.7 ± 1.7
18:0	7.1 ± 1.7	4.0 ± 0.0	5.5 ± 1.1
Σ SFA[a]	27.1 ± 2.6	20.4 ± 0.1	23.8 ± 3.6
16:1n-7	1.1 ± 0.9	0.2 ± 0.0	0.7 ± 0.4
18:1n-9	19.7 ± 1.9	24.1 ± 0.0	21.9 ± 2.2
18:1n-7	9.1 ± 0.9	5.4 ± 0.1	7.3 ± 1.8
Σ MUFA[b]	30.3 ± 0.2	30.7 ± 0.0	30.5 ± 0.2
16:3n-4	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.0
18:2n-6	5.6 ± 1.0	7.7 ± 0.1	6.7 ± 1.0
18:3n-3	18.3 ± 1.3	14.4 ± 1.6	16.4 ± 2.0
18:4n-3	2.1 ± 0.0	2.3 ± 0.1	2.2 ± 0.1
20:4n-6 (AA)	2.6 ± 0.2	1.6 ± 0.1	2.1 ± 0.5
20:5n-3 (EPA)	7.5 ± 0.6	8.7 ± 0.5	8.1 ± 0.6
22:6n-3 (DHA)	3.3 ± 1.8	9.6 ± 0.9	6.5 ± 3.2
Σ PUFA[c]	42.6 ± 2.5	48.9 ± 0.1	45.7 ± 3.2
DHA/EPA	0.4 ± 0.2	1.1 ± 0.0	0.8 ± 0.3
DHA/AA	1.4 ± 0.8	5.9 ± 0.2	3.7 ± 2.3
EPA/AA	3.0 ± 0.5	5.4 ± 0.0	4.2 ± 1.2

[a] includes ai-15:0, 15:0, i-17:0, 17:0, and 20:0
[b] includes 18:1n-5, 20:1n-9, 20:1n-7 and 24:1
[c] includes 16:2n-4, 16:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, 22:5n-6 and 22:5n-3

2.4.2 Growth and Survival

At week six, after two weeks of feeding on identically enriched *Artemia*, there were no differences between larvae in the DHA, DHA+EPA, or DHA+AA treatments in any of the growth parameters. However, larvae from the control group were still smaller than all other treatments in terms of standard length (Figure 2.8a, $p<0.05$), body depth (Figure 2.8b, $p<0.05$), and dry weight (Figure 2.8c, $p<0.05$). From week four to six, there was a dramatic increase in both body depth and dry weight when compared to the increase in standard length. This change in growth pattern coincided with the onset of metamorphosis and first observations of newly settled larvae.

From week six to week 13, larvae in all treatments had more than doubled in size (Figures 2.8). At this time, there were significant differences between tanks of the same treatment in all of the growth parameters. Therefore, differences between treatments were not statistically investigated. Interestingly, there was a significant relationship between the number of fish L^{-1} in each tank and their standard length ($r^2 = 76.6\%$, $p<0.005$, Figure 2.9a), body depth ($r^2 = 64.4\%$, $p<0.01$, Figure 2.9b) and dry weight ($r^2 = 77.2\%$, $p<0.005$, Figure 2.9c).

Survival from week four to 13 was variable between tanks within the same treatment and ranged from 4 -18% (Figure 2.10).

Figure 2.8: Morphometric measurements of yellowtail flounder larvae fed four types of differently enriched rotifers for the first four weeks post-hatch followed by identically enriched *Artemia*.
(a) Standard length, n=30 ($P<0.05$, $F_{3,116}$)
(b) Body depth, n=30 ($P<0.05$, $F_{3,116}$)
(c) Dry weight, n=6, ($P<0.05$, $F_{3,20}$).
* represents a significant differences between dietary groups (ANOVA, Tukey's Multiple Comparison). Data are mean ± SEM.

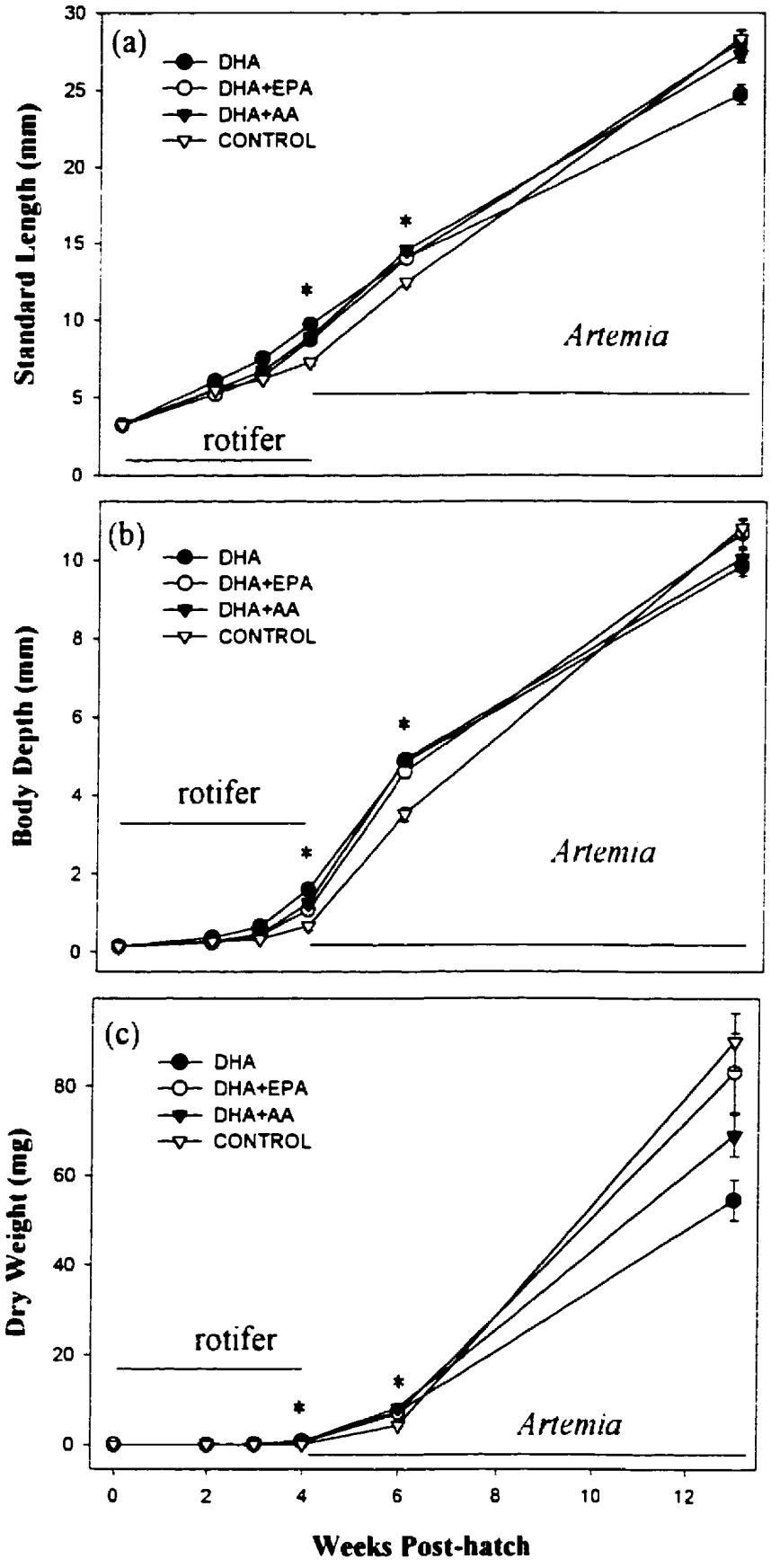

(a)
DHA
DHA+EPA
DHA+AA
CONTROL
Standard Length (mm)
rotifer
Artemia
(b)
Body Depth (mm)
(c)
Dry Weight (mg)
Weeks Post-hatch

Figure 2.9: The relationship between stocking density and (a) standard length, (b) body depth and (c) dry weight at the end of week 13. Data are tank means ± SEM (n=15 for standard length and body depth, n=3 for dry weight). Significance for regression analysis was alpha = 0 05, $F_{1,7}$.

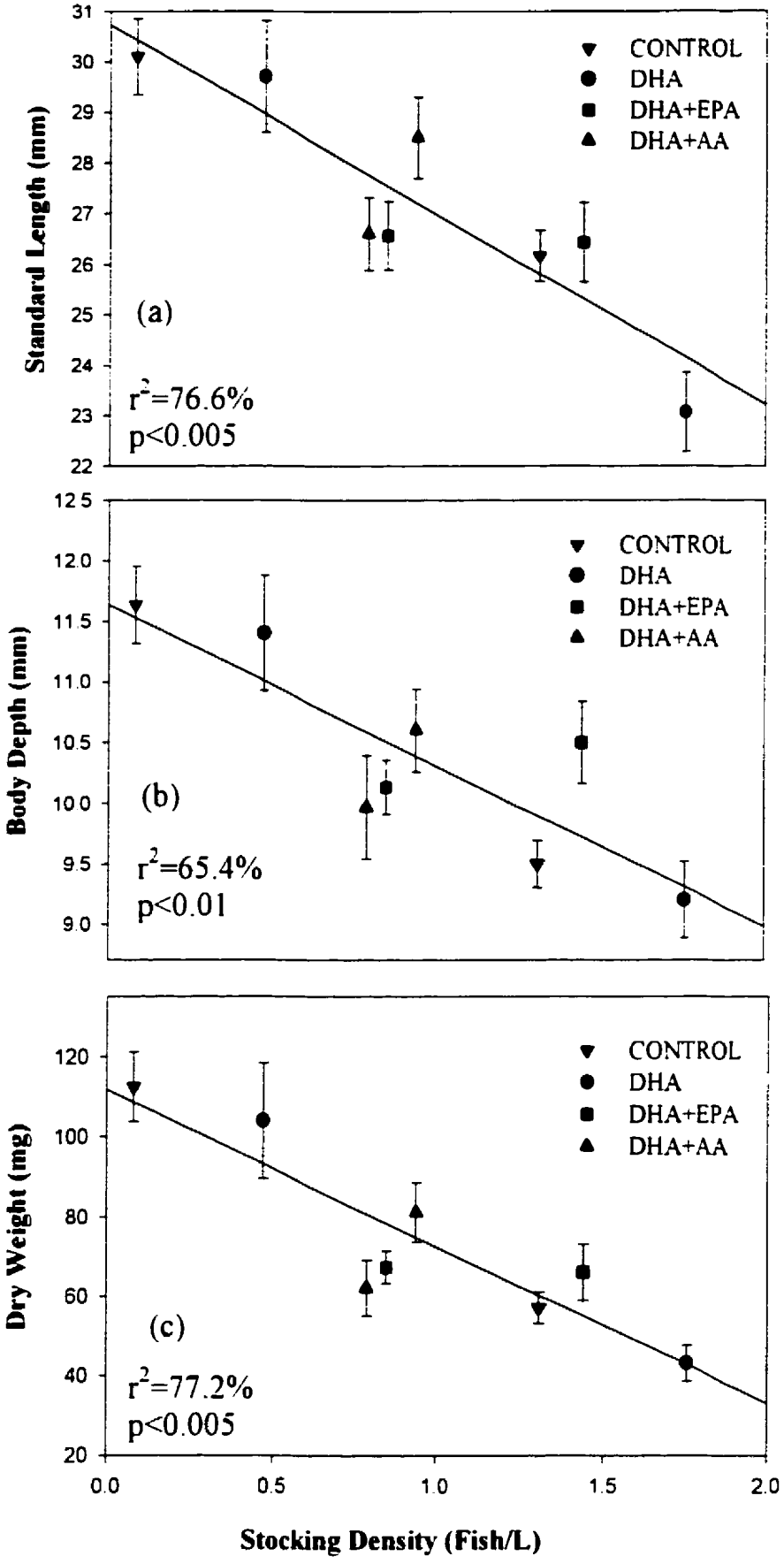
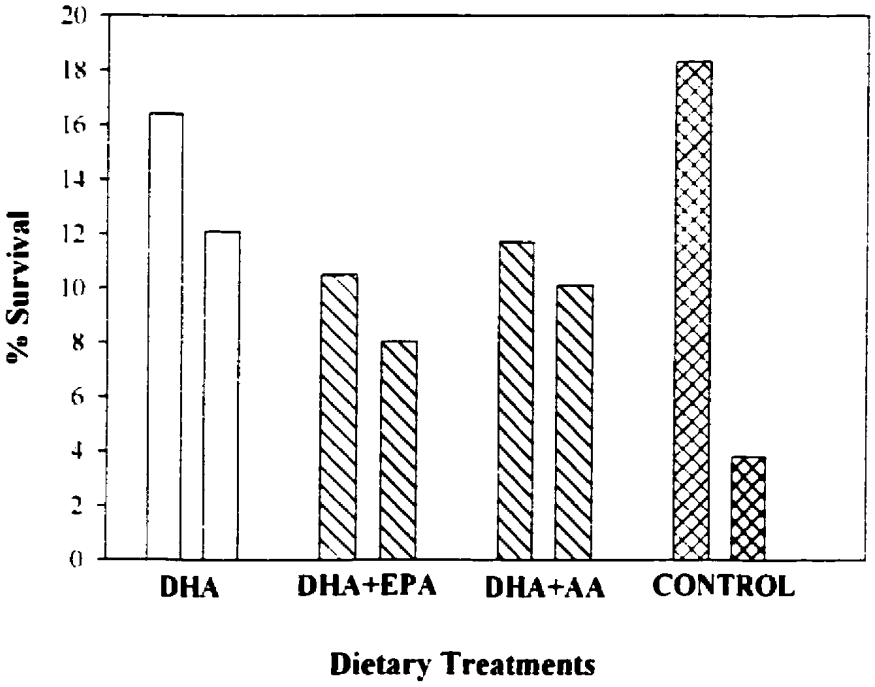

Standard Length (mm)
CONTROL
DHA
DHA+EPA
DHA+AA
(a)
r²=76.6%
p<0.005
Body Depth (mm)
(b)
r²=65.4%
p<0.01
Dry Weight (mg)
(c)
r²=77.2%
p<0.005
Stocking Density (Fish/L)

Fig 2.10: Percent survival of larvae from week 4 to week 13



Bars represent survival for individual tanks

2.4.3 Lipid class composition of larvae

At the end of the rotifer feeding period, larvae from the DHA and DHA+EPA diets had on average ~12.5% of their dry weight as lipid while larvae from the control and DHA+AA diets had ~8.8% (Results Part A, Table 2.9). However, after two additional weeks of feeding on identical *Artemia* all groups had between 12.8 to16.6% lipid (Figure 2.11a, Table 2.15). Larvae in the control treatment showed a dramatic increase from an average of ~8.8% at week four to 16.5% at week six.

After two weeks of feeding *Artemia*, there were no significant differences in the relative amounts of TAG or the TAG/ST ratio between larval groups ($p<0.05$, Table 2.15). At both weeks three and four, larvae from the control diet had lower TAG and TAG/ST ratio than all other treatments (Figure 2.11b & 2.11c). From week four to six, larvae originally fed the DHA and DHA+EPA diets showed a decreasing trend in their TAG/ST ratio, while larvae originally fed the DHA+AA diet showed an increasing trend, and larvae originally fed the control diet showed a dramatic increase from 1.2 to 2.6 (Figure 2.11c). At the end of week six, larvae in the control treatment had a higher percentage of PPL than larvae in all other groups (Table 2.15, Figure 2.11d, $p<0.05$).

Figure 2.11: Lipids in yellowtail flounder fed four types of differently enriched rotifers for the first four weeks post-hatch followed by two weeks of identically enriched *Artemia*.
(a) lipid dry weight^{-1}
(b) TAG (%)
(c) TAG/ST
(d) PPL (%)
* represents a significant difference between dietary groups (ANOVA, Tukey's Multiple Comparison test, $P<0.05$, $F_{3,20}$. Data are mean ± SEM (n=6).

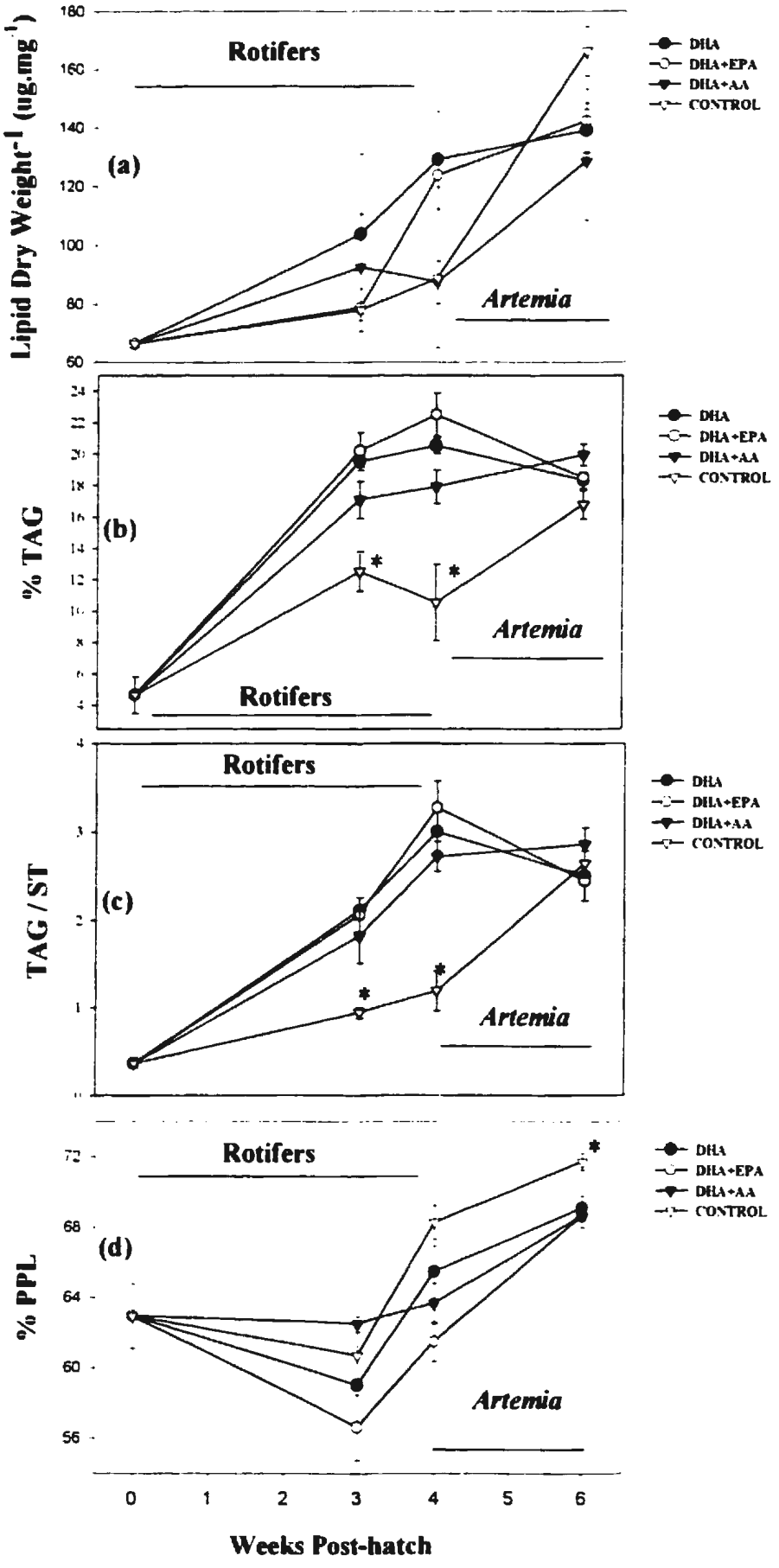

Lipid Dry Weight-1 (ug.mg-1)
Rotifers
(a)
Artemia
DHA
DHA+EPA
DHA+AA
CONTROL
% TAG
(b)
Artemia
Rotifers
TAG / ST
(c)
Rotifers
Artemia
% PPL
(d)
Rotifers
Artemia
Weeks Post-hatch

Table 2.15: Lipid class composition of week-six larvae fed differentially enriched rotifers for four weeks post-hatch followed by identically enriched *Artemia*. Data are mean ± SD (n=2).

	DHA	DHA+EPA	DHA+AA	CONTROL
Total lipid ($\mu g.mg^{-1}$)	138.9 ± 14.2	142.2 ± 15.3	128.5 ± 29.5	165.1 ± 5.2
Lipid class (% total lipid)				
Triacylglycerols	18.9 ± 0.4	18.5 ± 0.4	20.0 ± 0.7	16.8 ± 0.9
Free Fatty Acids	1.5 ± 0.7	0.6 ± 0.1	0.4 ± 0.1	0.8 ± 0.4
Sterols	7.6 ± 0.3	7.6 ± 0.6	7.0 ± 0.2	6.4 ± 0.1
Diacylglycerols	2.3 ± 0.2	2.6 ± 0.1	1.9 ± 0.2	2.1 ± 0.4
Acetone-Mobile Polar Lipids	1.7 ± 0.4	1.9 ± 0.1	1.7 ± 0.1	1.8 ± 0.2
Phospholipids	67.8 ± 0.9	68.6 ± 0.1	68.6 ± 0.7	71.8 ± 0.2
TAG/ST	2.5 ± 0.1	2.5 ± 0.2	2.9 ± 0.3	2.6 ± 0.2

2.4.4 Fatty acid composition of larvae

The fatty acid composition of larvae at the end of week six is shown in Table 2.16. The total fatty acids ranged from ~96 $\mu g.mg^{-1}$ in the DHA+AA group to a high of ~117 $\mu g.mg^{-1}$ in the control treatment. The control group had higher levels of total fatty acids per unit dry weight than larvae in all other treatments ($p<0.05$). However, larvae from the DHA+AA treatment still had a higher percentage of AA than larvae in all other treatments. Also, larvae from the control diet still had a lower relative amount of DHA in their bodies than all other groups ($p<0.05$, Table 2.16).

The most dramatic changes in larval fatty acid composition between week four and week six were in the PUFA (Figure 2.12a). The 18:3n-3 which was <1% of larval fatty acids at week four accounted for on average 14.5% of the total fatty acids, in all dietary groups, at week six (Table 2.16). The 18:4n-3 was also present at week six at ~2.5% of the total fatty acids whereas at week four this fatty acid made up <0.3% of the total. A relative decrease in the level of DHA was seen in larvae from the high PUFA groups while in larvae from the control diet this level stayed constant. The proportion of EPA in larval tissues increased from week four to week six in all treatments except the DHA+EPA group (Figure 2.12a). Similarly, the level of AA in larval tissue remained constant in all treatments except for larvae in the DHA+AA diet; where it decreased from ~8% at week four to ~3% at week six (Figure 2.12a).

There were differences in the fatty acid composition of the larvae compared to dietary levels found in *Artemia* (Figure 2.12b) The most dramatic difference was in the level of 22:5n-3 in the larvae compared to the diet. In all dietary groups 22:5n-3 was

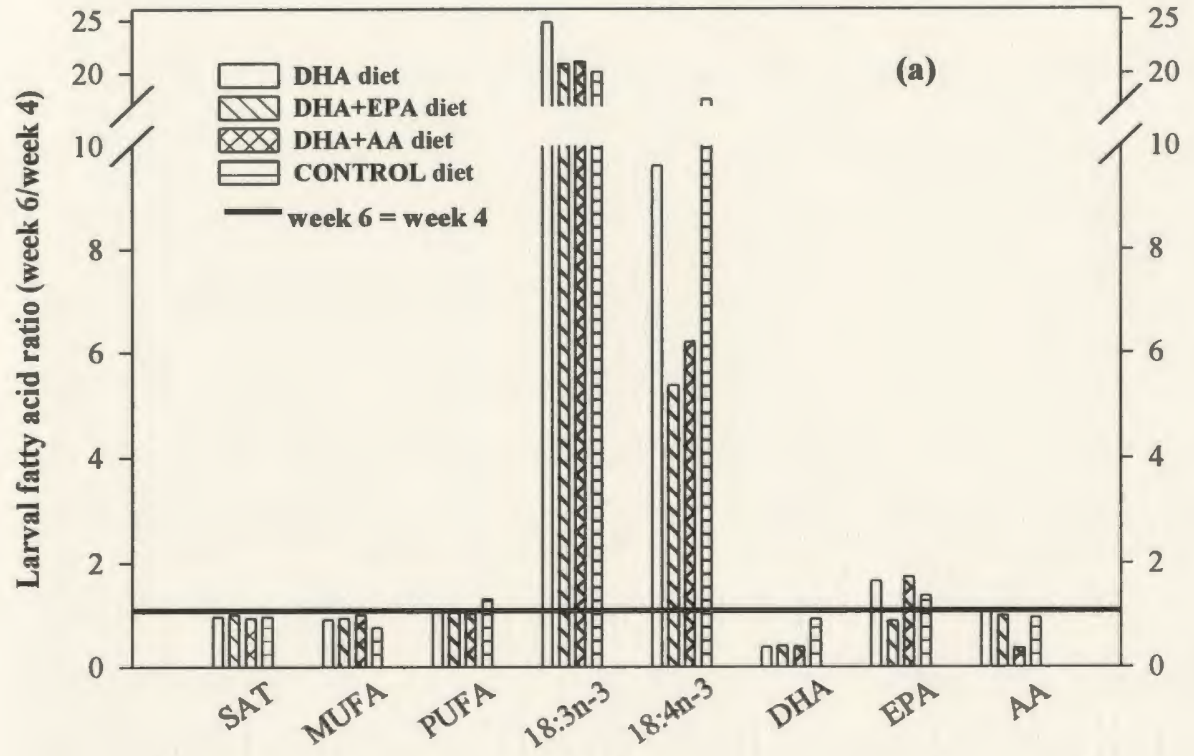
Table 2.16: Fatty acid composition of week-six larvae fed differentially enriched rotifers for four weeks post-hatch followed by identically enriched *Artemia*. Data are means ± SD (n=2).

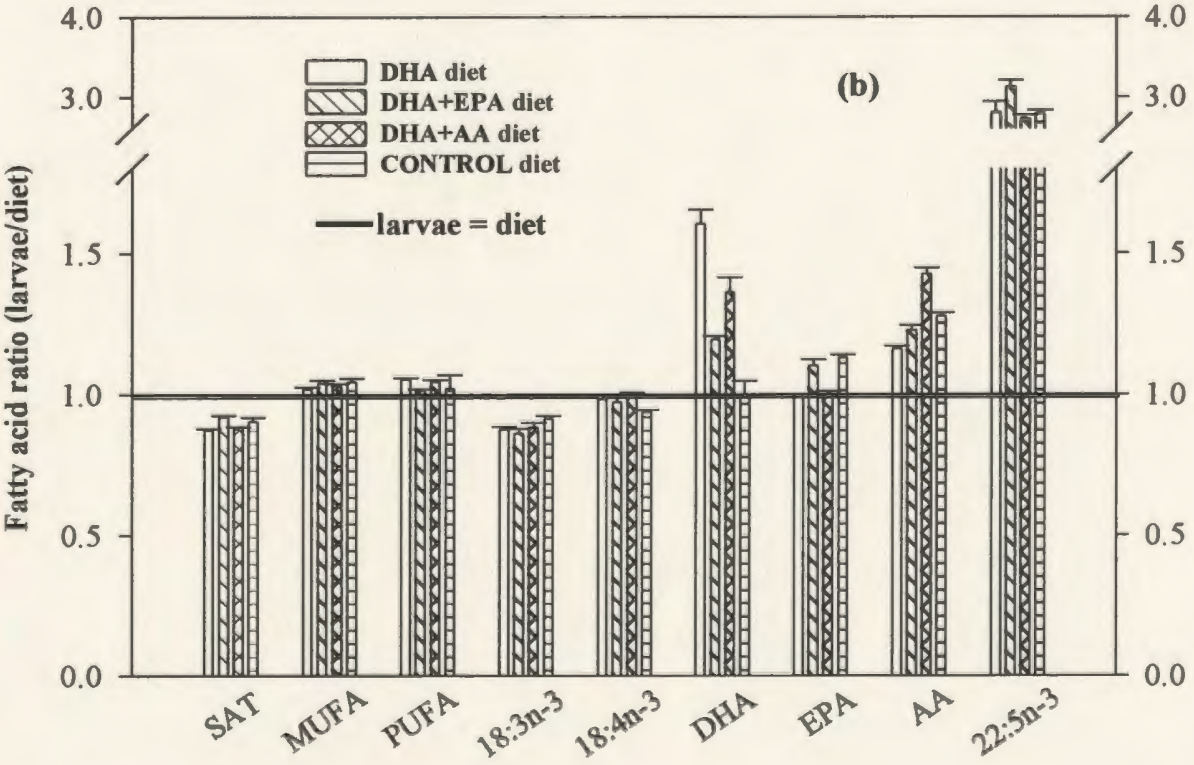
% fatty acids	DHA	DHA+EPA	DHA+AA	CONTROL
Total fatty acids ($\mu g.mg^{-1}$)	108.1 ± 5.1[a]	97.7 ± 5.1[a]	96.7 ± 6.3[a]	117.8 ± 3.3[b]
14:0	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
16:0	11.5 ± 0.2	12.4 ± 0.3	11.8 ± 0.1	12.3 ± 0.1
ai-17:0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
18:0	5.3 ± 0.0	5.4 ± 0.0	5.3 ± 0.1	5.3 ± 0.0
Σ SFA[1]	**20.8 ± 0.3[a]**	**21.9 ± 0.5[b]**	**20.9 ± 0.3[a]**	**21.5 ± 0.2[b]**
16:1n-7	3.4 ± 0.3	3.5 ± 0.1	3.7 ± 0.5	3.2 ± 0.3
18:1n-7	5.5 ± 0.1[a]	6.1 ± 0.1[ab]	5.6 ± 0.1[ab]	6.2 ± 0.2[b]
18:1n-9	19.0 ± 0.1	19.2 ± 0.1	19.0 ± 0.4	19.7 ± 0.2
20:1n-9	1.8 ± 0.0	1.8 ± 0.0	1.7 ± 0.0	1.7 ± 0.1
Σ MUFA[2]	**31.0 ± 0.7**	**31.7 ± 0.4**	**31.3 ± 1.0**	**31.8 ± 0.8**
16:3n-4	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
18:2n-6	5.5 ± 0.0[a]	5.5 ± 0.0[a]	5.6 ± 0.1[a]	5.9 ± 0.1[b]
18:3n-3	14.3 ± 0.2	14.1 ± 0.4	14.5 ± 0.6	15.0 ± 0.3
18:4n-3	2.2 ± 0.0	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.0
20:4n-6	2.4 ± 0.0[a]	2.6 ± 0.1[a]	3.0 ± 0.0[b]	2.7 ± 0.0[a]
20:5n-3	8.0 ± 0.1	8.9 ± 0.4	8.1 ± 0.1	9.1 ± 0.1
22:5n-6	0.9 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.1 ± 0.0
22:5n-3	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.0	1.3 ± 0.0
22:6n-3	10.4 ± 0.5[a]	7.8 ± 0.1[a]	8.8 ± 0.7[a]	6.5 ± 0.7[b]
Σ PUFA[3]	**48.2 ± 0.9**	**46.4 ± 1.3**	**47.7 ± 1.7**	**46.6 ± 1.3**
DHA/EPA	**1.3 ± 0.1[a]**	**0.9 ± 0.1[ab]**	**1.1 ± 0.1[ab]**	**0.7 ± 0.1[b]**
DHA/AA	**4.3 ± 0.2[a]**	**3.0 ± 0.1[b]**	**3.0 ± 0.2[b]**	**2.4 ± 0.2[b]**
EPA/AA	**3.3 ± 0.0[a]**	**3.5 ± 0.1[a]**	**2.7 ± 0.0[b]**	**3.4 ± 0.1[a]**

[1] includes 15:0 ai-15:0 i-17:0 17:0 20:0
[2] includes 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, 24:1
[3] includes 16:2n-4, 16:4n-3, 20:2n-6, 20:3n-6, 20:4n-3

[ab]Different subscripts represent a significant difference between dietary groups. One Way ANOVA, $F_{3,7}$, $p < 0.05$

Figure 2.12: Ratios of fatty acid proportions in yellowtail flounder fed differently enriched rotifers for four weeks post hatch followed by identically enriched *Artemia* for two weeks: a) Relative change in larval fatty acid composition from week four to week six. The solid line indicates where the proportions at week four were equal to those at week six. b) Relative proportions of specific fatty acids in larvae compared to dietary levels. Bars are means ± SEM (n=6). The solid line indicates where the proportions in the larvae are equal to those in the diet.


Larval fatty acid ratio (week 6/week 4)
25
20
10
8
6
4
2
0
DHA diet
DHA+EPA diet
DHA+AA diet
CONTROL diet
week 6 = week 4
(a)
SAT
MUFA
PUFA
18:3n-3
18:4n-3
DHA
EPA
AA


Fatty acid ratio (larvae/diet)
4.0
3.0
1.5
1.0
0.5
0.0
DHA diet
DHA+EPA diet
DHA+AA diet
CONTROL diet
larvae = diet
(b)
SAT
MUFA
PUFA
18:3n-3
18:4n-3
DHA
EPA
AA
22:5n-3

approximately three times higher in larval tissue than it was in *Artemia*. Enriched *Artemia* showed lower levels of AA than all larval groups. In the DHA+EPA and control diets, EPA was found at slightly higher levels in the larvae than in the diet while in the DHA and DHA+AA larvae, it was present in the same amount as in the diet. The control group was the only treatment that did not have higher levels of DHA in their bodies than was found in enriched *Artemia*. The 18:3n-3 was present in lower levels in larvae than in their diet.

The distribution of fatty acids in the neutral and polar lipids is given in Table 2.17. Similar to week four, all dietary groups showed a concentration of DHA in the polar lipid portion at the end of week six (Figure 2.13). However, in the high PUFA treatments this conservation was more dramatic than at week four. EPA was also concentrated in all groups in the polar lipid portion. This differs from week four when only the control group showed higher levels of EPA in the polar lipid. In all treatments 18:3n-3 and 18:1n-9 made up larger percentages of the neutral than the polar lipid (Table 2.17)

Table 2.17: Neutral and polar fatty acid composition of six-week-old yellowtail flounder larvae that were fed differently enriched rotifers for 4 weeks post-hatch followed by identically enriched *Artemia*. Data are means ± SD (n=2)

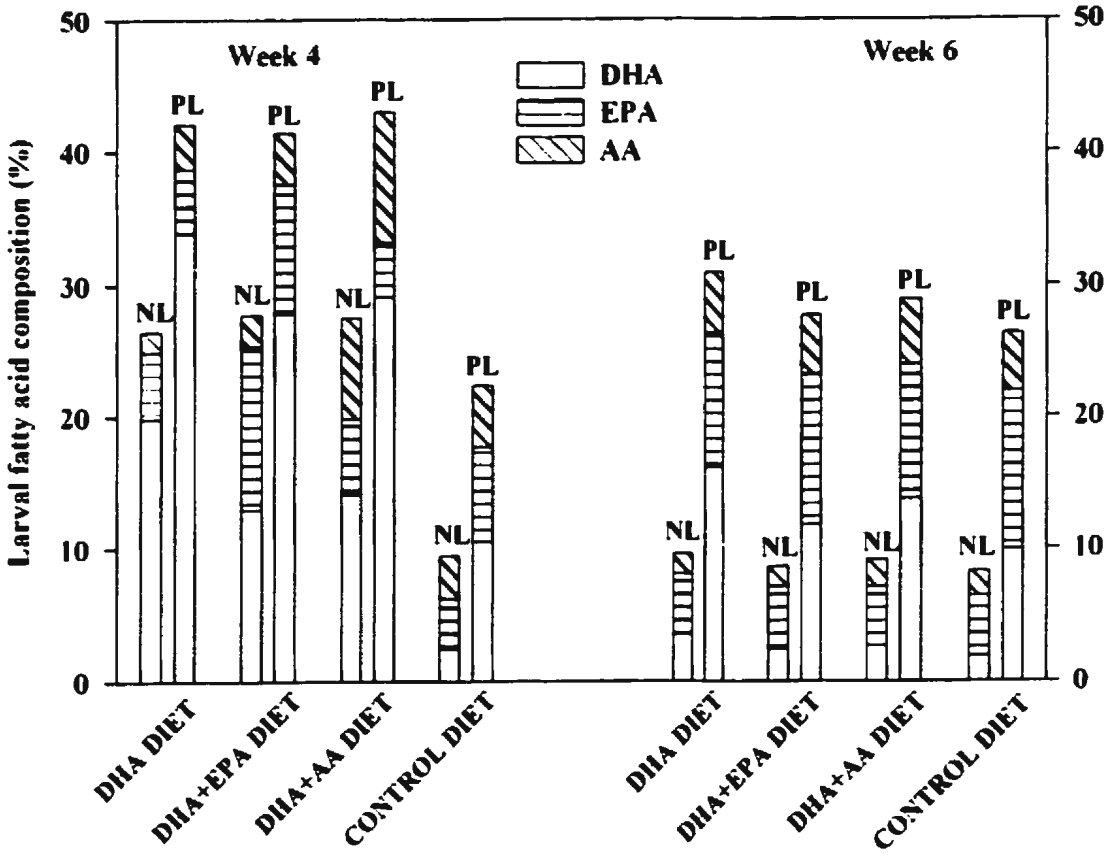
% of total fatty acids	DHA NEUTRAL	DHA POLAR	DHA+EPA NEUTRAL	DHA+EPA POLAR	DHA+AA NEUTRAL	DHA+AA POLAR	CONTROL NEUTRAL	CONTROL POLAR
14:0	1.7 ±0.0	0.9 ±0.0	1.7 ±0.0	0.9 ±0.0	1.6 ±0.0	0.9 ±0.1	1.7 ±0.1	0.9 ±0.0
16:0	7.6 ±0.1	12.9 ±0.3	8.5 ±0.3	12.6 ±0.1	7.4 ±0.2	12.1 ±0.1	8.2 ±0.3	12.8 ±0.1
18:0	4.2 ±0.1	6.4 ±0.2	4.4 ±0.0	6.3 ±0.0	4.4 ±0.1	6.6 ±0.1	4.3 ±0.0	6.4 ±0.1
Σ SFA[a]	**15.9 ±0.3**	**21.7 ±0.1**	**17.0 ±0.3**	**21.3 ±0.1**	**15.7 ±0.0**	**21.0 ±0.1**	**16.5 ±0.3**	**21.5 ±0.2**
18:1n-9	22.4 ±0.1	14.9 ±0.2	23.2 ±0.5	15.8 ±0.2	22.9 ±0.5	15.6 ±0.1	23.7 ±0.2	16.2 ±0.3
18:1n-7	6.7 ±0.2	5.2 ±0.1	7.4 ±0.1	5.8 ±0.1	7.1 ±0.0	5.6 ±0.1	7.6 ±0.1	6.0 ±0.1
16:1n-7	5.4 ±0.0	1.6 ±0.0	5.4 ±0.1	2.0 ±0.0	5.3 ±0.2	1.8 ±0.2	5.4 ±0.1	1.8 ±0.0
20:1n-9	2.3 ±0.0	1.3 ±0.0	2.7 ±0.1	1.4 ±0.1	2.5 ±0.0	1.4 ±0.0	2.4 ±0.1	1.3 ±0.1
Σ MUFA[b]	**38.5 ±0.3**	**23.8 ±0.3**	**40.4 ±0.7**	**25.7 ±0.4**	**39.5 ±0.2**	**25.1 ±0.1**	**40.7 ±0.2**	**25.9 ±0.5**
16:3n-4	1.4 ±0.0	0.7 ±0.0	1.4 ±0.0	0.7 ±0.0	1.4 ±0.0	0.7 ±0.0	1.4 ±0.0	0.7 ±0.0
18:2n-6	5.4 ±0.0	5.4 ±0.0	5.1 ±0.1	5.5 ±0.0	5.4 ±0.1	5.4 ±0.1	5.2 ±0.2	6.0 ±0.0
18:3n-3	19.2 ±0.3	9.7 ±0.2	18.1 ±0.6	10.5 ±0.1	19.1 ±0.6	10.5 ±0.3	18.7 ±0.7	11.3 ±0.0
18:4n-3	3.6 ±0.0	1.1 ±0.0	3.5 ±0.0	1.2 ±0.0	3.6 ±0.1	1.2 ±0.0	3.4 ±0.0	1.1 ±0.0
20:4n-6(AA)	1.5 ±0.3	4.6 ±0.3	1.4 ±0.1	4.5 ±0.2	2.0 ±0.1	4.9 ±0.1	1.9 ±0.4	4.4 ±0.1
20:3n-3	1.5 ±1.5	1.2 ±1.2	3.0 ±0.0	2.4 ±0.0	3.0 ±0.1	2.4 ±0.1	2.9 ±0.1	2.4 ±0.1
20:5n-3(EPA)	4.6 ±0.0	10.2 ±0.2	4.7 ±0.1	11.4 ±0.3	4.4 ±0.4	10.2 ±0.4	4.5 ±0.0	12.0 ±0.4
22:4n-6	0.1 ±0.1	0.0 ±0.0	0.1 ±0.1	0.0 ±0.0	0.2 ±0.0	0.0 ±0.0	0.1 ±0.0	0.0 ±0.0
22:5n-6	0.3 ±0.0	1.1 ±0.0	0.2 ±0.0	1.0 ±0.1	0.3 ±0.1	1.1 ±0.0	0.2 ±0.1	1.0 ±0.1
22:5n-3	0.9 ±0.0	1.5 ±0.0	1.0 ±0.0	1.9 ±0.1	0.9 ±0.1	1.6 ±0.0	0.8 ±0.0	1.7 ±0.0
22:6n-3(DHA)	3.5 ±0.4	16.1 ±0.2	2.5 ±0.2	11.8 ±0.3	2.7 ±0.5	13.7 ±0.7	1.9 ±0.1	9.9 ±0.9
Σ PUFA[c]	**44.2 ±1.4**	**53.4 ±1.0**	**42.7 ±1.0**	**53.1 ±0.3**	**44.8 ±0.2**	**53.9 ±0.1**	**42.8 ±0.6**	**52.6 ±0.7**
DHA/EPA	0.8 ±0.1	1.6 ±0.1	0.5 ±0.0	1.0 ±0.1	0.6 ±0.1	1.4 ±0.1	0.4 ±0.0	0.8 ±0.1
DHA/AA	2.5 ±0.7	3.5 ±0.2	1.7 ±0.2	2.6 ±0.0	1.4 ±0.3	2.8 ±0.2	1.1 ±0.3	2.3 ±0.2
EPA/AA	3.1 ±0.6	2.3 ±0.2	3.3 ±0.2	2.5 ±0.2	2.2 ±0.2	2.1 ±0.0	2.5 ±0.5	2.7 ±0.1

[a] includes 15:0 ai-15:0 i-17:017:0,20:0

[b] includes 18:1n-5, 20:1n-7, 22:1n-11, 22 1n-9, 24:1

c includes 16:2n-4, 16:4n-3, 20:2n-6, 20:3n-6, 20:4n-3

Figure 2.13: Comparison of the neutral and polar highly unsaturated fatty acid composition of yellowtail flounder larvae after four and six weeks of feeding on live-food.



PL= Polar lipid
NL= Neutral lipid

Bars are means (n=6 at week 4 and n=2 at week 6)

2.5: Discussion (Part A)

This is the first study to examine the effect of dietary lipid on the early growth and development of yellowtail flounder larvae. Due to limitations on the number of dietary treatments that could be investigated, diets were chosen to provide maximal information on not only the importance of DHA (22:6n-3) but also EPA (20:5n-3) and AA (20:4n-6).

2.5.1 Rotifer lipid composition

Rotifers enriched with the control emulsion (~70% 18:1n-9) had a significantly higher level of free fatty acid (FFA) than all other rotifer groups (Table 2.5). Rainuzzo et al. (1994) fed rotifers emulsions enriched with different lipid classes (triacylglycerol, wax ester, ethyl ester, or phospholipid) and found that rotifers maintained a constant level of phospholipid independent of the enrichment emulsion. Rotifers fed wax ester emulsions had elevated levels of TAG indicating that rotifers are able to hydrolyse wax esters and oxidise fatty alcohols into fatty acids. Further, rotifers were able to assimilate these fatty acids into the TAG lipid fraction (Rainuzzo et al., 1994)

The elevated level of FFA in our study could indicate that rotifers were able to hydrolyse the TAG from the emulsions but were unable to re-assimilate such high levels of 18:1n-9 into there own membrane or storage lipids. Oleic acid (18:1n-9) is commonly used as a control enrichment (Watanabe 1993; Watanabe & Kiron, 1994; Furuita et al., 1998) however lipid class data for other rotifers enriched with high levels of this fatty acid are not available.

A challenge to providing live-food with proper enrichment is the loss of lipid that can occur in first-feeding tanks prior to larval consumption (McEvoy et al., 1998). Olsen et al. (1993) examined the effect of both temperature (5-18°C) and time of starvation on the rate of lipid loss in rotifers. The rate was found to be low at temperatures < 8°C with essentially no loss of lipids up to four days. However, the rate of lipid loss was found to increase exponentially with temperatures above 8°C. Rainuzzo et al. (1989) also examined the rate of lipid loss in enriched rotifers and found that at 10°C they lost only 10% of their n-3 fatty acids per day. Temperatures during the rotifer-feeding period were <12°C here. Also, high flow rates (2-L min^{-1}) ensured that live-food did not remain in feeding tanks for prolonged periods. Approximately ten tank volumes were exchanged every 24 hours and observations of prey levels showed that <100 prey L^{-1} were available just two hours after feeding.

2.5.2 Larval growth and survival

Diet had a significant effect ($p<0.05$) on both growth and survival during the first four weeks post-hatch. After four weeks, larvae in the DHA diet were significantly larger while larvae in the control diet were significantly smaller than all other treatments (Figure 2.1). Rabe & Brown (2000) examined a pulse feeding strategy for rearing yellowtail flounder. They found that after four weeks of feeding on rotifers which were maintained on culture Selco and enriched with microalgae (*T-Isochrysis*), larvae reached a standard length of ~9 mm. Here larvae ranged between 7.3 - 9.7 mm in standard length

after four weeks. This demonstrates that the experimental emulsions produced growth comparable to trials using more typical enrichment methods.

Trends in survival reflected those seen in growth, with larval survival in the DHA diet the highest (~22%) and that in the control diet the lowest (~5%). Survival is often low and variable for new cold-water aquaculture species. Rabe & Brown (2000) reported survival for yellowtail flounder during the first two weeks post-hatch to range between 1.4 and 17.3% and survival from weeks 2 to 7 ranged from 1.5 to13.8%. These studies represent findings for larvae that were reared in small containers (30-300 L) and it is probable that higher survival would easily be achieved using larger first-feeding tanks.

A significant relationship between dietary DHA:EPA and larval growth and survival was found in this study (Figure 2.7). The dietary DHA:EPA ratio ranged from ~8:1 in the DHA diet to ~1:1 in the control diet. Rodriguez et al. (1997) investigated the effect of dietary ratios of DHA:EPA on early development of larval gilthead seabream (*Sparus aurata*). They used DHA:EPA ratios in the range 1.4:1 – 0.3:1 and found a significant positive relationship between the DHA:EPA ratio in the larval polar lipids and larval total length after two weeks. Furuita et al. (1999) examined the effect of EPA and DHA on the growth, survival and salinity tolerance of Japanese flounder *(Paralichthys olivaceus)* larvae. They found no difference in growth in relation to EPA and DHA. However, larvae receiving the high DHA diet demonstrated better 'vitality' after exposure to stress tests (high salinity water, 65 ppt). Estevez et al. (1999) also tested a range of DHA:EPA ratios (0.1-3.1) on the early development of turbot *(Scophthalmus*

maximus) larvae and found that there was no significant difference between dietary groups in either growth or survival.

The biological significance of dietary DHA:EPA can be viewed in terms of competitive interactions between fatty acids for incorporation into phospholipids, specifically competition for the enzymes that esterify fatty acids onto the glycerophospho-base backbone. Previous studies have shown that high levels of di-22:6 n-3 species are present in the eyes and brains of Atlantic cod (*Gadus morhua*), sea bass (*Dicentrarchus labrax*), turbot and herring (*Clupea harrengus*) (Bell & Dick, 1991; Mourente et al., 1991; Bell et al., 1995; Bell et al., 1996). Diets deficient in DHA have been shown to change the fatty acid composition of neural tissue and decrease foraging efficiency at low light intensities in juvenile herring (Bell et al., 1995). Yellowtail flounder larvae are also visual predators and it is therefore probable that dietary composition could affect their early foraging behaviour and thus growth and survival.

Examination of the lipid composition of eggs/yolk has been suggested as a method for determining the nutritional requirements of newly hatched larvae. Typically a dietary DHA:EPA ratio of 2:1 is found in marine species and has thus been suggested as adequate for larval feeding (Tocher & Sargent, 1984; Sargent, 1995; Sargent et al., 1999). However, analysis of eggs and just hatched larvae here resulted in a DHA:EPA ratio of 1.4:1 and 1.5:1 respectively (Table 2.8). These eggs were collected from fish that were captured only a week prior to spawning and therefore, the egg composition should reflect a wild broodstock diet. There is no previously published data on the lipid composition of yellowtail flounder eggs and larvae. Still, for other marine species, evidence points to a

connection between lipid composition and egg and early larval survival. Navas et al. (1997) reported that decreased TAG and increased n-3 fatty acids were correlated with higher egg quality in European sea bass (*Dicentrarchus labrax*). Similarly, Zhu (1999) found that decreased TAG and increased EPA were associated with better fertilisation success in Atlantic halibut. Fernandez-Palacios et al. (1995) found that for sea bream improved fecundity, hatching success and larval survival were obtained using broodstock diets with 1.6% n-3 HUFA (of the total fatty acids), while 3.2% caused decreased fecundity and yolk sac hypertrophy in newly hatched larvae.

This is the first study to demonstrate a relationship between dietary DHA:EPA and increased rearing success using a ratio as high as 8:1. The elevated DHA used here was achieved by utilising a speciality TAG oil. This oil was produced by a heterotrophic dinoflagellate, *Crypthecodinium cohnii*, which contains DHA as the sole HUFA (Martek BioSci Columbia, MD. USA). The majority of studies in larval nutrition use emulsions based on fish oils which typically have a DHA:EPA ratio of < 2:1; with tuna orbital oil providing the highest ratio at ~3.6:1 (McEvoy et al., 1996; Estevez et al., 1999). This relationship between extraordinarily high dietary DHA:EPA and increased rearing success is new and calls into question the theory of 'nature knows best'. Further studies using novel DHA enrichment products, similar to that used here, may show this relationship in other marine species.

2.5.3 Pigmentation

There were high levels of mal-pigmention in all dietary treatments. However, larvae that received high levels of dietary AA during the first four weeks post-hatch show a significantly lower proportion of normal pigmention (8%) than all other dietary groups (>39%, Table 2.12). Although pigmentation can only be categorised after metamorphosis, the factors that determine pigmentation are influential during the larval phase. These results indicate that the sensitive period for determination of pigmentation in yellowtail flounder is within the first four weeks post-hatch. Flexion of the notochord (45° upward) and an increase in body depth relative to standard length both occurred during the fourth week. Both of these observations indicate that week four represented the onset of metamorphosis. At week six, the beginning of eye migration and the first incidence of settling behaviour were observed.

Seikai et al. (1987) estimated the larval stage at which nutrition had an impact on later pigmentation in Japanese flounder. Live-feed known to induce albinism (*Artemia* nauplii and rotifers) or normally pigmentation (copepods and rotifers) were fed in different sequences in order to deduce the 'pigmentation window'. This critical period was found to occur at about 8 mm standard length during the pre-metamorphic period when larvae began to increase in body depth. Naess & Lie (1998) conducted a similar experiment with Atlantic halibut *(Hippoglossus hippoglossus)* and also found that the sensitive period was the initial stage of metamorphosis: standard length 16 mm and dry weight 2.8 mg. In agreement with this, the 'pigmentation window' for yellowtail

flounder also occurs during the pre-metamorphic stage, standard length < 9 mm and body depth <1.6 mm.

Relatively little is known about the significance of AA on the early growth and development of larval fish and the existing results point to species-specific and age-specific requirements. I found that AA had no positive effect on growth or survival and had a negative effect on pigmentation, when included at 0.8% dietary dry weight (7% of total fatty acids). Castell et al. (1994) found that AA had growth promoting effects in juvenile turbot when included at between 0.3 and 1.0% of dietary dry weight. Bessonart et al. (1999) also found a growth and survival promoting effect of dietary AA when included at between 1.0 and 1.8% of dietary dry weight. However, this effect was masked when the DHA:EPA ratio was altered.

McEvoy et al. (1998) and Estevez et al. (1999) investigated the effects of EPA and AA on pigmentation in Atlantic halibut and turbot. In both these studies, high levels of DHA were present in all diets and the EPA:AA ratio was varied. It was concluded that given adequate DHA, pigmentation was dependent on AA and not EPA. A high incidence of mal-pigmentation was found in turbot larvae that received diets containing EPA:AA ratios of 1.4:1 and 0.3:1. Here, the DHA+AA diet also produced high levels of mal-pigmentation and the EPA:AA ratio was 0.5:1. Estevez et al. (1999) noted that mal-pigmentation occurred when levels of AA and EPA increased and decreased respectively in the phosphatidylinositol (PI) fraction of the brain. Given that AA is the preferred dietary precursor for the production of eicosanoids (Bell et al, 1994), elevated albinism was hypothesised to result from the effects of increased brain eicosanoid production on

both the nervous and endocrine functions associated with metamorphosis. Notably, Estevez et al. (1997) also found that increased dietary AA had a positive influence on pigmentation in Japanease flounder, which reiterates that there are species specific dietary requirements.

Sargent et al. (1999) hypothesised that increased levels of dietary AA may cause a generalised state of stress due to elevated eicosanoid production. Although our levels of mal-pigmentation were highest in the DHA+AA group, levels of mal-pigmentation were still high in all dietary treatments. Larval survival was estimated during week four and this process involved vigorous aeration and stirring of tank water. Therefore, it is possible that this stressful event may have influenced pigmentation in all treatments. It is likely that stressful activities such as enumeration or transferring of larvae should be avoided until well after the 'pigmentation window'. If high levels of mal-pigmentation can be attributed to generalised stress, then improvements in pigmentation of yellowtail flounder may be achieved using lower stocking densities, lower water exchange (turbulence), larger tanks, lower light intensity, increased tank algae concentrations and antibiotics baths.

2.5.4 Larval lipid composition

Lipid class analysis showed that the control treatment had significantly ($p<0.05$) lower levels of TAG and TAG/ST ratios than larvae in all other treatments (Figure 2.3). Relative improvements in larval condition in other species, such as herring and Atlantic cod have been attributed to elevated total lipid, TAG per dry weight and TAG/ST ratios

(Fraser, 1989; Lochman et al., 1995). However, larvae from the DHA+EPA and DHA+AA treatments did not differ in growth or survival despite larvae from the DHA+EPA group tending towards higher total lipid dry weight^{-1} ($p<0.09$).

Yellowtail larvae fed the high PUFA diets had lipid compositions which generally reflected dietary levels, while larvae in the control diet had elevated level of PUFA and reduced levels of 18:1n-9 compared to dietary levels (Figure 2.5). The high levels of AA (~4x), DHA (~4x), and EPA (~2.5x) show evidence for preferential retention of these fatty acids and point to their dietary essentiality, while high levels of 22:5n-3 (~2.5x) may represent evidence for chain elongation. Larvae in the PUFA enriched treatments (DHA present as 22-28% of dietary fatty acids) did not show any preferential retention of DHA compared to dietary levels, indicating that the actual requirement for DHA may be lower than 22%. Interestingly, all treatments except for the DHA+EPA had higher levels of EPA in the larvae than in the diet. This could indicate that EPA is not needed at the high levels found in the DHA+EPA enrichment (11%). Similarly, AA was also conserved in all groups but to a lesser extent in larvae receiving a diet containing higher levels of AA. Curiously, larvae with the DHA enriched diet had higher incorporation efficiency for AA (~1.8x) than larvae in the DHA+EPA (~1.2x) enriched group which could indicate a competitive interaction between EPA and AA for incorporation into phospholipids.

Elevated levels of specific fatty acids in larval tissue compared to dietary levels have been referred to as incorporation efficiency (Castell et al., 1994). However, it is difficult to differentiate whether this elevation is due to preferential retention/utilisation or chain elongation/desaturation. Tocher (1993) examined the metabolism of EPA and

18:3n-3 in primary cultures of brain astrocytes from juvenile turbot and found very low levels of both Δ5 and Δ4 desaturase activity. Therefore, it was concluded that very little DHA could result from the elongation and desaturation of 18:3n-3 and EPA. However, Tocher et al. (1992) also found significant elongation of EPA to DHA in mixed brain cell culture from juvenile turbot. When juvenile herring were fed a pelleted diet devoid of 22:5n-3, DHA and low in EPA, there was significant incorporation of 22:5n-3 in the eye phospholipid with no increase in DHA after four weeks (Bell et al., 1995). This indicates that herring have the ability to chain elongate 18:3n-3 and EPA to 22:5n-3 but cannot complete the final desaturation step to form DHA. The ability of larval yellowtail flounder to desaturate and elongate 18:3n-3 or EPA to DHA has not yet been investigated. Nevertheless, based on trends for other carnivorous marine organisms it is probable that they have very low levels of desaturase activity and are dependent on pre-formed dietary DHA.

Examination of the neutral and polar fatty acid composition of the larvae demonstrated that PUFA, 18:1n-9, 16:0 and 18:0 were the major fatty acids in the polar lipid, while monounsaturated fatty acids were generally found at much higher levels in the neutral lipid (Table 2.11). In particular, DHA was incorporated into the polar lipids and in the control treatment this was particularly evident (5x higher than in neutral lipids). The control treatment was the only group that showed higher levels of EPA and 22:5n-3 in their polar lipid fraction. These results indicate that yellowtail flounder have similar molecular species composition in the polar lipid to that of other cold water marine species. Bell & Dick (1991) described the molecular species of the major phospholipids

in the muscle, liver, retina and brain of Atlantic cod. They found that 16:0/22:6 and 16:0/20:5 accounted for 18% and 22% respectively of the PC fraction of the muscle while 22:6/22:6 and 18:1/22:6 made up 20% and 13% respectively of the PE fraction. The PS fraction was found to be particularly high in 18:0/22:6 which accounted for 36% of the total.

2.5.5 Conclusions

Based on the results of this study it can be concluded that dietary PUFA have a pronounced effect on early growth, survival, and pigmentation in yellowtail flounder larvae. High dietary levels of DHA relative to EPA had growth promoting effects and this relationship was observed throughout a wide range of DHA:EPA ratios (1:1 to 8:1). Elevated dietary AA caused an increase in mal-pigmentation and therefore, the 'pigmentation window' was concluded to be within the first-four weeks post hatch. Further investigations into the role of husbandry induced stress on pigmentation are recommended, as high levels of mal-pigmentation were observed in all treatments. Enrichment of live-food with DHA is essential for this species, however further studies are needed to confirm whether enrichment with EPA and AA is necessary. Based on the incorporation efficiency of PUFA into larval tissue and on the relative amounts of PUFA in the neutral and polar lipid fractions, a ratio of DHA:EPA:AA of 10:1.5:1 could be used as a starting point for further investigations into the dietary requirements of yellowtail flounder larvae.

2.6: Discussion (Part B)

This section focuses on the changes in growth and lipid composition that occurred when larvae of differing nutritional status were fed one diet of enriched *Artemia*. Although factors such as stocking density and prey levels were not controlled, dramatic changes in larval lipid class and fatty acid composition were observed.

2.6.1 *Artemia* lipid composition

The PUFA composition of the enriched *Artemia* was very different from that of the enriched rotifers (Table 2.13). High levels of 18:3n-3 were found in *Artemia* enriched with both Algamac and DHA Selco. Navarro et al. (1993) reported that freshwater nauplii generally have elevated levels of 18:3n-3 and very low amounts of EPA and DHA (< 0.5%). Enrichment of *Artemia* with highly unsaturated fatty acids (HUFA) is also complicated by retro-conversion of DHA to EPA within the animal (Navarro et al., 1999). Levels of DHA were low and the average DHA:EPA ratio in enriched *Artemia* was 0.8:1. *Artemia* had 15% and 19% lipid dry weight^{-1} respectively, when enriched with Algamac or DHA Selco. These results are within the range reported in other studies (Blair et al., 1998; Olsen et al., 1999). There is considerable variation in the literature for live-food lipid levels following enrichment with commercial products. This variation between studies could be due to physical differences in enrichment parameters or variations in *Artemia*-cyst batch quality.

2.6.2 Growth and Survival

Stocking density significantly affected growth during the *Artemia*-stage, particularly in the DHA and control treated larvae (Figure 2.9). Due to differential survival during the first four weeks, there were different stocking densities during the *Artemia*-stage. Stocking density was not adjusted to the lowest level of survival (5%) in all treatments because adequate numbers of fish were required at the end of the experiment to determine pigmentation rates.

There is little available information on the effect of stocking density on early larval growth and survival. However, stocking density has been shown to affect growth and survival in a few species when food availability is limiting. Baskerville-Bridges & Kling (2000) examined the effects of stocking density on Atlantic cod larvae. They found that cod reared at 150 and 300 larvae L^{-1} showed no difference in growth during rotifer feeding. However, after two weeks of feeding on *Artemia*, larvae at the lower density had significantly higher growth rates. In a second experiment, larvae were reared at four different densities (50-300 larvae L^{-1}) and food levels were constantly adjusted to 3-prey ml^{-1}. This resulted in no differences in growth or survival. Therefore, it was concluded that differences in growth observed in the first experiment were a result of food limitation at high stocking densities.

Daniels et al. (1996) also found that stocking density had no effect during the early larval phase in southern flounder (*Paralichthys lethostigma*) but significantly affected growth and survival during metamorphosis. Based on this study, a two step hatchery rearing protocol with high densities during the initial larval period and reduced

densities during metamorphosis was recommended. No published data are currently available for the effect of stocking density on yellowtail flounder during their larval period. However, studies on juvenile fish showed that yellowtail flounder (0+, ~10 g) stocked at 90% bottom coverage had lower growth rates and higher gross food conversion ratios than fish stocked at 23% and 45%. (Boyce, 2000).

Food levels were not adjusted to match stocking densities during the *Artemia*-feeding stage and all tanks received *Artemia* at a rate of 4000 prey L^{-1} day^{-1}. At the beginning of the *Artemia*-feeding, this resulted in the control group receiving ~1330 *Artemia* $larvae^{-1}$ day^{-1} while the DHA treatment received ~300 *Artemia* $larvae^{-1}$ day^{-1}. From weeks six to thirteen, it was observed that tanks with higher densities had more pelagic larvae. However, more research is required to elucidate whether these results are due to a behavioural response resulting from limited surface area for settlement or due to slower growth and development as a result of food limitation.

A rapid increase in growth was observed during the *Artemia*-feeding stage (Figure 2.8). Enhanced growth rates during the *Artemia*-phase have previously been reported for yellowtail flounder larvae (Puvandndran & Brown, 1995; Rabe & Brown, 2000). Improved foraging efficiency, increased prey size, and better digestive efficiency likely influenced the observed increase in growth. Rabe & Brown (2000) showed that yellowtail flounder became more efficient predators during the first seven weeks post-hatch. While, Morris (1997) investigated prey size selection in yellowtail larvae and found that larvae <4.5 mm selected small rotifers, >4.5 mm larvae selected larger rotifers, and >8 mm larvae selected *Artemia*. Consumption increased rapidly after 8 mm in

standard length with ~2 and 24 *Artemia* found in the gut at 8.2 mm and 10.9 mm respectively. Studies on ingestion rates and prey selection have shown that in both turbot and halibut larger larvae also prefer larger prey items (Cunha & Planas, 1993; Olsen et al., 2000). Digestive capacity likely also increased during the first few weeks post-hatch due to both considerable enzymatic and gut morphological development (Baglole et al., 1997, Baglole et al., 1998). Enriched *Artemia* contained very low levels of DHA and therefore, it seems counterintuitive that lipid nutrition alone could explain the observed increase in growth. A more reasonable explanation is presumably a combination of behavioural, physiological, and morphological development.

2.6.3 Larval lipid composition

Larvae in the control treatment showed a period of 'lipid recovery' from week four to six (Figure 2.11). They demonstrated dramatic increases in both relative levels of TAG (10-16%) and lipid dry wt^{-1} (88-165 $\mu g.mg^{-1}$). It is likely that this compensatory accumulation of lipid is due to the previously low PUFA composition of control rotifers. Larvae possibly had difficulty assimilating lipid from the control rotifers given that they were high in 18:1n-9 and low in PUFA (20%), DHA (2%) and EPA (2%). As previously discussed, cold-water marine larvae show a high level of membrane specificity for PUFA, particularly in eye and neural tissue (Bell & Dick, 1991; Bell et al., 1995). The *Artemia* diet had higher levels of PUFA (45%), DHA (6.5%) and EPA (8.1%) than found in the control rotifers. Therefore, it is possible that control larvae showed a compensatory effect in terms of membrane formation. At week six, control larvae had

higher levels of PPL dry wt^{-1} (~119 $\mu g.mg^{-1}$) than larvae in other dietary groups (~88 $\mu g.mg^{-1}$), which supports this hypothesis.

Larvae quadrupled their dry weight from week four to six and therefore, it is interesting that significant differences in larval fatty acids still remained between dietary treatments (Table 2.16). These differences reflected rotifer lipids, with larvae in the high PUFA diets still having significantly higher levels of DHA than the control treatment. Also, larvae in the DHA+AA groups still had significantly higher levels of AA. These data agree with previous studies which have found that marine larvae conserved DHA and AA during early larval development and periods of starvation, thus reconfirming their essentiality (Rainuzzo et al., 1994, Sargent, 1995).

The level of 18:3n-3 present in *Artemia* (~18%) is much higher than that found in natural food items such as copepods, ~1-2% (Norsker & Støttrup, 1994; Nanton & Castell, 1999). The 18:3n-3 accounted for ~14% of the total fatty acids in yellowtail after only two weeks of feeding on *Artemia*. Sargent et al. (1999) discussed the problem of elevated 18:3n-3 given that this fatty acid can compete with DHA and EPA for the transacylases and acylases that esterify fatty acids onto phospholipid backbones. Here yellowtail flounder had ~10% of the fatty acids in their polar lipid present as 18:3n-3 after only two weeks of *Artemia*-feeding. However, given the high levels of PPL found in control larvae, which had the lowest levels of DHA and highest levels of 18:3n-3, elevated 18:3n-3 did not inhibit the formation of phospholipids. However, the effect of 18:3n-3 on fluidity and function of phospholipids has yet to be investigated.

The total level of HUFA (DHA, EPA, and AA) in larval tissue decreased during *Artemia*-feeding and larvae directed relatively more HUFA into the polar lipid than into the neutral lipid (Figure 2.13). Levels of HUFA dropped in the polar lipid from ~ 42-30% and in the neutral lipid from 28-9% from week four to six. Most of this decrease was due to lower levels of DHA. Decreasing HUFA coupled with increasing 18:3n-3 demonstrates a trend towards decreased levels of tissue unsaturation. Nonetheless, these molecular changes occurred during a period of increased growth. This suggests high levels of HUFA may be unnecessary during later larval development.

Decreased demand for HUFA during late larval development could be due to a relative decrease in the amounts of neural tissue, such as eyes and brain, which typically require elevated levels of HUFA (Mourente et al., 1991, Bell et al., 1995). For example, the ratio of eye diameter (mm): body depth (mm) decreased in this study from ~1:1, 1:3, and 1:6 respectively at weeks three, four and six. Another possible explanation for decreased HUFA requirements during *Artemia*-feeding is the relative increase in culture temperatures. High levels of unsaturation are typically associated with fish living in colder environments and are believed to aid in maintaining membrane fluidity and structure (Hazel & Williams, 1990; Sargent et al., 1995; Dunstan et al., 1999). During early larval development (< four weeks) culture temperatures in this study averaged ~12°C however, during the first two weeks of *Artemia* feeding, temperatures were routinely ~16°C.

2.6.4 Conclusions:

The transition from rotifer feeding to *Artemia* feeding was characterised by a period of 'lipid recovery' in the control larvae and a period of decreased unsaturation in all other treatments. Larvae demonstrated a dramatic increase in size despite decreased levels of dietary HUFA. Given poor lipid nutrition, increased foraging and digestive capacity with age likely explain the acceleration in growth. Therefore, it is concluded that high levels of HUFA may not be as essential during later larval development as during initial stages of first-feeding. However, further studies are required to determine the exact dietary HUFA requirements during the late larval period. A significant effect of stocking density on growth was observed. Further studies are recommended to clarify the role of both stocking density and prey availability on growth and settling behaviour during metamorphosis.

Chapter 3 - Comparison of the lipid composition of mal-pigmented and normally pigmented newly settled yellowtail flounder (*Limanda ferruginea*).

3.1 Introduction

Mal-pigmentation is common in a number of cultured flatfish species (Seikai et al., 1987; Rainuzzo et al., 1994; Baker et al., 1998; Gara et al., 1998; Naess & Lie, 1998). Pigmentation abnormalities reduce the market value of fish and consequently represent a significant challenge to the aquaculture industry. In addition, juveniles produced for enhancement of wild stocks are likely more vulnerable to predation as they are unable to blend into their natural benthic environments (Godin, 1997). Recently, it has also been suggested that abnormally pigmented Atlantic halibut are more susceptible to skin damage from ultraviolet radiation than normally pigmented fish (Bricknell et al., 1996).

The exact mechanism behind the development of abnormal pigmentation is not known. However, environmental/husbandry, nutritional, and neuroendocrinal activity are thought to be important (Kanazawa, 1993; Denson & Smith, 1997; Venizelos & Benetti, 1999; Estevez et al., 2001). Numerous studies have shown that early lipid nutrition affects later pigmentation in several flatfish species (See Section 2.5.3). Further, wild copepods have been shown to be more effective in inducing normal pigmentation than rotifers and *Artemia* (Naess et al., 1998; McEvoy et al., 1998). Rainuzzo et al. (1994) found that the DHA:EPA ratio in the polar lipid of turbot larvae was positively correlated with levels of normal pigmentation. However, other dietary factors such as the AA:EPA ratio, and levels of vitamin A, phospholipid, and DHA have also been correlated with pigmentation development (Kanazawa, 1993; McEvoy et al., 1998; Estevez et al., 1999).

The lipid composition of normally pigmented (NP) and mal-pigmented (MP) fish has been compared in both Atlantic halibut (McEvoy et al., 1998) and Japanese flounder (Estevez & Kanazawa, 1996). NP Atlantic halibut fry had significantly higher amounts of DHA and EPA present in the phosphatidylcholine (PC) fraction of the eye than MP fry. While in Japanese flounder, MP fish showed better growth and higher neutral lipids in the body. Normal pigmentation was also associated with higher relative amounts of PUFA present in the polar lipid of larval brains and eyes.

This study was designed to compare the lipid composition of MP and NP juvenile yellowtail flounder. Lipid classes and fatty acids were examined in different body zones to account for known variations between the body and neural tissue. The objectives of this study were two-fold: 1) to elucidate a possible connection between lipids and pigmentation and 2) to provide baseline data on the lipid composition of different body zones within a new aquacultured species.

3.2 Materials and Methods

3.2.1 Live-food

Rotifers were cultured on baker's yeast and culture Selco (INVE, Belgium) for five days prior to enrichment. They were then taken from stock cultures and placed into 300-L enrichment vessels at a density of 3 X 10^5 rotifers L^{-1}. Rotifer batches were enriched for approximately 18 hours using 0.3 g of Algamac-3010 per 10^6 rotifers. Algamac was blended in 3 L of fresh water and was added to rotifer enrichment vessels at time zero and after nine hours of enrichment. Rotifers were sampled in triplicate for lipid analysis twice during this experiment.

Second instar stage *Artemia* were stocked into a 300-L tanks at a density of 2 X 10^5 animals L^{-1}. During enrichment, the temperature was maintained at 26°C and vigorous bottom aeration was applied. Algamac 3010 was added at a concentration of 2 g per 10^6 animals. After 12 hours of enrichment, *Artemia* were transferred to a new enrichment vessel to receive a second 12-hour enrichment. *Artemia* were sampled in triplicate twice during the experimental period for lipid analysis.

3.2.2 Larviculture

Eggs for this experiment were obtained from yellowtail flounder broodstock between July 4 and 9, 1999 and were pooled to obtain the required quantity. They were then incubated in a 300-L cylindro-conical upwelling tank and hatched at approximately 65-degree-days. At 100 % hatch, larvae were transferred into two 300-L cylindro-conical upwelling tanks at a density of 30 larvae L^{-1}. Water flow was set at 2-L min^{-1} and one air

stone placed in the centre of each tank provided aeration. Larvae were reared under ambient temperatures (12°-18°C) using a 24-hour photoperiod with the light intensity of ~800 lux.

Enriched rotifers were added to tanks twice per day at a density of 7000 prey L^{-1} for the first four weeks post-hatch. Tanks were 'greened' daily with 5 L of *Isochrysis galbana*. Larvae were transferred into a 3000-L tank after four weeks and *Artemia* were fed twice per day at a density of 2000 prey L^{-1}. The water level was reduced to ~1000 L from week eight until the end of week 12 in order to aid with settling behaviour.

3.2.3 Lipid and Morphometric Samples

Larvae were sampled at the end of twelve weeks as this was the earliest time at which pigmentation and eye migration could be defined. Also, this was the point at which ~100% of the fish demonstrated settling behaviour. At this time, juveniles were sampled for both morphometric measurements and lipid analysis. Pigmentation was defined as in Section 2.2.8. Five normally pigmented (classification 5) and five mal-pigmented juveniles (classification 1) were first sampled for standard length and body depth. After this, their eyes and heads were dissected and measurements of wet weight, dry weight and ash-free dry weight were taken on these three body zones (See Section 2.2.5 for morphometric sampling). These data were also compared to morphometric data collected from 13-week old fish in 1998 (Section 2.2.5).

Ten normally pigmented (NP) and ten mal-pigmented (MP) fish were sampled for lipid analysis. Juveniles were sacrificed using an overdose of MS-222 and their standard length, body depth, and wet weights were recorded. The eyes, heads and bodies were dissected on ice and the parts from two fish were pooled into each lipid sample. This resulted in 5 samples of NP and 5 samples of MP fish for eye, head, and body zones.

Lipid samples were stored, extracted, and analysed as described in Section 2.2.7. In addition, a phospholipid class separation was performed using thin layer chromatography with flame ionisation detection (TLC/FID) and a MARK V Iatroscan (Iatron Laboratories, Tokyo, Japan). Lipid extracts were spotted on silica gel coated Chromarods and a two stage development system was used to separate phospholipid classes. The first separation consisted of two developments in acetone. The first development was for 12 minutes followed by a second development for 10 minutes. The rods were then dried and all lipid classes except for the phospholipids were scanned. A development of 35 minutes in 70:35:3.5 (v/v/v) chloroform:methanol:water was used to separate phospholipids classes. Rods were scanned and a chromatogram of phospholipid classes was obtained using T-data scan software (RSS Inc., Bemis, TN, USA). A calibration curve was used to convert peak areas into lipid mass values. The standards used in the calibration curve were: phosphatidylcholine, phosphatidyletholamine, phosphatidyl-L-serine, sphingomyelin, and lysophosphatidylcholine. All standards were obtained from Sigma Inc. (St. Louis., MO, USA).

In order to explain discrepancies between the lipid class composition of newly settled yellowtail flounder and other flatfish species (McEvoy et al., 1998; Estevez &

Kanazawa, 1996), lipid class data collected from week-6 larvae in 1998 (Section, 2.4.6) were also discussed.

3.2.5 Statistical Analysis

Differences in size and lipid parameters between mal-pigmented and normally pigmented fish were analysed using a T-test (α= 0.05). Residuals versus fitted values were examined to check for normality and heteroscedasticity. Certain percentage data were arcsine-square root transformed in order to meet these assumptions.

Principle components analysis was used to simplify this multivariate data set by transforming correlated variables into a set of uncorrelated principal components (Minitab, version 10.2). This technique was employed using 15 fatty acid and 3 lipid class parameters from the eyes, bodies and heads on MP and NP fish. Two coordinates were described that accounted for the largest and second largest variance among the samples. This allowed a display of the major trends within the data set without significant loss of total original variation.

3.3. Results

3.3.1 Live-food

A summary of lipids present in both enriched rotifers and *Artemia* is shown in Table 3.1. Rotifers had 106 µg of lipid per mg dry weight while *Artemia* had higher levels of lipid per dry weight at 165 $\mu g.mg^{-1}$. The major lipid class in both rotifers and *Artemia* was TAG which accounted for 45% and 52% respectively of the total lipids. Rotifers contained ~20% SFA, 25% MUFA, and 51% PUFA while *Artemia* had higher relative amounts of SFA (26%) and MUFA (30%) and lower levels of PUFA (42%). The major PUFA in rotifers was DHA (29%), while this fatty acid was present at low levels in *Artemia* (4%). The major PUFA in *Artemia* was 18:3n-3, which accounted for 18% of the total fatty acids.

3.3.2 Size of normally and mal-pigmented juveniles

Ten mal-pigmented (MP) and ten normally pigmented (NP) fish were measured at the end of the experiment to determine differences in size. The wet weight of NP and MP fish was on the average 0.6 g and 0.4 g respectively (Figure 3.1a). NP fish had an average body depth of ~15 mm while MP fish were on average 14 mm (Figure 3.1b). Figure 3.1c shows that the standard length of NP fish (35 mm) was significantly longer than that of MP fish, 32 mm ($T_{1,28} = 2.2$, $p=0.04$). Differences in the size of NP and MP fish were also observed in the 1998 production season (Chapter 2.2.5, final morphometric sampling at week 13, 15 fish per tank). Figure 3.2a shows that 1998 NP fish had a higher wet weight at the end of the experiment than MP fish. NP fish also had greater body depths

Table 3.1: Summary data on the lipid composition of rotifers and *Artemia* enriched with Algamac for 18 hours.

	Rotifers	Artemia
Lipid dry wt^{-1} ($\mu g.mg^{-1}$)	106.0 ± 13.5	165.4 ± 14.2
% total lipid		
Triacylglycerol	44.9 ± 2.4	51.5 ± 3.3
Sterol	10.7 ± 7.6	6.8 ± 2.8
Phospholipid	26.3 ± 4.6	20.4 ± 5.5
% total fatty acids		
Σ SFA	19.8 ± 0.4	26.4 ± 1.4
Σ MUFA	24.8 ± 0.4	30.1 ± 1.2
18:3n-3	0.3 ± 0.0	17.5 ± 2.0
AA	3.3 ± 0.0	2.0 ± 0.3
EPA	3.9 ± 0.1	8.0 ± 1.0
DHA	28.8 ± 0.6	4.0 ± 0.2
Σ PUFA	50.9 ± 1.0	42.0 ± 0.6

Data are mean ± SEM, n=6.

Figure 3.1: The (a) wet weight, (b) body depth and (c) standard length of normally pigmented fish compared to mal-pigmented fish at the end of week 12 (1999). Data are mean ± SEM (N=15 larvae per group, significance for T-test was alpha = 0.05, $T_{1,28}$).

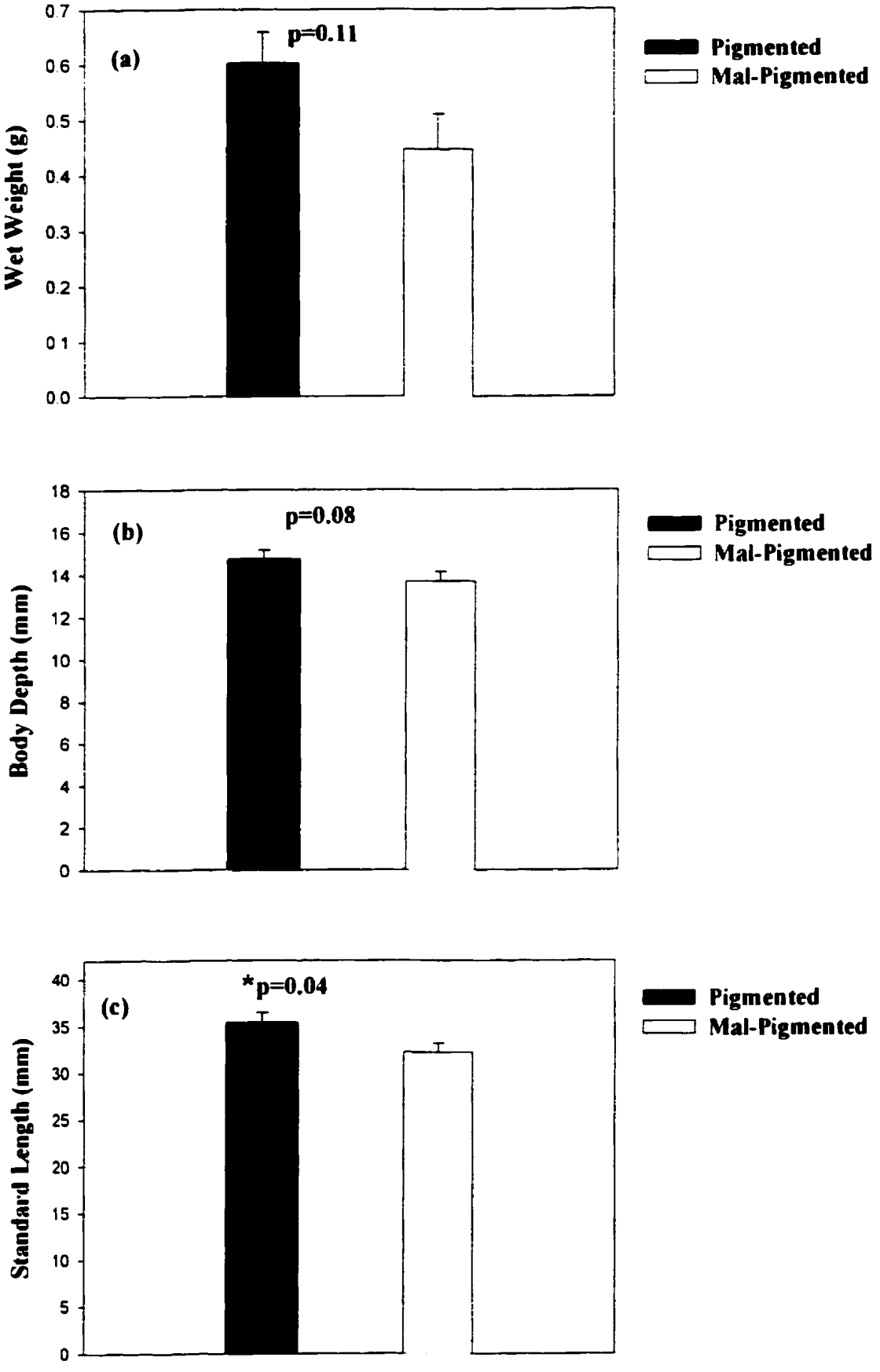
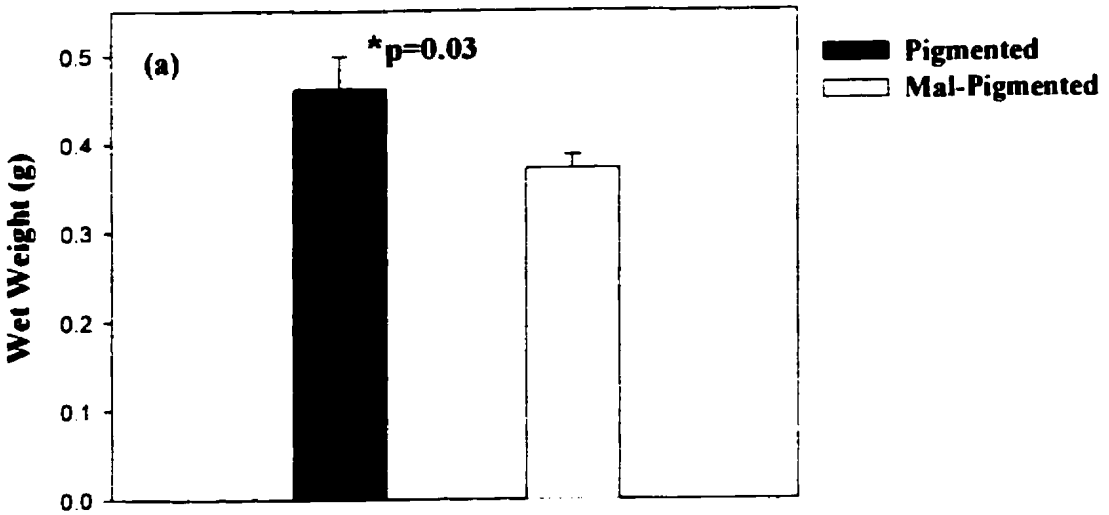
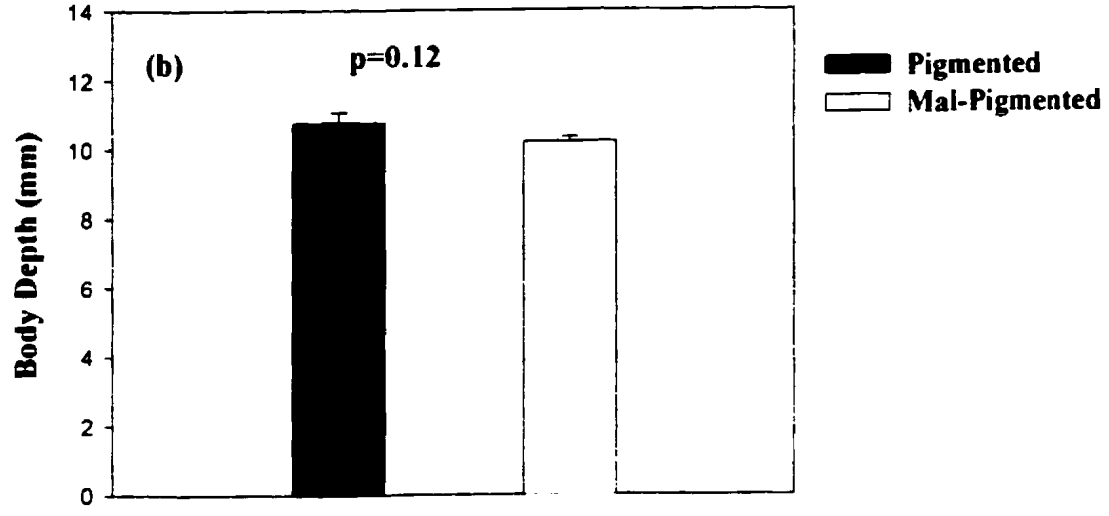
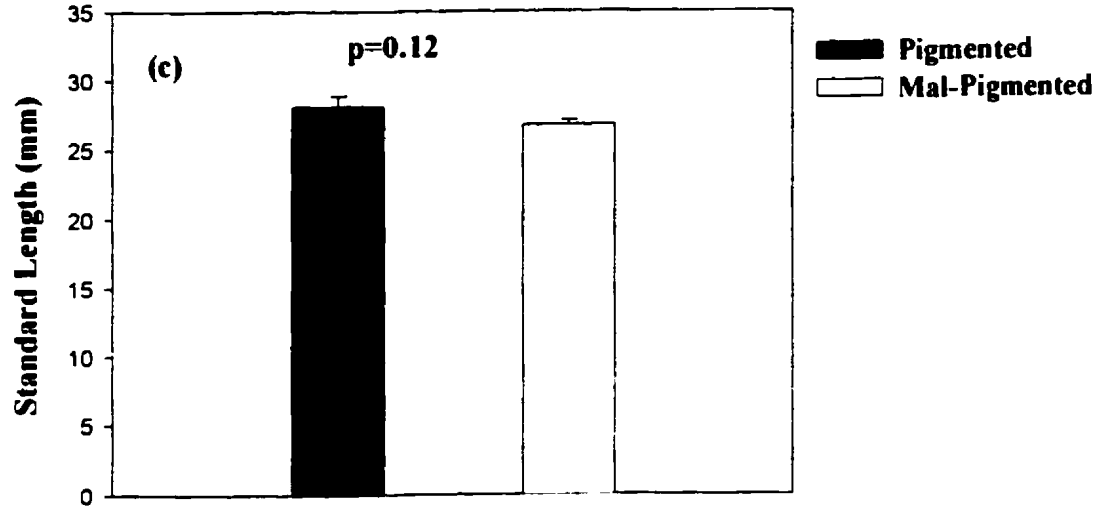

(a)
p=0.11
Wet Weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Pigmented
Mal-Pigmented
(b)
p=0.08
Body Depth (mm)
18
16
14
12
10
8
6
4
2
0
Pigmented
Mal-Pigmented
(c)
*p=0.04
Standard Length (mm)
40
35
30
25
20
15
10
5
0
Pigmented
Mal-Pigmented

Figure 3.2: The (a) wet weight, (b) body depth and (c) standard length of normally pigmented fish compared to mal-pigmented fish at the end of week 13 (1998). Data are means ± SEM (N=120 larvae total, 35 normally pigmented, 85 mal-pigmented, alpha = 0.05).


(a)
*p=0.03
Wet Weight (g)
0.0
0.1
0.2
0.3
0.4
0.5
Pigmented
Mal-Pigmented


(b)
p=0.12
Body Depth (mm)
0
2
4
6
8
10
12
14
Pigmented
Mal-Pigmented


(c)
p=0.12
Standard Length (mm)
0
5
10
15
20
25
30
35
Pigmented
Mal-Pigmented

and standard lengths than MP fish although this difference was not significant (Figure 3.2b & 3.2c).

3.3.3 Lipid class composition of juveniles

The lipid class composition of the bodies from NP and MP fish is shown in Table 3.2. MP and NP fish contained 24% and 23%, respectively of their dry weight as lipid. The major lipid class in both MP and NP fish was TAG although significantly more of the total lipid was comprised of TAG in NP fish (77%) than in MP fish (70%)($T_{1,8}$= -2.8, p=0.02). Neutral lipids accounted for significantly more of the total lipid in NP than in MP individuals ($T_{1,8}$ = -3.6, p=0.007). The relative amount of phospholipid was significantly higher in MP fish than in NP fish ($T_{1,8}$ = 3.5, p=0.008). Phosphatidylcholine (PC) was the major phospholipid in both types of fish, accounting for 6-8% of the total lipids.

The head lipid class composition of NP and MP fish is shown in Table 3.3. Lipid accounted for 13% and 15% of the dry weight respectively in MP and NP fish. As in their bodies, the major lipid class was TAG with NP fish having 33% TAG and MP 26%. MP fish contained significantly lower levels of neutral lipids than NP animals ($T_{1,8}$ = -3.8, p=0.005). Conversely, MP fish had relatively higher levels of phospholipids ($T_{1,8}$ = 4.1, p=0.004) and total polar lipid ($T_{1,8}$ = 3.8, p=0.005) than NP fish.

Eye tissue contained lower levels of lipid per dry weight than both the bodies and heads (Table 3.4). MP fish had eyes with 8.5% lipid while NP fish contained 8.9% lipid. The major lipid class in the eye was phospholipid and MP fish contained significantly

Table 3.2: Comparison of the body lipid class composition of normally and mal-pigmented newly settled yellowtail flounder. Mean ± SEM, n=5.

	Mal-pigmented	Normally Pigmented
Total Lipids per Dry Weight ($\mu g.mg^{-1}$)	237.7 ± 25.4	225.6 ± 20.6
Total Lipid ($mg.carcass^{-1}$)	21.3 ± 3.0	35.6 ± 8.6
(% total lipid)		
Triacylglycerols	70.3 ± 1.9*	77.1 ± 1.5
Free Fatty Acids	1.6 ± 0.5	2.0 ± 0.3
Sterols	5.6 ± 0.7	4.5 ± 0.5
Diacylglycerols	0.9 ± 0.9	1.3 ± 0.6
Total Neutral Lipid	78.3 ± 1.4*	84.9 ± 1.1
Acetone Mobile Polar Lipids	5.1 ± 0.5	4.0 ± 0.6
Phosphatidycholine	8.4 ± 0.5	5.8 ± 0.3
Phosphatidyethanolamine	5.7 ± 0.2	3.5 ± 0.3
Sphingomyeline	0.3 ± 0.0	0.5 ± 0.2
Phosphatidylserine	1.0 ± 0.2	0.6 ± 0.2
Lysophosphatidylcholine	0.8 ± 0.2	0.6 ± 0.1
Total Phospholipids	16.2 ± 1.3*	11.0 ± 0.7
Total Polar Lipids	*21.3 ± 1.6	15.0 ± 1.2

* T-test, $p<0.05$ mal-pigmented fish were significantly different from normally pigmented fish

Table 3.3: Comparison of the head lipid class composition of normally and mal-pigmented newly settled yellowtail flounder. Mean ± SEM.

	Mal-pigmented	Normally Pigmented
Total Lipids per Dry Weight ($\mu g.mg^{-1}$)	134.3 ± 15.2	153.6 ± 16.4
Total Lipid ($mg.head^{-1}$)	2.3 ± 0.3	3.1 ± 0.3
(% total lipids)		
Triacylglycerols	26.3 ± 1.6	33.05 ± 4.0
Free Fatty Acids	2.3 ± 0.6	3.58 ± 1.0
Sterols	14.2 ± 0.8	14.26 ± 1.3
Diacylglycerols	0.6 ± 0.6	4.75 ± 0.4
Total Neutral Lipid	43.4 ± 1.9*	55.6 ± 2.1
Acetone Mobile Polar Lipids	6.5 ± 0.3	6.0 ± 0.5
Phosphatidycholine	29.5 ± 1.1*	20.9 ± 1.6
Phosphatidyethanolamine	5.2 ± 1.3	6.4 ± 1.3
Sphingomyeline	2.9 ± 0.5	1.7 ± 0.3
Phosphatidylserine	6.1 ± 0.6	6.1 ± 1.7
Lysophosphatidylcholine	5.0 ± 0.4	2.9 ± 0.5
Total Phospholipids	16.2 ± 1.3*	11.0 ± 0.7
Total Polar Lipids	55.2 ± 2.0*	44.0 ± 2.2

* T-test, $p<0.05$ mal-pigmented fish were significantly different from normally pigmented fish

Table 3.4: Comparison of the eye lipid class composition from normally and mal-pigmented newly settled yellowtail flounder. Mean ± SEM.

	Mal-pigmented	Normally Pigmented
Total Lipids per Dry Weight ($\mu g.mg^{-1}$)	85.9 ± 2.9	89.0 ± 6.4
Total Lipid ($\mu g.eye^{-1}$)	79.5 ± 5.0	79.5 ± 8.3
(% total lipids)		
Triacylglycerols	8.9 ± 0.9*	14.8 ± 1.5
Free Fatty Acids	-	-
Sterols	15.4 ± 0.6*	18.3 ± 0.6
Diacylglycerols	2.0 ± 0.1	1.5 ± 0.5
Total Neutral Lipid	28.5 ± 1.1*	35.9 ± 1.0
Acetone Mobile Polar Lipids	2.1 ± 0.1	1.2 ± 0.2
Phosphatidycholine	42.8 ± 7.4	42.5 ± 6.7
Phosphatidyethanolamine	4.8 ± 1.5	4.7 ± 1.4
Sphingomyeline	2.1 ± 0.7	0.6 ± 1.1
Phosphatidylserine	18.3 ± 3.0	15.3 ± 7.8
Lysophosphatidylcholine	3.5 ± 3.1	1.0 ± 1.0
Total Phospholipids	71.5 ± 1.2*	64.1 ± 1.2
Total Polar Lipids	73.7 ± 5.0*	65.4 ± 1.0

* T-test, $p<0.05$ mal-pigmented fish were significantly different from normally pigmented fish

higher relative amounts of phospholipid than NP fish ($T_{1.8} = 3.6$, $p=0.007$). Conversely, NP fish contained higher relative amounts of neutral lipids and in particular TAG ($T_{1.8} = -2.7$, $p=0.026$). Generally, NP fish contained high relative amounts of TAG than MP fish while MP fish had significantly higher levels of phospholipids present in all body parts.

3.3.4 Fatty acid composition of juveniles

Table 3.5 shows the neutral and polar fatty acid composition from both MP and NP larvae. The eyes were not separated into neutral and polar lipids due to the limited amount of lipid present in these samples. Therefore, the eye fatty acid data is expressed as a percent of the total fatty acids. However, phospholipids represented ~80% of the total acyl lipid in the eye and, therefore, the fatty acid composition of the total lipid is likely similar to the composition of just the polar lipid.

SFA were present in the bodies, heads, and eyes of larvae at ~20%, 25%, and 40% respectively (Figure 3.3). The major SFA present was 16:0, however, 18:0 was present at high levels in the polar lipids of the head and in the total lipids of the eye (Table 3.5). Generally, neutral lipids contained higher levels of MUFA and lower levels of PUFA than the polar lipids. MUFA were present at higher levels in bodies and heads than in the eyes (Figure 3.3).

PUFA were seen at higher levels in the polar lipids than in the neutral lipids but this trend was not observed in the head lipids of MP fish. The major PUFA present in the neutral lipids was 18:3n-3, while DHA was the major PUFA in the polar lipids of the body and head as well as the total lipids of the eye. DHA was found at higher levels in

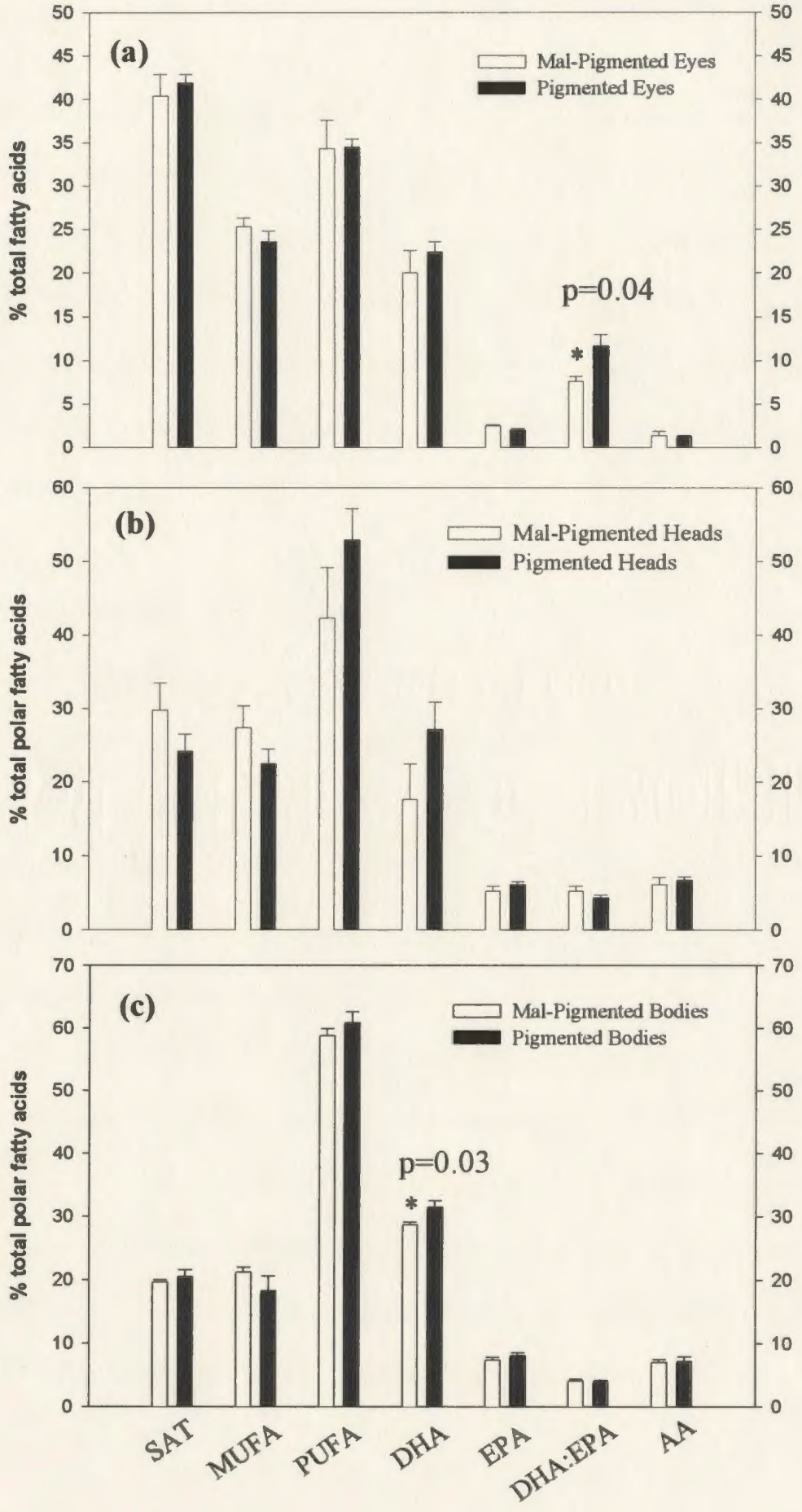
Table 3.5: Neutral and polar fatty acid composition of mal-pigmented and normally pigmented newly settled yellowtail flounder larvae

	Body				Head				Eyes	
	Mal-pigmented		Normally Pigmented		Mal-pigmented		Normally Pigmented		Mal-pigmented	Normally Pigmented
	NL	PL	NL	PL	NL	PL	NL	PL	Total	Total
14:0	3.7	1.1	2.9	1.2	2.8	1.8	3.8	1.2	1.8	1.8
16:0	11.3	7.7	10.4	8.1	18.2	11.3	11.9	9.0	22.4	23.6
18:0	2.7	9.4	2.8	9.9	2.9	14.1	3.0	12.1	12.7	12.1
Σ SFA[a]	19.3	19.6	18.1	20.5	25.8	29.8	20.9	24.2	40.4	41.9
18:1n-9	17.5	10.6	17.6	9.8	16.0	16.6	16.9	13.2	14.9	12.9
18:1n-7	5.5	4.8	6.4	3.6	5.7	5.7	5.9	4.9	4.7	5.6
16:1n-7	5.9	1.8	4.3	1.8	5.1	2.2	5.9	1.7	3.4	3.5
20:1n-9	3.7	2.7	3.2	2.7	3.1	2.9	3.6	2.7	0.8	0.6
Σ MUFA[b]	36.4	21.7	33.2	18.6	31.2	27.9	34.0	22.9	25.4	23.6
18:2n-6	6.4	3.6	5.8	4.2	5.2	3.6	5.8	3.6	2.2	1.9
18:3n-3	13.8	3.5	17.6	3.5	12.5	2.8	12.3	2.4	2.7	2.3
20:4n-6(AA)	1.8	7.0	1.9	7.2	2.8	6.1	2.9	6.7	1.4	1.3
20:5n-3(EPA)	7.0	7.3	6.8	8.0	7.2	5.2	8.1	6.2	2.5	2.0
22:5n-3	1.3	1.8	1.2	1.9	1.0	1.2	1.4	1.3	0.6	0.7
22:6n-3(DHA)	9.6	28.7	9.7	31.5	9.9	17.7	10.2	27.2	19.2	22.8
Σ PUFA[c]	44.3	58.8	48.7	60.9	43.0	42.3	45.1	52.9	34.3	34.5
DHA/EPA	1.4	4.0	1.4	3.9	1.4	3.4	1.3	4.4	7.6	11.7
DHA/AA	5.2	4.1	5.1	4.3	3.5	2.9	3.7	4.1	14.2	17.2
EPA/AA	3.9	1.0	3.6	1.1	2.6	0.9	2.8	0.9	1.8	1.5

[a] includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, and 20:0
[b] includes 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, and 24:1
[c] includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, and 22:4n-6

Figure 3.3: Summary of the fatty acid composition in the different body zones of newly settled mal-pigmented versus normally pigmented yellowtail flounder.
(a) Eye total lipid
(b) Head polar lipid
(c) Body polar lipid

* represents a significant difference between normally and mal-pigmented fish (T-test, $P<0.05$, $T_{1,8}$. Data are means ± SEM. (n=5).


(a)
Mal-Pigmented Eyes
Pigmented Eyes
% total fatty acids
p=0.04
*
(b)
Mal-Pigmented Heads
Pigmented Heads
% total polar fatty acids
(c)
Mal-Pigmented Bodies
Pigmented Bodies
% total polar fatty acids
p=0.03
*
SAT
MUFA
PUFA
DHA
EPA
DHA:EPA
AA

the membranes of NP fish than MP fish. However, this relationship was only significant in the polar lipid fraction of the body (Figure 3.3c, p=0.03). EPA was present equally in both the neutral and polar lipids and this resulted in a higher DHA:EPA ratio in the polar lipids than in the neutral lipids of the head and bodies. The total lipids of the eye contained low levels of EPA, which resulted in a high DHA:EPA ratio in the eye. This ratio was significantly higher in the eyes of NP fish (~12:1) than in MP fish (~8:1)(Figure 3.3a, p=0.04).

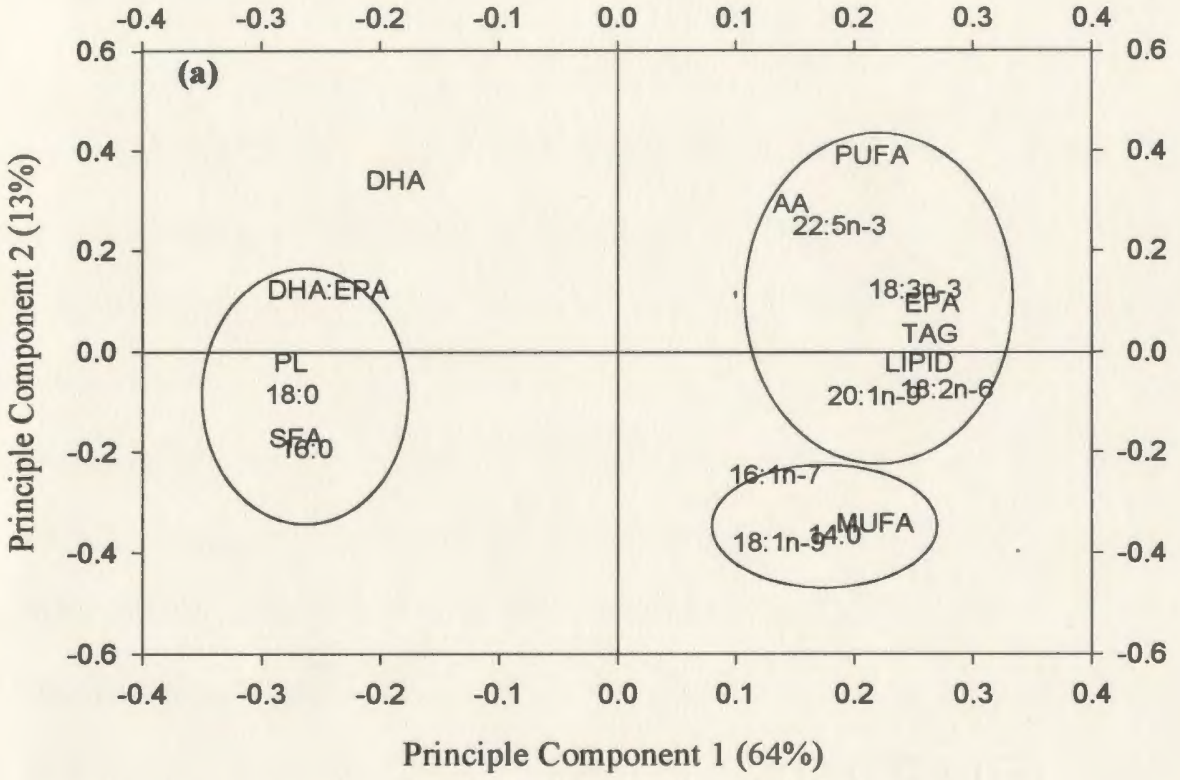
3.3.5 Principal Components Analysis

Principle component analysis allowed the description of the majority of the variance in the data set using 15 fatty acid parameters and 3 lipid class parameters. Figure 3.4a shows the coefficients plotted for the first two principle components. These principle components cumulatively accounted for 77% of the total variance. The first PC accounted for 64% of the total variance and separated lipid parameters into those associated with membranes and those associated with storage. Lipids associated with membranes such as phospholipids, DHA, 18:0 and 16:0 loaded negatively onto this axis. Conversely, lipid parameters associated with storage such as percent TAG, 18:3n-3, and lipid dry weight^{-1} loaded positively onto the PC 1 axis. The PC 2 axis accounted for only 13% of the total variance and separated lipid parameters based on the level of unsaturation. PUFA, DHA, AA, and 22:5n-3 were positively loaded on this axis while MUFA, SFA, 18:1n-9 and 14:0 were negatively loaded along PC 2.

Figure 3.4: Principle components analysis (PCA) of lipid data from eyes, heads, and bodies of MP and NP juvenile yellowtail flounder. Five samples of MP and NP fish tissue were analyzed from each body zone. The fatty acid parameters used were: 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 20:1n-9, 20:4n-6 (AA), 20:5n-3 (EPA), 22:5n-3, 22:6n-3 (DHA), DHA:EPA, ΣSFA, ΣMUFA, ΣPUFA, TAG, PPL, LIPID per dry weight ($\mu g.mg^{-1}$).

(a) Lipid parameter coefficients for PC1 and PC2

(b) Eyes, heads and bodies scores for PC1 and PC2


(a)
DHA
DHA:ERA
PL
18:0
SFA
16:0
PUFA
AA
22:5n-3
18:3n-3
EPA
TAG
LIPID
20:1n-9
18:2n-6
16:1n-7
18:1n-9
14:0
MUFA
Principle Component 2 (13%)
Principle Component 1 (64%)

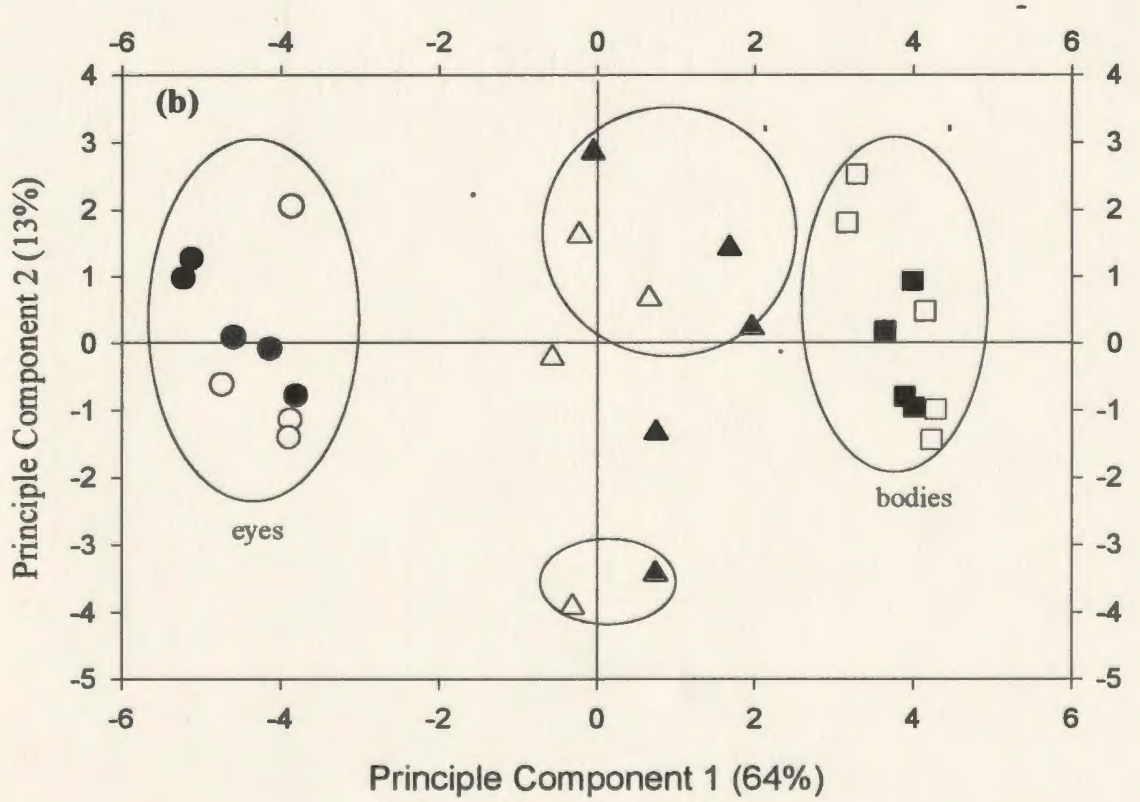

(b)
eyes
bodies
Principle Component 2 (13%)
Principle Component 1 (64%)

Figure 3.4b shows the scores for the eyes, heads, and bodies of NP and MP fish plotted along PC1 and PC2. Eyes grouped together on the negative side of the axis while bodies grouped together on the positive side of PC1. The grouping of eyes on the negative segment of the axis was expected, as eyes have high levels of polar lipid present in the form of highly unsaturated phospholipids. Bodies were associated with very high levels of lipid and, in particular, TAG which resulted in their position on the positive side of PC1. Heads were located near the origin of PC1 indicating their intermediate lipid composition, consisting of approximately equal amounts of membrane and storage lipids. No separation between MP and NP fish into clusters was observed. This indicated that differences between body zones were greater than between NP and MP fish within a given zone.

3.4: Discussion

3.4.1 Size of juveniles

In both 1998 and 1999, normally pigmented (NP) yellowtail flounder were significantly larger than mal-pigmented (MP) fish. McEvoy et al. (1998) examined differences in both lipid composition and size between NP and MP Atlantic halibut. Differences were compared between MP and NP fish fed Super Selco enriched *Artemia* or a copepod diet. The copepod treatment resulted in higher levels of NP fish (92%) than the *Artemia* reared group (66%). Fish were also significantly larger in the copepod treatment. However, NP and MP fish within the *Artemia* treatment showed no differences in size.

On the other hand, a similar study by Estevez & Kanazawa (1996) showed opposite results. Japanese flounder fed enriched rotifers/*Artemia* resulted in variable sized NP and MP fish. MP fish, however, were significantly larger than NP fish at day 50 post-hatch. This increased size was hypothesised to result from differences in visual abilities between NP and MP fish. Further, MP fish were stated to grow faster due to their 'better vision under bright illuminations'. The light intensity used (600-1000 lux) was thought to be too stressful for the NP fish and thus caused a relative reduction in growth. A similar level of light intensity was used here with no adverse effects observed on the growth of NP yellowtail flounder.

Purchase (1997) examined the effects of three different photoperiods on the growth and survival of MP and NP 1+ year old (5.0 cm standard length) yellowtail flounder and found no differences in growth between NP and MP fish. This disagrees

with the data presented here. However, this study examines the size of newly settled larvae while Purchase (1997) used 1+ fish. The differences in age may be a possible explanation for these variable results. As in other flatfish species, such as winter flounder, compensatory juvenile growth has been observed in fish that grew slowly as larvae (Bertram et al., 1993; Bertram et al., 1997). Therefore, it is possible that the MP fish studied by Purchase (1997) were also smaller at settlement but these differences were masked by a year of compensatory growth.

3.4.2 Lipid class composition of juveniles

Lipid accounted for ~ 23% of the dry weight in the body in both NP and MP fish. Whalen (1999) compared the lipid composition in wild juvenile (1+ year class) yellowtail flounder to that of cultured juveniles. Cultured fish were found to have much higher levels of lipid per dry weight in both the liver (14%) and muscle (8.4%) than wild fish (3.6%, 1.3% respectively). In this study, TAG was the main lipid class in the body of MP and NP juveniles, accounting for 70% and 77% of the total lipid respectively. Whalen (1999) also found TAG was the major lipid class in the flesh of both cultured (87%) and wild fish (60%). Therefore, both studies indicate that cultured yellowtail flounder use their flesh as a major storage sight for neutral lipids.

Newly settled yellowtail flounder had higher levels of lipid dry weight^{-1} (23%) and TAG (~74%) than reported for other flatfish species. Estevez & Kanazawa (1996) reported that lipids accounted for ~10% of the dry weight in newly settled Japanese flounder with only ~16% of the total lipid as TAG. McEvoy et al. (1998) reported that

newly settled Atlantic halibut contained only ~31% of the total lipids in the carcass as TAG. However, the measurements on both Japanese flounder and Atlantic halibut were taken at a younger age/developmental stage than those for yellowtail. Japanese flounder were measured on day 50 post-hatch (~65 mg wet weight) while Atlantic halibut were measured on day 43 past first-feeding (~140 mg wet weight). Here, yellowtail flounder were sampled on day 84 past first feeding when larvae weighed ~400 mg and were 100% settled.

Yellowtail flounder of a similar age/developmental stage to Japanese flounder and Atlantic halibut, cited above, were sampled in 1998 (Section 2.4.3). On day 43 past first-feeding, yellowtail larvae had on average 14 % of their dry weight as lipid and ~18% present as TAG. Therefore, differences in lipid composition between yellowtail analysed in this chapter and other flounder species can likely be explained by variation in the age at sampling. Further, days 43-84, which represents the six weeks following the first observations of settling behaviour, may be a time during which yellowtail flounder dramatically increase their neutral lipid storage.

Comparisons of the lipid composition of NP and MP fish revealed that levels of TAG were significantly higher in both the body and eye lipids of NP fish (Table 3.2 and 3.4). However, the opposite trend was observed in Japanese flounder where MP fish had higher relative amounts of TAG in body lipids than NP fish (Estevez & Kanazawa, 1996). No differences in the carcass lipid class composition were found between NP and MP *Artemia*-fed Atlantic halibut juveniles (McEvoy et al., 1998).

Comparisons between the different body zones showed that more lipid dry weight^{-1} was present in the bodies, ~230 μg.mg^{-1}, than in the head, ~143 μg.mg^{-1}, or eyes, ~87.5 μg.mg^{-1} for both NP and MP fish. Most of this increase in lipid can be accounted for by increased relative levels of TAG in the body (~73%) compared to the head (43%) or eyes (10%) (Tables 3.2-3.4, Figure 3.4). However, Japanese flounder showed the opposite trends with higher percentages of lipid dry weight^{-1} in the brain (~28%) and eyes (18%) than in the body (10%). They also had higher percentages of TAG in the eye (35%) than in either the brain (11%) or body (16%) (Estevez & Kanazawa, 1996). Atlantic halibut showed similar percentages of TAG in both their eyes and carcass, ~30% of total lipid (McEvoy et al., 1998). As discussed previously, differences in the amount of lipid and percentage of neutral lipid storage seem to vary depending on both the species and developmental stage/nutritional status.

3.4.3 Fatty acid composition of juveniles

Both NP and MP fish preferentially retained PUFA in their polar lipid rather than their neutral lipid and this trend was upheld in the body, head and eyes (Table 3.5). Within the HUFA, DHA and AA were found at higher levels in the polar lipid while EPA was found equally in neutral and polar (Previously discussed in Section 2.5.4 & 2.6.3). Interestingly, the percent PUFA present in the eye was lower than that in the body and head. This was due to reduced relative levels of 18:2n-6, 18:3n-3, AA, and EPA. As a result, there were higher ratios of DHA:EPA in the eye tissue than observed in either the head or body.

The eyes contained higher levels of SFA than in other tissues. The major SFA present in the eyes were 16:0 and 18:0, while the major PUFA present was DHA. Bell & Dick (1993) described the molecular species composition of the phospholipids present in the eyes of juvenile herring. In the PC fraction of the eye the phospholipid molecular species, 16:0/22:6, 18:0/22:6, and 22:6/22:6 were present as 30%, 10% and 15% respectively. Similarly, Bell & Dick (1991) described the molecular species present in the retinal phospholipids of Atlantic cod and found that 16:0/22:6, 18:0/22:6, and 22:6/22:6 were present at 23%, 9%, 30% respectively in the PC fraction. Therefore, the proportions of the fatty acids present in the eye suggest the molecular species composition of yellowtail flounder eyes are similar to those reported for other marine fish species.

A significantly higher percentage of DHA was found in the body polar lipids from NP (23%) fish compared to MP fish (19%). This trend, although not significant, was also observed in comparisons of head and eye tissue (Figure 3.3). Similarly, Estevez & Kanazawa (1996) reported significantly higher levels of DHA present in the polar lipids of the head and eyes in NP Japanese flounder compared to MP fish. However, McEvoy et al. (1998) found higher absolute amounts of DHA present in the eyes of NP Atlantic halibut than MP fish. I found that the DHA:EPA ratio was also significantly higher in NP fish eyes than in eyes from MP fish. This was not observed in Japanese flounder or Atlantic halibut as EPA was also found in higher relative and absolute levels in NP fish eyes.

In yellowtail flounder, Japanese flounder, and Atlantic halibut differences between NP and MP fish were related to either higher relative or absolute amounts of DHA present in neural tissues, particularly in the eye. Further, Bell & Dick (1993) correlated the levels of di-DHA phospholipids in the eye to the appearance of rods in the retina of juvenile herring. Rod outer segment membranes have been found to be particularly high in di-DHA phospholipids in other vertebrates (Stinson et al., 1991).

Rods and cones are the two types of photoreceptor cells present in the retina of larval fish with rods playing an important role in vision at low light intensities. Both herring and sole (*Solea solea*) larvae hatch with all cone retinas and rods are recruited during larval development, especially during the onset of metamorphosis (Sandy & Blaxter, 1980). This increase in rod concentration co-occurs with behavioural changes such as a switch from pelagic to benthic life in flatfish or the onset of schooling behaviour in pelagic species. Interestingly, rod recruitment also coincides with the sensitive period for the influences of nutrition on later pigmentation in flatfish, the 'pigmentation window' (previously discussed in Section 2.5.3).

Kanazawa (1993) proposed a nutritionally based hypothesis for the occurrence of abnormal pigmentation in hatchery-reared flatfish. He stated that vitamin A, DHA, and phospholipids were important in the formation of rhodopsin in the eye and that a lack of rhodopsin impaired vision. Further, impairment of vision was hypothesized to cause a deficiency in neural (central nervous system) and thus hormonal stimulation (melanophore stimulating hormone) which are essential for the formation of melanophores. However, there is currently no evidence linking decreased rhodopsin

levels in the eye or melanophore-stimulating hormone in the integument to abnormal pigmentation. Furthermore, a lack of DHA rich phospholipids may impair not only visual membrane function, but also general neural function and, in particular, melanophore cell-membrane function.

Estevez et al. (2001) investigated a possible mechanism behind the nutritional influence on pigmentation development in Japanese flounder. The roles of adrenocorticotropin (ACTH) and melanocyte-stimulating hormone (MSH) were investigated in relation to the development of pigmentation in Japanese flounder. Flounder were fed a diet high in AA, known to induce high levels of MP fish, and a control diet of tuna orbital oil, known to induce high levels of normally pigmented fish (See Section 2.5.3 for discussion of the importance of dietary AA:EPA ratio in pigmentation). ACTH and MSH were then measured, however, no differences in volumes of ACTH and MSH producing cells or total pituitary volume was detected in relation to increased levels of MP fish.

Conclusions

The NP fish were significantly larger than MP fish at the time of 100% settlement. Generally, yellowtail flounder juveniles had high levels of lipid per dry weight both compared to younger larval stages and other species. There were differences between the lipid composition of different body zones and between NP and MP fish within these zones. NP fish had a higher percentage of their lipid as TAG while MP fish had higher relative amounts of phospholipids. NP fish showed higher levels of DHA in

the polar lipid of the body and higher ratios of DHA:EPA in their eyes. Higher relative and absolute amounts of DHA in the neural tissues of NP fish have been reported for other marine species.

These data tend to support previous proposals for the importance of DHA in visual and thus neural and hormonal development. However, behavioural and histological studies are required to validate whether DHA actually affects visual function during the 'pigmentation window'. Further, nutrition has yet to be directly linked to any of the mechanisms involved with pigmentation development, such as levels of rhodopsin in the eye, numbers of rods in the retina, or levels of MSH in the larvae.

Chapter 4 - Summary of Experiments and Suggestions for Further Research

This study is the first investigation of the essential fatty acid requirements of yellowtail flounder larvae. Purified algae lipid extracts were used in rotifer enrichments which allowed the investigation of extreme dietary ratios of DHA:EPA:AA. Dietary PUFA significantly affected growth and survival during the first four weeks post-hatch; with larvae in the DHA diet showing the highest and those in the control diet the lowest growth and survival. Both growth and survival were positively correlated with unusually high DHA:EPA ratios (8:1). Therefore, the theory 'nature knows best' (2:1 ratio) may not describe optimum dietary lipid levels for early larval development. Further research is required using high DHA products to validate this finding in other marine species.

The 'pigmentation window' was determined to be within the first four weeks post-hatch. High dietary AA (8%) during this period increased the incidence of mal-pigmentation. The effect of AA on pigmentation is interesting in terms of a functional effect of diet on pigmentation development. This result has been demonstrated in other flatfish species and is thought to result from increased stress due to elevated eicosanoid activity, particularly in the brain. However, the role of eicosanoids in pigmentation development has not yet been shown on a molecular level. High levels of mal-pigmentation in all dietary groups indicate that husbandry factors other than diet should also be investigated in relation to pigmentation.

Growth during the *Artemia* phase (weeks 6-13) was affected by factors other than dietary PUFA. Stocking density was negatively correlated with size at the end of week 13. However, it is not clear whether this result is due to food limitation or a behavioural

response to overcrowding. An examination of the role of stocking density on both growth and settling behaviour should be completed in order to design optimum rearing protocols for this species.

During the *Artemia* phase, larvae in the control diet showed a period of 'lipid recovery' and compensatory growth while all other groups showed decreased levels of PUFA in their tissues. However, all treatments showed increased growth despite decreased dietary HUFA. Therefore, it was concluded that increased foraging and digestive capacity with age explained the observed acceleration in growth. High levels of dietary HUFA may not be as essential during this developmental stage. Further nutritional investigations to determine the optimal dietary HUFA requirements could result in the elimination of live-food enrichments for late larval stages.

A comparison of the lipid composition between MP and NP juveniles showed significant differences in both lipid class and fatty acid composition. NP fish had high DHA in their polar body lipids and a higher DHA:EPA ratio in the eye total lipid. NP fish were also larger and had higher relative amounts of neutral lipid storage than MP fish. Higher levels of DHA in the polar lipids of the eye have been reported for other species of marine flatfish. The connection between HUFA in neural tissue and pigmentation development has not yet been fully investigated.

References

Arts, M.T., Ackman, R.G., and Holub, B.J., 2001. "Essential fatty acids" in aquatic ecosystems: a crucial link between diet and human health and evolution. Canadian Journal of Fisheries and Aquatic Sciences 58: 122-137.

Baglole, C.J., Murray, H.M., Goff, G.P., and Wright, G.M., 1997. Ontogeny of the digestive tract during larval development of yellowtail flounder: a light microscopic and mucous histochemical study. Journal of Fish Biology 51: 120-134.

Baglole, C.J., Goff, G.P., and Wright, G.M., 1998. Distribution and ontogeny of digestive enzymes in larval yellowtail and winter flounder. Journal of Fish Biology 53: 767-784.

Baker, E.P., Alves, D., and Bengtson, D.A., 1998. Effects of rotifer and *Artemia* fatty-acid enrichment on survival, growth and pigmentation of summer flounder *Paralichthys dentatus* larvae. Journal of the World Aquaculture Society 29: 494-498.

Barrows, F.T. and Rust, M.B., 2000. Larval feeding-Fish. In: Encyclopedia of Aquaculture (Ed. R.R. Stickney), pp 465-469. Wiley & Sons, New York, USA.

Baskerville-Bridges, B., and Kling, L.J., 2000. Larval culture of Atlantic *cod (Gadus morhua)* at high stocking densities. Aquaculture 181:61-69.

Bell, M.V., Henderson, J.R., and Sargent, J.R., 1986. The role of polyunsaturated fatty acids in fish. Comparative Biochemistry and Physiology 83B: 711-719.

Bell, M.V., and Dick, J.R., 1991. Molecular species composition of the major diacylglycerophospholipids from muscle, liver, retina, and brain of cod (*Gadus morhua*). Lipids 26:565-573.

Bell, M.V., and Dick, J.R., 1993. The appearance of rods in the eyes of herring and increased Di-docosahexaenoyl molecular species of phospholipids. Journal of Marine Biology 73: 679-688.

Bell, J.G., Tocher, D.R., and Sargent, J.R., 1994. Effect of supplementation of 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. Biochemistry and Biophysiology. Acta 1211: 335-342.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., and Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). Lipids 30:443-449.

Bell, M.V., McEvoy, L.A., and Navarro, J.C., 1996. Deficit of didocosahexaenoyl phospholipid in the eyes of larval sea bass fed an essential fatty acid deficient diet. Journal of Fish Biology 49:941-952.

Bertram, D.F., Chambers, R.C., and Leggett, W.C., 1993. Negative correlations between larval and juvenile growth rates in winter flounder: implications of compensatory growth for variation in size-at-age. Marine Ecology Progress Series 96:209-215.

Bertram, D.F., Miller, T.J., and Leggett, W.C., 1997. Individual variation in growth and development during the early life stages of winter flounder, *Pleuronectes americanus*. Fisheries Bulletin 95:1-10.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernandez-Cruz, C.M., Gonzalez, M.M., Fernandez-Palacios, H., 1999. Effect of dietary arachidonic acid levels on growth and survival of gilthead sea bream (*Sparus aurata* L.) larvae. Aquaculture 179:265-275.

Blair T., Powell, F., Brooking, P., and Castell, J., 1998. Evaluation of commercial enrichment media for enhancing nutritional value of *Artemia* for larval halibut (*Hippoglossus hippolgossus*) culture. Bulletin of the Aquaculture Association of Canada 98-4: 21-26.

Boyce, L.B., 2000. Feeding and on-growing strategies for yellowtail flounder *Limanda ferruginea* (storer). Memorial University of Newfoundland, St. John's, Newfoundland. 80p.

Bricknell, I.R., Bruno, D.W., Bowden T.J., and Smith, P., 1996. Fat cell necrosis syndrome in Atlantic halibut, *Hippoglossus hippoglossus* L. Aquaculture 144: 65-69.

Brodie, W.B., and Walsh, S.J., 1994. Changes in the distribution of yellowtail flounder on the Grand Banks during the late-1980s and early-1990s. NAFO SCR Doc. 94/46, June, 1994.

Brodie, W.B., Walsh, S.J. and Atkinson, D.B., 1998. The effect of stock abundance on range contraction of yellowtail flounder (*Pleuronectes ferruginea*) on the Grand Bank of Newfoundland in the Northwest Atlantic from 1975 to 1995. Journal of Sea Research, 39: 139-152.

Brown, J.A., Helm, M., and Moir, J., 1995. New-candidate species for aquaculture. In: Cold-water Aquaculture in Atlantic Canada (Ed. A.D. Boghen), pp. 341-362. The Canadian institute for research on regional development, Sackville, Canada.

Brown, J.A., 2000. Status of new candidate finfish species for marine aquaculture in Newfoundland. In: Seafood in Health and Nutrition (Ed. F. Shahidi), pp. 435-448. ScienceTech Publishing, St. John's, Canada.

Budge, S.M., 1999. Fatty acid biomarkers in a cold water marine environment. Ph.D. thesis. Memorial University of Newfoundland, St. John's, Newfoundland. 197p.

Buzzi, M., Henderson, R.J., and Sargent J.R., 1996. The desaturation and elongation of linolenic acid and eicosapentaenoic acid by hepatocytes and liver microsomes from rainbow trout (Oncorhyncus mykiss) fed diets containing fish oil or olive oil. Biochemistry and Biophysiology. Acta, 1299: 235-244.

Canadian Department of Fisheries and Ocean. Statistical Services, Aquaculture. http://www.ncr.dfo.ca/communic/statistics/aquacult/Aqua_E.htm.

Castell, J.D., Bell, J.G., Tocher, D.R., and Sargent, J.R., 1994. Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth, and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). Aquaculture 128:315-333.

Copeman, L.A., 1996. Effects of induced ovulation on larval behaviour and size in the yellowtail flounder (*Pleuronectes ferrugineus*). B.Sc. Honours thesis. Memorial University of Newfoundland, St. John's, Newfoundland.

Cunha, I., and Planas, M., 1993. A study on the maximisation of ingestion rates of turbot larvae using different sized live prey. ICES 29.

Daniels, H.V., Berlinsky, D.L., Hodson, R.G., and Sullivan, C.V., 1996. Effects of stocking density, salinity, and light intensity on growth and survival of Southern flounder *Paralichthys lethostigma* larvae. Journal of the World. Aquaculture Society 27: 153-159.

Denson, M.R., and Smith, T.J., 1997. Diet and light intensity effects on survival, growth and pigmentation of southern flounder *Paralichthys lethostigma*. Journal of the World Aquaculture Society 28:366-373.

Dunstan, G.A., Olley, J. and Ratkowsky, D.A., 1999. Major environmental and biological factors influencing the fatty acid composition of seafood from Indo-Pacific to Antarctic waters. Recent Research Developments in Lipids Research 3 63-86

Estevez, A., and Kanazawa, A., 1996. Fatty acid composition of neural tissues of normally pigmented and unpigmented juveniles of Japanese flounder using rotifer and *Artemia* enriched in n-3 HUFA. Fisheries Science 62: 88-93.

Estevez, A., Ishikawa, M., and Kanazawa, A., 1997. Effects of arachidonic acid on pigmentation and fatty acid composition of Japanese flounder, *Paralichthys olivaceus*. Aquaculture Research 28: 279-289.

Estevez, A., McEvoy, L.A., Bell, J.G., and 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.

Estevez, A., Kaneko, T., Seikai, T., Tagawa, M., and Tanaka, M., 2001. ACTH and MSH production in Japanese flounder (*Paralichthys olivaceus*) larvae fed arachidonic acid-enriched live prey. Aquaculture 192:309-319.

Evjemo, J.O., and Olsen, Y., 1997. Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. In: Hagiwara, A., Snell, T.W., Lubzens, E., and Tamaru, S. (Eds.), Live food in Aquaculture. Hydrobiologia 358: 159-162.

Fernandez-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M., and Vergara, J.M., 1995. Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.). Aquaculture 132: 325-337.

FOA, 1999. The state of world fisheries and aquaculture, 1998: Outlook: expected trends in supply and demand. Food and agriculture organization of the United Nations, Rome, Italy.

Folch, J., Lees, M., and Sloane, S.G. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 22: 497-509.

Fraser, A.J., Gamble, J.C., and 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*). Marine Biology 99:307-313.

Fraser, A.J., 1989. Triacylglycerol content as a condition index for fish, bivalve, and crustacean larvae. Canadian Journal of fisheries and Aquatic Sciences 46:1868-1873.

French, K.J., 1995. The growth and behavioural development of yellowtail flounder, *Pleuronectes ferrugineus*. B.Sc. Honours thesis. Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

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

Furuita, H., Konishi, K., and Takeuchi, T., 1999. Effect of different levels of eicosapentaenoic acid and docosahexaenoic acid in *Artemia* nauplii on growth, survival and salinity tolerance of larvae of the Japanese flounder, *Paralichthys olivaceus*. Aquaculture 170: 59-69.

Gara, B., Shields, R.J., and McEvoy, L., 1998. Feeding strategies to achieve correct metamorphosis of Atlantic halibut, *Hippoglossus hippoglossus* L., using enriched *Artemia*. Aquaculture Research 29: 935-948.

Godin, J.G.J, 1997. Evading predators. In: Behavioural Ecology of Teleost Fishes (Ed. J. Godin), pp. 192-236. University Press, Oxford.

Hazel, J.R. and Williams, E.E., 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Progress in Lipids Research 29: 167-227.

Hunter, J.R. 1981. Feeding ecology and predation of marine fish larvae. In: Marine Fish Larvae (Ed. R.Lasker), pp. 34-77. Washington Sea Grant Program, University of Washington.

Hutchings, J.A. Walters, C., and Haedrich, R.L., 1997. Is scientific inquiry incompatible with government information control? Canadian Journal of Fisheries and Aquatic Sciences 54: 1198-1210.

Kanazawa, A., 1993. Nutritional mechanisms involved in the occurrence of abnormal pigmentation in hatchery-reared flatfish. Journal of the World Aquaculture Society 24:162-166

Kattner, G., and Krause, M., 1987. Changes in lipids during the development of *Calanus finmarchicus* s.l. from copepodid I to adult. Marine Biology 96: 511-518.

Koven, W.A., Tandler, A., Sklan, D., and Kissil, G.W., 1993. The association of eicosapentaenoic and docosahexaenoic acids in the main phospholipids of different-age *Sparus aurata* larvae with growth. Aquaculture 116: 71-82.

Laurence, G.C., and Howell, W.H., 1981. Embryology and influence of temperature and salinity on early development and survival of yellowtail flounder *Limanda ferruginea*. Marine Ecology Progress Series 6: 11-18.

Lavens, P., Sorgeloos, P., Dhert, P., and Devresse, B., 1995. Larval Foods. In: Broodstock management and egg and larval quality (Eds. N. Bromage & R. Roberts), pp. 398-414. University Press, Cambridge.

Leaf, A., and Weber, P., 1988. Cardiovascular effects of n-3 fatty acids. The New England Journal of Medicine 318: 549-557.

Le Ruyet, J.P., Alexandre, J.C., Thebaud, L., and Mugnier, C., 1993. Marine fish larvae feeding; Formulated diets or live prey? Journal of the World Aquaculture Society 24: 211-223.

Lochman, S.E., Maillet, G.L., Frank, K.T., and Taggart, C.T., 1995. Lipid class composition as a measure of nutritional condition in individual larval Atlantic cod (*Gadus morhua*). Canadian Journal of Fisheries and Aquatic Sciences 52: 1294-1306.

Manning A.J. and Crim L.W., 1998. Maternal and inter-annual comparison of the ovulatory periodicity, egg production and egg quality of the batch-spawning yellowtail flounder. Journal of Fish Biology 53: 954-972.

May, R.C. 1973. Larval mortality in marine fishes and the critical period concept. In: The early life history of fish (Ed. J.H.S. Blaxter), pp. 3-21. Springer-Verlag, New York.

McEvoy, L.A., Navarro, J.C., Hontoria, F., Amat, F., and Sargent, J.R., 1996. Two novel *Artemia* enrichment diets containing polar lipid. Aquaculture 144: 339-352

McEvoy, L.A., Estevez, A., Bell, J.G., Shields, R.J., Gara, B., and Sargent, J.R., 1998. Influence of dietary levels of eicosapentaenoic and arachidonic acids on the pigmentation success of turbot (*Scophthalmus maximus* L.) and halibut (*Hippoglossus hippoglossus* L.). Bulletin of the Aquaculture Association of Canada 98-4: 17-20.

McEvoy, L.A., Naess, T., Bell, J.G., and Lie, O., 1998. Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. Aquaculture 163: 237-250.

Morris, N.J., 1997. Prey size selection in yellowtail flounder (*Pleuronectes ferrugineus*): A laboratory study. B.Sc. Honours thesis. Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

Morrison, W.R., and Smith, L.M., 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride methanol. Journal of Lipid Research 5: 600-608.

Mourente, G., Tocher, D.R., and Sargent, J.R., 1991. Specific accumulation of docosahexaenoic acid (22:6n-3) in brain lipids during development of juvenile turbot *Scophthalmus maximus* L. Lipids 26:871-877.

Naess, T., and Lie, O., 1998. A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. Aquaculture Research 29: 925-934.

Nanton, D.A., and Castell, J.D., 1998. The effects of dietary fatty acids on the fatty acid composition of the harpacticoid copepod, *Tisbe* sp., for use as a live food for marine fish larvae. Aquaculture 163: 251-261.

Nanton, D.A, and Castell, J.D., 1999. The effects of temperature and dietary fatty acids on the fatty acid composition of harpacticoid copepods, for use as a live food for marine fish larvae. Aquaculture 175: 167-181.

Navarro, J.C., Amat, F., and Sargent, J.R., 1993. The lipids of the cysts of freshwater- and marine-type *Artemia*. Aquaculture 109: 327-336.

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

Navas, J.M., Bruce, M., Thrush, M., Farndale, B.M., Bromage, N., Zanuy, S., Carrillo, M., Bell, J.G., and Ramos, J., 1997. The impact of seasonal alteration in the lipid composition of broodstock diets on egg quality in the European sea bass. Journal of Fish Biology 51: 760-773.

Nes, W.R., 1974. Role of sterols in membranes. Lipids 9:596-612.

Newton, I.S., 2000. Long chain fatty acids in health nutrition. In: Seafood in Health and Nutrition (Ed. F. Shahidi), pp. 15-28. ScienceTech Publishing, St. John's, Canada.

Norsker, N.H., and Støttrup, J.G., 1994. The importance of dietary HUFAs for fecundity and HUFA content in the harpacticoid, *Tisbe holothu*riae Humes. Aquaculture 125: 155-166.

Oie, G., Makridis, P., Reitan, K.I., and Olsen Y., 1997. Protein and carbon utilization of rotifers (*Brachionus plicatilis*) in first feeding of turbot larvae (*Scophthalmus maximus* L.). Aquaculture 153: 103-122.

Olsen Y., Reitan, K.I., and Vadstein, O., 1993. Dependence of temperature on loss rates of rotifers, lipids, and ω3 fatty acids in starved *Brachionus plicatilis* cultures. Hydrobiologia 255-256: 13-20.

Olsen, A.I., Attramadal, Y., Jensen, A., and Olsen, Y., 1999. Influence of size and nutritional value of *Artemia franciscana* on growth and quality of halibut larvae (*Hippoglossus hippoglossus*) during the live feed period. Aquaculture 179: 475-487.

Olsen Y., Evjemo J.O., and Olsen A. 1999. Status of the cultivation technology for production of Atlantic halibut (*Hippoglossus hippoglossus*) juveniles in Norway/Europe. Aquaculture 176: 3-13.

Olsen, A.I., Attramadal, Y., Reitan, K.I., and Olsen, Y., 2000. Food selection and digestion characteristics of Atlantic halibut (*Hippoglossus hippoglossus*) larvae fed cultivated prey organisms. Aquaculture 181: 293-310.

O'Rielly, A., 2000. Transformation in fisheries and aquaculture: An industry perspective. In: Seafood in Health and Nutrition (Ed. F. Shahidi), pp 1-6. ScienceTech Publishing, St. John's, Canada.

Parrish, C.C., 1987. Separation of aquatic lipid classes by Chromarod thin-layer chromatography with measurement by Iatroscan flame ionization detection. Canadian Journal of Fisheries and Aquatic Science 44: 722-731.

Parrish, C.C., 1988. Dissolved and particulate marine lipid classes: a review. Marine Chemistry 23:17-40.

Parrish, C.C., 1998. Determination of total lipid, lipid classes and fatty acids in aquatic samples. In: Lipids in Freshwater Ecosystems (Eds. M.T. Arts and B.C. Wainman), pp. 4-12. Springer-Verlag, New York.

Purchase, C.F., 1997. Growth of juvenile yellowtail flounder (*Pleuronectes ferrugineus*) under three photoperiods. Honours thesis. Memorial University of Newfoundland. St John's, Newfoundland. 40p.

Purchase, C.F., Boyce, D.L., and Brown, J.A., 2000. Growth and survival of juvenile yellowtail flounder *Pleuronectes ferrugineus* (Storer) under different photoperiods. Aquaculture Research 31: 547-552.

Puvanendran, V., and Brown, J.A., 1995. Preliminary experiments on the effect of prey concentration on the survival and growth of yellowtail flounder larvae (*Pleuronectes ferrugineus*). In Larvai '95. European Aquaculture Society, Special Publication No. 24, Gent, Belgium.

Rabe, J., 1999. The behaviour, growth, and survival of witch flounder and yellowtail flounder larvae in relation to prey availability. M.Sc. thesis. Memorial University of Newfoundland, St. John's, Newfoundland, Canada. 134pp.

Rabe, J., and Brown, J.A., 2000. A pulse feeding strategy for rearing larval fish: an experiment with yellowtail flounder. Aquaculture 191: 289-302.

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

Rainuzzo, J.R., Reitan, K.I., Jorgensen, L., and Olsen, Y., 1994. Lipid composition in turbot fed live feed cultured by emulsions of different lipid classes. Comparative Biochemistry and Physiology 107A: 699-710.

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

Rodriguez, C., Perez, J.A., Diaz, M., Izquierdo, M.S. Fernandez-Palacios, H., and Lorenzo, A., 1997. Influence of EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture 150: 77-89.

Ronnestad, I., Thorsen, A., and Finn, R.N., 1999. Fish larval nutrition: a review of recent advances in the roles of amino acids. Aquaculture 177: 201-216.

Sandy, J.M., and Blaxter, J.H.S., 1980. A study of retinal development in larval herring and sole. Journal of Marine Biology 60: 59-71.

Sargent, J.R., Eilertsen, H.C., Falk-Pertersen, S., and Taasen, J.P., 1985. Carbon assimilation and lipid production in phytoplankton in northern Norwegian fjords. Marine Biology 85: 109-116.

Sargent, J.R. 1995. Origins and functions of egg lipids: nutritional implications. In: Broodstock management and egg and larval quality. (Eds. N.R. Bromage and J.R. Roberts), pp. 353-372. Oxford University Press, Cambridge, UK.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J., and Tocher, D., 1999. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture 179: 217-229.

Scott, W.B., and Scott, M.G., 1988. Atlantic Fishes of Canada. Canadian Bulletin of Fisheries and Aquatic Sciences. Special Publication. No. 219.

Seikai, T., Shimozaki, M., and Watanabe, T., 1987. Estimation of larval stage determining the appearance of albinism in hatchery-reared juvenile flounder *Paralichthys olivaceus*. Nippon Suisan Gakkaishi 53:1107-1114.

Stinson, A.M., Wiegand, R.D. and Anderson, R.E., 1991. Fatty acid and molecular species composition of phospholipids and diacylglycerols from rat retinal membranes. Experimental Eye Research 52: 213-218.

Stottrup, J.G., and Norsker, NH., 1997. Production and use of copepods in marine fish larviculture. Aquaculture 155: 231-247.

Stottrup, J.G., 2000. The elusive copepods: their production and suitability in marine aquaculture. Aquaculture Research 31: 703-711.

Takeuchi, T., 1997. Essential fatty acid requirements of aquatic animals with emphasis on fish larvae and fingerlings. Reviews in Fisheries Science 5: 1-25.

Tilseth, S., 1990. New marine fish species for cold-water farming. Aquaculture 85: 235-245.

Tocher, D.R., and Sargent, J.R., 1984. Analysis of lipids and fatty acids in ripe roes of some Northwest European marine fish. Lipids 19: 492-499.

Tocher, D.R., Mourente, G., and Sargent, J.R., 1992. Metabolism of [1-14C] docosahexaenoate (22:6n-3), [1-14C] eicosapentaenoate (20:5n-3), [1-14C] linolenate (18:3n-3) in brain cells from juvenile turbot *Scophthalmus maximus*. Lipids 27: 494-499.

Tocher, D.R., 1993. Elongation predominates over desaturation in the metabolism of 18:3n-3 and 20:5n-3 in turbot (*Scophthalmus maximus*) brain astroglial cells in primary culture. Lipids 28: 267-272.

Venizelos, A. and Benetti., D., 1999. Pigment abnormalities in flatfish. Aquaculture 176: 181-188.

Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H., 1991. The metabolism of 7, 10, 13, 16, 19-docosapentaenoic acid to 4, 7, 10, 13, 16, 19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. Journal of Biological Chemistry 266: 19 995- 20 000.

Walsh, S.J., 1992. Factors influencing distribution of juvenile yellowtail flounder (*Limanda Ferruginea*) on the Grand Banks of Newfoundland. Netherlands Journal of Sea Research 29: 193-203.

Wantanabe, T., 1993. Importance of docosahexaenoic acid in marine larval fish. Journal of the world aquaculture society 24: 152-161

Watanabe, T. and Kiron, V. 1994. Review Prospects in larval fish dietetics. Aquaculture 124: 223-251.

Whalen, K., 1999. Lipid utilization and feeding of juvenile yellowtail flounder (*Pleuronectes ferrugineus*). M.Sc. thesis. Memorial University of Newfoundland, St. John's, Newfoundland Canada. pp. 161.

Yang, Z., 1995. Development of a gas chromatographic method for profiling neutral lipids in marine samples. M.Sc. Thesis. Memorial University of Newfoundland, St. John's, Newfoundland, 96 pp.

Zhu, P., 1998. Biochemical composition of eggs and larvae of captive Atlantic halibut (*Hippoglossus hippoglossus*). M.Sc Thesis. Memorial University of Newfoundland, St. John's, Newfoundland, 120p.

Appendix A1: Lipid class composition of week- three larvae fed differentially enriched rotifers (mean ± SEM, n=6)

	DHA	DHA + EPA	DHA + AA	Control
Total lipid (μg/mg)	103.8 ± 15.4	78.7 ± 18.0	88.9 ± 9.8	78.0 ± 6.4
Lipid class (% of total lipid)				
Triacylglycerols	19.5 ± 0.3[a]	20.2 ± 0.6[a]	16.9 ± 0.7[a]	12.5 ± 0.8[b]
Free Fatty Acids	5.1 ± 0.4	5.9 ± 0.4	4.8 ± 0.4	8.7 ± 1.0
Sterols	9.3 ± 0.34	9.8 ± 0.3	9.8 ± 0.5	12.3 ± 0.3
Diacylglycerols	1.8 ± 0.5	1.1 ± 0.4	2.2 ± 0.8	1.9 ± 0.4
Acetone-Mobile Polar Lipid	2.8 ± 0.2[a]	5.2 ± 0.4[b]	3.0 ± 0.3[a]	2.4 ± 0.4[a]
Phospholipid	59.0 ± 1.2	56.6 ± 1.3	62.4 ± 1.0	60.7 ± 0.9
TAG/ST	2.1 ± 0.1[a]	2.1 ± 0.2[a]	1.8 ± 0.3[a]	0.9 ± 0.1[b]

HC, Steryl/wax esters, KET, and GE present at < 1%.

Appendix A2: Fatty acid composition of week-three larvae fed differentially enriched rotifers (mean ± SEM, n=6)

%of total fatty acids	DHA	DHA+EPA	DHA+AA	CONTROL
14:0	4.3 ± 0.3	3.1 ± 0.1	3.5 ± 0.0	1.3 ± 0.1
16:0	11.8 ± 0.4	11.5 ± 0.1	11.9 ± 0.4	12.3 ± 0.2
18:0	5.3 ± 0.2	6.3 ± 0.1	5.9 ± 0.2	6.0 ± 0.1
Σ SFA[a]	**23.3 ± 0.1**	**22.5 ± 0.1**	**22.8 ± 0.7**	**21.8 ± 0.3**
16:1n-7	5.4 ± 0.1	5.8 ± 0.1	5.3 ± 0.0	6.5 ± 0.2
18:1n-11	1.1 ± 0.1	1.5 ± 0.0	1.2 ± 0.1	0.0 ± 0.0
18:1n-9	19.0 ± 0.2	17.1 ± 0.2	18.6 ± 0.1	26.9 ± 1.4
18:1n-7	2.4 ± 0.1	3.0 ± 0.0	2.4 ± 0.0	3.4 ± 0.1
20:1n-9	2.0 ± 0.1	2.1 ± 0.0	2.0 ± 0.0	2.3 ± 0.0
ΣMUFA[b]	**31.8 ± 0.1**	**31.9 ± 0.4**	**31.7 ± 0.0**	**41.3 ± 1.8**
16:3n-4	0.5 ± 0.0	1.4 ± 0.9	0.4 ± 0.0	1.1 ± 0.4
18:2n-6	3.8 ± 0.0	3.8 ± 0.0	4.5 ± 0.1	10.3 ± 0.4
20:4n-6	2.4 ± 0.1	2.7 ± 0.1	7.9 ± 0.1	2.7 ± 0.1
20:5n-3	5.5 ± 0.1	10.0 ± 0.2	4.3 ± 0.0	7.2 ± 0.3
22:5n-6	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	1.1 ± 0.5
22:5n-3	2.3 ± 0.1	3.6 ± 0.1	2.2 ± 0.0	3.9 ± 0.3
22:6n-3	27.7 ± 0.1	20.2 ± 0.6	22.8 ± 0.9	7.1 ± 0.4
ΣPUFA[c]	**44.9 ± 0.1**	**45.6 ± 0.4**	**45.5 ± 0.6**	**36.9 ± 1.6**
DHA/EPA	5.1 ± 0.1	2.0 ± 0.0	5.3 ± 0.2	1.0 ± 0.0
DHA/AA	11.7 ± 0.4	7.5 ± 0.1	2.9 ± 0.1	2.6 ± 0.1
EPA/AA	2.3 ± 0.0	3.7 ± 0.1	0.6 ± 0.0	2.6 ± 0.0

[a] includes ai-15:0, 15:0, i-17:0, ai-17:0, 17:0, and 20:0
[b] includes 18:1n-5, 20:1n-7, 22:1n-11, 22:1n-9, and 24:1
[c] includes 16:2n-4, 16:3n-4, 16:4n-3, 18:3n-3, 18:4n-3, 20:2n-6, 20:3n-6, 20:4n-3, and 22:4n-6