# FINAL REPORT: LIPIDS, EGG AND LARVAL QUALITY IN COD

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## EXECUTIVE SUMMARY

This project consisted of two studies on lipid quality and performance of cod broodstock, eggs and larvae. The first study aimed to provide comparative data on lipid composition in relation to egg quality in eggs from farmed and wild broodstock. This was necessary to investigate the cause of generally poor egg quality from farmed cod broodstock in UK in order to reduce reliance on wild-caught or imported eggs.

The second study aimed to compare lipid composition of rotifers enriched using four different commercial enrichment products, and to investigate the effect of feeding enriched rotifers on lipid composition, growth and survival of cod larvae. Losses of cod larvae may exceed 90% during early development. Optimisation of the nutritional composition of feed to improve survival and growth is important to minimise losses in commercial production.

In the first study, egg fertilisation rates and cell division symmetry scores, total lipid, lipid class composition and fatty acid profiles were determined for eggs collected from spawning cod of three different origins: 'Farmed' (hatchery reared), 'Wild' (wild caught, held in tanks for several months prior to spawning) and 'True wild' (wild caught at the start of the spawning season). Two batches of 'Farmed' and two batches of 'Wild' eggs were incubated to hatch. Egg fertilisation rates, symmetry scores and rates of survival to hatch were better in eggs from 'Wild' and 'True wild' than 'Farmed' broodstock. No differences were detected in total lipid content of eggs, and there were few differences in lipid class composition, but eggs from 'Farmed' fish were found to have significantly lower concentrations of the phospholipids, phosphatidylinositol and phosphatidylglycerol/cardiolipin than eggs from 'Wild' and 'True wild' fish. Concentrations of the polyunsaturated fatty acid, arachidonic acid (ARA), and ARA/EPA ratios were lower in 'Farmed' eggs than in both 'Wild' and 'True wild' eggs and a strong positive correlation was found between egg quality measures and ARA content and ARA/EPA ratios in the eggs. Astaxanthin was present in eggs from all sources, but canthaxanthin was detected only in 'Wild' eggs. Total carotenoid concentrations were variable, but significantly lower in 'Farmed' than in 'Wild' eggs. The data suggest that arachidonic acid and carotenoid pigment concentrations in eggs from farmed fish may be sub-optimal and further analysis of commercial egg batches is underway to investigate whether a general correlation exists between egg quality and content of these nutrients. Further studies are planned to investigate the effect of ARA and carotenoid pigment supplementation of broodstock diets on egg quality in cod.

In the second study, triplicate groups of cod larvae were reared from day 1 to day 30 post-hatch using rotifers enriched with one of four enrichment media: (A)'SV12': SV12 Chlorella, a commercially-produced concentrated suspension of *Chlorella vulgaris* supplemented with Vitamin B12, DHA and EPA (produced in Japan and supplied by Docosa Ltd, Germany), (B) 'IoA Enrichment': a new oil emulsion product prepared from mackerel oil, capelin phospholipid, soya lecithin, protein omega concentrate (from shrimp by-products), vitamin E and ascorbyl palmitate, (C) 'DHA-PS': DHA Protein Selco (Inve Aquaculture NV, Belgium), a commercial protein enrichment with a minimum DHA/EPA ratio of 2.5, and (D) 'MBlowARA': Multiboost Low ARA (Dana Feed, Denmark). A commercial enrichment diet containing 15% DHA, 1.5% EPA and 0.1% ARA.

Survival, standard length, dry weight, swim bladder inflation rate, feeding rate and incidence of deformity were assessed and fatty acid profiles were determined for rotifers and larvae at intervals during the course of the trial. At the end of the feeding period, mean standard length ranged from 7.97 mm in group A ('SV12') to 7.1 mm in group B ('IoA enrichment') and mean dry weight ranged from

6.10 mg in group D (‘MBlowARA’) to 4.0 mg in group B (‘IoA enrichment’). Significant differences in standard length were detected between larvae in group A (‘SV12’) and those in group B (‘IoA enrichment’) at 15, 20, 26 and 30 days, and between larvae in group A (‘MBlowARA’) and group D (IoA enrichment) at 20, 26 and 30 days. Similarly, on day 29, dry weight of larvae in group A (‘SV12’) and group D (‘MBlowARA’) was significantly different to that in group B (‘IoA enrichment’). Survival rates ranged from 11.9 % in group D (‘MBlowARA’) to 5.1% in group B (‘IoA enrichment’). Survival rates in Group D (‘MBlowARA’) and Group C (‘DHA-PS’) were significantly different to those in group A (‘SV12’) at day 13. There were no significant differences in swimbladder inflation success or incidence of deformity. No deformed larvae were identified.

Rotifers enriched with ‘MBlowARA’ had a significantly higher percentage of ARA than all the other treatments, and a significantly higher percentage of DHA than rotifers unenriched and enriched with *Nannochlorpsis* sp., ‘SV12’ and ‘DHA-PS’. The EPA/ARA ratio was significantly lower and the DHA/EPA ratio was significantly higher in rotifers enriched with ‘MBlowARA’ when compared to all of the other treatments.

The lipid profile of larvae reflected the composition of the live feed. On days 11, 22 and 29, larvae in Group A (Multiboost) had significantly higher levels of ARA and DHA, and lower levels of EPA, than all the other treatments. The EPA/ARA ratio in was significantly lower and DHA/EPA ratio was significantly higher in this group than in all of the other treatments. Larvae in group D had significantly higher EPA concentrations and a higher EPA/ARA ratio than other groups. ARA concentrations and the DHA/EPA ratio in Group D were similar to those in Groups B and C.

The requirements of cod larvae for essential fatty acids are not well-defined but the results of this and other studies indicate that relatively high DHA concentrations and DHA/EPA ratios, low EPA concentrations and EPA/ARA ratios, and high ARA concentrations are advantageous. On the basis of fatty acid profile, ‘MBlowARA’ appeared to be the most suitable of the four products tested, but performance of larvae reared on all except ‘IoA enrichment’ was similar and factors such as cost and ease of use should be considered when selecting a suitable enrichment for commercial larval rearing.

The significance of these results is discussed in relation to practical problems of egg and larval quality affecting the emerging cod farming industry in UK.

## 1. Introduction

This project consisted of two studies on lipid quality and performance of cod broodstock, eggs and larvae. The first study aimed to provide comparative data on egg quality from wild and farmed broodstock to evaluate the role of lipid nutrition in any differences observed. The second study aimed to compare state-of-the-art live feed enrichment products, and to provide data on lipid quality in enriched rotifers and cod larvae, to investigate possible bases for any differences observed in larval survival and growth. Both studies address the Seafish policy goal to develop and refine sustainable and efficient production systems for cod, and also provide welfare and economic benefits. Data generated by the project is intended to promote further development of broodstock and larval rearing feeds for the cod farming industry.

### 1.1 Essential polyunsaturated fatty acids in marine fish

In common with other vertebrates, fish have a requirement for three polyunsaturated fatty acids for normal growth and development: arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These long-chain fatty acids play a general role in maintaining the structure and function of cell membranes, but also have specific roles as precursors for eicosanoids. Eicosanoids are a range of highly active C20 compounds involved in a wide range of homeostatic functions at biochemical and physiological levels. Most vertebrates, including many freshwater fish species, have the capacity to produce DHA and EPA from linolenic acid (LNA, 18:3 n-3) and ARA from linoleic acid (LA, 18:2 n-6). However, all marine fish species so far studied do not

have this capacity and dietary sources of DHA, EPA and ARA are therefore considered essential for survival, growth and normal development (Sargent *et al*. 1999).

ARA is the predominant long-chain polyunsaturated fatty acid (PUFA) in cell membranes of terrestrial animals in all tissues except neural tissue and is also the main precursor for eicosanoids in both fish and terrestrial animals. In contrast, EPA and DHA are abundant in microalgae, zooplankton and marine fish, and are conserved through the marine food chain as the major PUFAs present in cellular membranes in fish. DHA is present in high concentrations in neural tissues in terrestrial animals and fish. These fatty acids have received particular attention in the context of marine fish egg quality and broodstock and larval nutrition.

The marked chemical similarities of ARA, EPA and DHA lead to competitive interaction in a wide range of physiological processes and it is therefore important to consider the relative as well as absolute amounts of these fatty acids. The particular significance of DHA/EPA and EPA/ARA ratios in marine broodstock feeds, fish eggs and larvae is discussed by Sargent et al. (1999).

## 1.2. Study 1: Lipids in relation to egg quality differences between farmed and wild cod broodstock

Farm observations indicate that wild-caught cod tend to produce better quality eggs and larvae than farmed broodstock and wild caught fish currently provide the bulk of eggs stocked in UK hatcheries. However, reliance on wild broodstock creates a risk of introduction of disease, raises environmental concerns over the potential use of non-native stocks, prevents stock improvement by artificial selection, and causes commercial uncertainty regarding the cost and reliability of egg supplies. It is well known that diet has a major influence on egg composition, and egg quality from farmed broodstock can be improved by manipulation of broodstock diets and feeding regimes (eg. Czesny and Dabrowski 1998; Ashton *et al*. 1993; Gallagher *et al*. 1998; Sargent *et al.* 2002). In general, wet fish based broodstock feeds which most closely simulate the nutritional composition of the natural diet produce best egg quality from farmed broodstock, but these impose a risk of disease introduction. On the other hand, existing formulated dry diets, whilst adequate for growth and survival of production stock, appear to be nutritionally sub-optimal for feeding broodstock. For example, Pavlov *et al*. (2004) considered that low fertilisation rates in cod eggs, and poor egg and larval quality, were directly related to broodstock diet composition. Dietary n-3 and n-6 PUFA have been shown to influence fecundity, egg quality, hatching success, and incidence of malformation in larvae (Pavlov *et al.* 2004). Recent studies have confirmed that the fatty acid composition of cod fed commercial dry diets reflects the composition of feed (Morais *et al*. 2001). Supplementation of broodstock feed with marine fish oil rich in specific PUFAs can lead to an increase in levels of these fatty acids in the developing eggs and, in the case of sea bass and halibut, this has been shown to have a measurable impact on egg quality (Bruce *et al.* 1999; Mazorra *et al*. 2003). The possibility therefore exists to improve egg quality by manipulation of the lipid composition of diets for broodstock cod.

Study 1 aimed to compare eggs from wild and farmed broodstock with respect to their lipid/ fatty acid composition and to relate this to egg quality where possible.

## 1.3 Study 2. Role of polyunsaturated fatty acids in relation to performance of larvae fed rotifers enriched using different enrichment products

Cod eggs hatch in around 10 days at 7ºC and larvae begin exogenous feeding shortly thereafter. During the period of approximately 60 days between first feeding and weaning onto dry feed, mortality rates in excess of 90 % routinely occur, growth is variable and skeletal deformities and other abnormalities are common. Poor survival rates in larval rearing of cod are a major cause of uncertainty in planning commercial production and it is evident that there are considerable opportunities for improvement of performance, economy and fish welfare during larval rearing.

Many factors can influence larval survival rates, including egg quality, husbandry techniques, and physical and microbiological environmental conditions. In relation to feed and nutrition, an adequate supply of suitable feed is necessary and the nutritional quality of the feed must be adequate to provide

appropriate nutrients for normal development, ensure good feed consumption and growth, and promote disease resistance and tolerance of environmental stressors.

Rotifers are used in commercial hatcheries since they can be reared reliably in the numbers required and are of a size suitable for feeding to newly hatched cod larvae but they require enrichment in order to meet larval nutritional requirements. Enrichment products are used to enhance the nutritional value of live feed organisms for marine fish larvae, particularly with respect to PUFA content (Sargent *et al*. 1999), and many studies have demonstrated that the lipid composition of rotifers (and *Artemia*) can be successfully manipulated using enrichments in various forms (eg Vismara *et al*. 2003). Consequential effects on the chemical composition of larvae, and/or effects on growth, survival and incidence of developmental abnormalities have been demonstrated but there are significant differences between species (see for example, Mourente *et al.* 1991; Estevez *et al.* 1999; Sargent *et al.* 1999).

Live feed enrichment products come in various forms, including algae (various species, as fresh, liquid concentrate or paste); oil emulsions; dry, yeast-based products and microcapsules. Many different commercial formulations are available and these frequently contain supplements of essential fatty acids and vitamins to improve nutritional composition and bioavailability. The aim of Study 2 was to evaluate larval performance and to examine the fatty acid composition of larvae and rotifers enriched using four different enrichment products.

## 2. Materials and methods

### 2.1 Study 1

#### 2.1.1 Study design

Three stocks of cod broodstock, one farmed and two wild, were conditioned for spawning under similar environmental and husbandry conditions. Egg batches were collected during the natural spawning season in spring 2004. Egg quality was assessed using a range of indices including fertilisation rate and egg symmetry. Samples of eggs were collected for lipid analysis. Several egg batches were incubated to evaluate survival and hatch rate and lipid analysis was carried out on egg samples to identify changes in lipid profiles during early development.

#### 2.1.2 Broodstock origin

Three groups of broodstock fish were used for the study:
Group A (‘Farmed’): Hatchery reared farmed fish, hatched in spring 2002 reared in tanks at the Marine Environmental Research Laboratory (MERL) and Machrihanish Marine Farm (MMF), Machrihanish, UK. Group A1 consisted of a population of around 100 fish in one tank at MMF. Group A2 consisted of 200 fish in one tank at MERL.
Group B (‘Wild’): Wild fish captured in and around the Clyde estuary. Group B1 consisted of approximately 180 fish which arrived progressively on site between July 2003 and January 2004 and were pooled in one tank at MMF Group B2 consisted of 175 fish captured in February 2004 which were held in one tank at MERL.
Group C (‘True wild’): On 6 April 2004, a new batch of freshly caught wild cod arrived at MERL and eggs were collected from fish not fed artificial feed. These fish stopped spawning two days after their arrival, probably because of the stress of environmental change. As a consequence, only two batches of eggs were sampled from this group.

2.1.3 Broodstock holding and husbandry

Broodstock tanks at MMF were 40 $m^3$; tanks at MERL were 13$m^3$. All were supplied with a continuous flow of seawater. During the spawning period, from February to May 2004, tanks were supplied with chilled seawater at 8±1ºC and fitted with a secondary outlet to collect eggs from the water surface. The density of fish in groups A1 and B1 (held in 40 $m^3$ broodstock tanks at MMF) was approximately 15kg/$m^3$. The density of fish in groups A2 and B2 was approximately 30 kg/$m^3$, and the density of group C was around 15 kg/$m^3$. Fish in groups A and B were fed using the same feeding regime whilst held on site. Prior to the onset of maturation, fish were fed twice daily using a commercial dry formulated diet (DANA Feed AS, Denmark; 15mm, 58% crude protein, 17% crude lipid) at a rate of approximately 0.33% biomass per day. From one month before the start of the spawning season until the end of the study period, fish were fed to satiation twice daily, with dry pellets only in the morning and with a commercial broodstock paste (Breed M, INVE, Belgium; 62% crude protein, 16% crude lipid) in the afternoon. Acceptance of the paste diet was particularly good and the daily ration consisted of around 40% dry pellets and 60% paste.

2.1.4 Egg collection and examination

Eggs were spawned and fertilised within the tanks and collected in egg collectors on surface overflows over a period of 24 hrs. Each morning, eggs from the collectors were transferred into buckets of clean seawater and left for about ten minutes to allow separation: live eggs float, dead eggs sink. When fully separated, a sample of around 1000 floating eggs was examined under a binocular microscope. Egg batches were assessed using the following parameters:

A. Fertilisation rate (%), estimated by counting the relative numbers of fertilised and apparently unfertilised eggs in a sub-sample of 100 eggs ('Farmed' n=8'; 'Wild' n=17; 'True wild' n=2)
B. Egg symmetry score (Table 1) ('Farmed' n=7'; 'Wild' n=11; 'True wild' n=2).
C. Egg lipid analysis ('Farmed' n=7'; 'Wild' n=13; 'True wild' n=2)

Table 1: Egg symmetry scoring

| Estimation | Description | Score |
|---|---|---|
| *Very poor* | Very few eggs have an acceptable symmetry | 1 |
| *Poor* | Most of the eggs are asymmetric | 2 |
| *Average* | About 50% of the eggs have a good symmetry | 3 |
| *Good* | The majority of eggs have a good symmetry | 4 |
| *Very good* | No asymmetry observed | 5 |

2.1.5 Egg incubation

Two 70L conical incubators, supplied with filtered, UV treated, seawater were used for egg incubation. The incubators were fitted with airstones, banjo-type outlet screens and waste purge valve, and were cleaned, disinfected and rinsed before stocking with each batch of eggs. Daily husbandry involved recording water temperature, and stopping the water and air in order to allow separation of the dead and live eggs. After the eggs had separated, samples were taken if required, and each incubator was flushed into a net via the purge valve in order to collect the dead eggs. Any remaining dead eggs were removed by siphon. Dead eggs were weighed daily and mortality calculated.

Four batches of eggs were incubated as follows:

F1 = farmed stock; batch 1
F2 = farmed stock; batch 2
W1 = wild stock; batch 1
W2 = wild stock; batch 2

Eggs hatched at around 90 degree-days, equivalent to 12.8 days at 7ºC. When all the larvae had hatched, survival at hatching was calculated by subtracting the recorded daily mortality from the initial wet weight of eggs stocked in the incubator.

2.1.6 Collection of samples for lipid analysis

*Daily egg batch assessment*. Samples from daily egg production were taken in triplicate for lipid analysis. Approximately 1g of eggs was placed in 2ml glass vials previously filled with 1ml chloroform/methanol (2:1) plus 0.01% BHT. Samples were stored at -20°C prior to analysis. The purpose of this type of sample was to collect data to compare eggs post-spawning, and to observe any changes during the course of the spawning period.

*Egg development*. Eggs in the incubators were sampled every other day: triplicate samples were placed in chloroform/methanol as described above. These samples were used to follow each batch through egg development.

*Dry weight determination*. Each time a new batch of eggs was incubated, 20-30 eggs with as little water as possible were sampled in triplicate into plastic Eppendorf tubes. The samples were stored frozen at -20°C prior to analysis.

2.1.7 Sample analysis

*Total lipid content*. Lipids were extracted from eggs or larvae samples using the Folch-Lee extraction method (Folch *et al.* 1957). Samples were homogenised using a rotating Teflon probe in a glass tube and lipids were extracted in chloroform/methanol (2:1). Samples were then centrifuged (1500rpm, 5 min, Jouan C312) and filtered (Whatman no.1 filter paper) before the solvent was evaporated under a stream of oxygen-free nitrogen (OFN) and total lipid quantified gravimetrically.

*Lipid class analysis*. High Performance Thin-Layer Chromatography (HPTLC) was used to determine the lipid class composition of the egg samples (Henderson and Tocher 1992). Samples were applied to a 10x10cm HPTLC plate (Merck, silica gel 60) and subjected to one-dimensional double-development chromatography. Two solvents were used: the first to move the polar lipids up to 5.5cm high on the plate, the second to move the neutral lipids up to 9cm high. The plate was then sprayed with copper acetate and phosphoric acid reagent, and charred by heating in an oven at 160 °C to make the lipids visible. The amount of each lipid class is proportional to the amount of charring which is measured with a scanning densitometer (Camag TLC Scanner 3).

*Fatty acid composition*. Samples, including a free fatty acid standard, were methylated using 1% methanol-sulphuric acid methylating reagent for 16 hours at 50°C (Christie, 1982). Resultant fatty acid methyl esters (FAME) were purified on a 20x20cm TLC (Thin-Layer Chromatography) plate (Tocher and Harvie 1988). Once the FAMEs were purified and silica removed, they were re-suspended in isohexane and injected into the GC equipped with a 30m x 0.32µm Gas Chromatography column (GC, Chrompack CP WAX 52CB) in order to identify and quantify the fatty acids as described by Ackman (1980). The carrier gas was hydrogen, and the temperature was 50°C at injection, then firstly increased at 40°C/min to 150°C, secondly at 2°C/min to 225°C where it stayed for 5 min.

*Pigment analysis*. During egg sampling, a clear difference in pigmentation was observed between the wild and farmed eggs. As a consequence, pigments were analysed. 1ml of the total lipid extracts was solvent evaporated under OFN and re-dissolved in 500µl isohexane before 50µl were injected and

analysed by High Performance Liquid Chromatography (Barua *et al*., 1993). Carotenoid (astaxanthin and canthaxanthin) standards were also run in order to identify the retention time in the column and calculate the sample concentration by comparison of the surface areas on the chromatogram. Samples were also analysed for total pigments, reading absorbance at 470nm with a spectrophotometer (Jenway 6405, UV/Vis).

2.1.8 Statistical analysis

All data were presented as means ± SD. Differences in fertilisation rate and symmetry were analysed using one-way ANOVA with Tukey's post test using GraphPad Prism (v4.00 for Windows, GraphPad Software, San Diego California USA). The Day 0 samples were treated the same way, in order to compare the three origins of eggs for their respective lipid content, lipid class or fatty acid profiles.

Two-way ANOVA was used to process the data from the incubated samples, with egg batches as first factor, and incubation time as second factor. Data from lipid class and fatty acid analysis were treated this way. Mean values were considered significantly different if $P<0.05$, unless otherwise stated.

2.2 Study 2

2.2.1 Study Design

Four enrichment products were tested using triplicate groups of larvae reared from hatching to around 30 days post-hatch. Enrichments were fed to rotifers which were then used to feed triplicate tank groups of larvae. Larval survival, growth and incidence of deformity were used as indices of larval quality. Samples of enrichment products, enriched live feeds and larvae were collected for fatty acid analysis.

2.2.2 Enrichment products

The products tested were as follows:

a) 'SV12': SV12 Chlorella, a commercially-produced concentrated suspension of *Chlorella vulgaris* supplemented with Vitamin B12, DHA and EPA (produced in Japan and supplied by Docosa Ltd, Germany). This product was stored refrigerated for the duration of the study.
b) 'IoA Enrichment': a new oil emulsion product prepared from mackerel oil, capelin phospholipid, soya lecithin, protein omega concentrate (from shrimp by-products), vitamin E and ascorbyl palmitate. Since the stability and storage of this product is relatively low, it was prepared just before the start of the experiment.
c) 'DHA-PS': DHA Protein Selco (Inve Aquaculture NV, Belgium). A commercial protein enrichment with a minimum DHA/EPA ratio of 2.5.
d) 'MBlowARA': Multiboost Low ARA (Dana Feed, Denmark). A commercial enrichment diet containing 15% DHA, 1.5% EPA and 0.1% ARA.

2.2.3 Rotifers

*Rotifer production.* Rotifers for enrichment were supplied by Machrihanish Marine Farm Ltd, (Argyll, UK). Rotifers were *Brachionus plicatilis* (large strain) with a mean length of 225µm and a mean width of 150µm.

*Rotifer enrichment.* Rotifers were enriched in 15 L buckets containing 10 litres of seawater (34‰) at 20-25°C. Strong aeration was provided by an air stone placed into each bucket. As the requirement for rotifers increased during the trial according to the larval growth, the density of rotifers for the enrichment period increased accordingly. At the start of the study, the density was 158 rots/ml, and increased gradually to reach 900 rots/ml by the end. The mean density was 570 rots/ml during the period of the trial. Rotifer enrichments were set up each day at 5pm for a similar enrichment time of 15 hours. This standard protocol differed from manufacturer's recommendations but enabled larvae to be fed the following morning with freshly enriched rotifers, and twice in the afternoon with recently

stored rotifers. The concentration of enrichment used varied between each treatment (Table 2) based on manufacturers recommendations. 'DHA-PS', 'MBlowARA and 'IoA enrichment' were blended for 3 to 5 minutes in warm salt water with a hand held kitchen blender, and poured into the buckets. 'SV12' was added directly into the bucket.

Table 2: Concentration of enrichment for the four treatments (g or ml per million rotifers)

| MBlowARA | DHA-PS | IoA enrichment | SV12 |
|---|---|---|---|
| 0.24g/Mrots | 0.5g/Mrots | 2g/Mrots | 2ml/Mrots |

*Rotifer counts*. Rotifers were counted using a counting cell under the microscope (40x). One drop of Lugol's iodine was added to kill the rotifers and to colour them so as to count them more easily. A sample of 100µl to 200µl was taken from each bucket before the enrichment period to calculate the amount of enrichment needed, and after the enrichment period to know how many rotifers were available to be fed to the larvae. The oxygen level and the temperature were checked every morning.

*Rotifer harvest and storage.* Rotifers were harvested underwater using a concentrator/rinser. Aeration was used during the concentration of rotifers to avoid the obstruction of the mesh. Rotifers were concentrated into a volume of 6 litres per bucket. One third was fed immediately and the rest was stored for use later in the day An ice pack was added to each bucket so as to maintain the temperature below 6°C during the day, and aeration maintained.

2.2.4 Larval rearing

*Fish origin.* Larvae originated from eggs collected from broodstock held at Machrihanish Marine Farm Ltd. The larvae were stocked into the experimental tanks on day 1. Then, the larval density was estimated (by counting the number of larvae with a 10ml pipette several times) and the larvae were evenly distributed into the twelve tanks. The starting number of larvae was around 3,385 per tank. The four different treatments were allocated randomly to the twelve tanks, and three replicates were made for each treatment.

*Larval rearing facilities.* Larvae were reared in 75L insulated tanks supplied with seawater filtered to 5um and treated with UV. A banjo filter with a 500µm mesh was used for the tank outlets and aeration was used to maintain larvae in suspension in the water column.

*Environmental conditions.* Larvae were held under constant light provided by fluorescent tubes. Air temperature was controlled by an air conditioning unit to maintain seawater at external temperature. The oxygen ratio varied accordingly, but remained well above critical levels. The salinity stayed constant during the 30 days of the trial (34‰).

*Husbandry.* Water temperature and dissolved oxygen were recorded in the tanks every morning. Dead larvae, faeces, uneaten food and bacterial slime were siphoned daily from day 16, and the edges as well as the bottom of the tanks were cleaned to remove algae and bacteria. Larvae were fed at 10am, 1pm and 5pm. 4 ml of *Nannochloropsis* sp. (algal paste; Aquamer: $5.6 \times 10^{10}$ cells/ml) were added per tank to provide "green water" at 10am and 5pm.

2.2.5 Larval assessment.

Standard length, swim bladder inflation, feeding rate and deformities were assessed at day 5, day 10, day 15, day 20, day 26 and day 30. 10 larvae per tank (30 per treatment) were removed randomly using a beaker and placed in a petri dish under a dissecting microscope. Standard length was determined under the dissecting microscope with a 10 mm eyepiece graticule, from the end of the notochord to the tip of the upper jaw (Larsen, 2002). Swim- bladder inflation, feeding rate and deformities were assessed by looking at the larvae under the dissecting microscope. Each factor was assessed a "1" or a "0" (inflated swim bladder or not, larvae feeding or not and larvae with deformity

or not). Deformities were not taken into account in the results as only two deformed larvae were found (at day 30). Survival was assessed at day 2, day 8, day 13, day 18, day 23 and day 28: larval density was estimated by counting the number of individuals fished with a one litre beaker 20 different times. The average number was then multiplied by the volume of the tank, i.e. 75L.

Dry weight of larvae was measured at day 2, day 6, day 11, day 22 and day 29. Ten larvae were sampled randomly from each tank. They were placed in pre weighed plastic Eppendorf tubes, and stored frozen. At the end of the trial period, the tubes were dried for 24 hours in an oven at 100°C. Once dried, the tubes were reweighed to calculate the dry weight of the 10 larvae. 10 additional larvae were collected at each similar time point for the lipid samples, so as to relate the dry weight of the larvae to the lipid content of the larvae.

2.2.6. Sample collection

*Larvae.*Ten larvae were collected for lipid analyses as described above. Larvae were placed into 2 ml glass vials containing 1ml of chloroform/methanol + 0.01% BHT. Samples were stored at –20°C.

*Rotifers.* Rotifers were collected before and after enrichment on day 15, day 18 and day 19. Approximately 160,000 rotifers were collected on a 54µm mesh and excess water was removed to give a wet weight of approximately 0.25g. Rotifers were stored at –20°C in 2ml glass vials containing 1ml of chloroform:methanol + BHT for subsequent lipid analyses. The same amount of rotifers was placed at the same time into pre weighed plastic Eppendorf tubes, and stored at –20°C. At the end of the trial period, the tubes were dried for 24 hours in an oven at 100°C. Once dried, the tubes were reweighed to calculate the dry weight of the rotifers.

*Lipid analysis.* Total lipids were extracted and measured using the Folch-Lee method. Preparation, purification and analysis of fatty acid methyl esters (FAME) using GLC was carried out as described in section 2.1.7.

2.2.7 Statistical analysis

Mean and standard deviations (SD) were calculated. Comparisons between treatments were made using ANOVA using SPSS v.11.5. Growth rates and condition parameters were analysed with LSD's Post Test, whereas fatty acid compositions were analysed with Tukey's Post Test. Data were significantly different when $P<0.05$. The highest significance value was assigned an a and each significant lower value was assigned b and then c etc. Values that were not significantly different were assigned one similar letter or no letter.

## 3. Results

3.1 Study 1

3.1.1 Egg fertilisation rate and symmetry

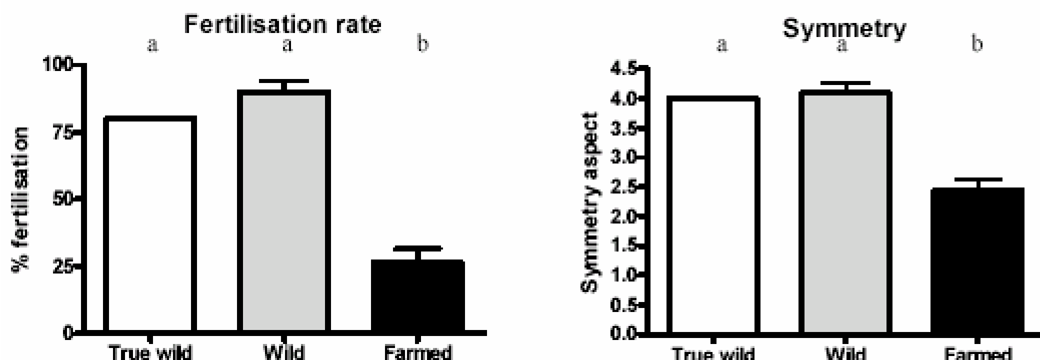


Figure 1. Mean fertilisation rates and symmetry scores of collected eggs on day 0. Note: values with different superscript letters are significantly different, [a] shows the highest level.

Figure 1 shows mean fertilisation rates and symmetry scores for eggs from each of the three broodstock groups. For both fertilisation rate and symmetry, the 'True wild' eggs did not differ from the 'Wild' eggs (P>0.05), whereas the fertilisation rate and symmetry score in 'Farmed' eggs were significantly different (P<0.001). Both 'Wild' and 'True wild' eggs had better fertilisation rate and symmetry than 'Farmed' eggs.

3.1.2 Egg survival and hatching

Survival through incubation showed a similar pattern. Incubated 'Wild' eggs had relatively high fertilisation rates, symmetry scores and survival rates, whereas 'Farmed' eggs performed less well (Table 3). Regardless of egg origin, there was a positive correlation between fertilisation rate, symmetry score and survival to hatch. No deformed larvae were observed at hatching in any batch.

Table 3: Performance of incubated eggs

| Batch | Wild 1 | Wild 2 | Farmed 1 | Farmed 2 |
|---|---|---|---|---|
| *Fertilisation rate* | 70.0% | 100.0% | 50.0% | 17.0% |
| *Symmetry* | 4 | 5 | 3 | 2 |
| *Survival to hatch* | 46.2% | 57.2% | 46.1% | 17.4% |

3.1.3 Egg lipid analysis

*Total lipid content*. The total lipid content of eggs sampled on day 0 was similar in the three groups of eggs. The overall mean was 0.63±0.09 % based on the egg wet weight.

*Lipid class analysis*. Results of lipid class analysis are summarised in Figures 2 and 3. Three of the four main lipid classes found in the day 0 samples (phosphatidylethanolamine, cholesterol, and triacylglycerol), showed no significant difference between the three groups (P>0.05). The same pattern was observed for the total neutral and total polar lipid classes. However, phosphatidylcholine was found in significantly greater amounts in 'Farmed' eggs than in 'True wild' eggs. More significant differences between origins of eggs were found in the quantitatively minor lipid classes phosphatidylinositol and phosphatidylglycerol/cardio-lipin (P<0.01), 'True wild' eggs had the highest fraction of these classes and 'Farmed' eggs the lowest.

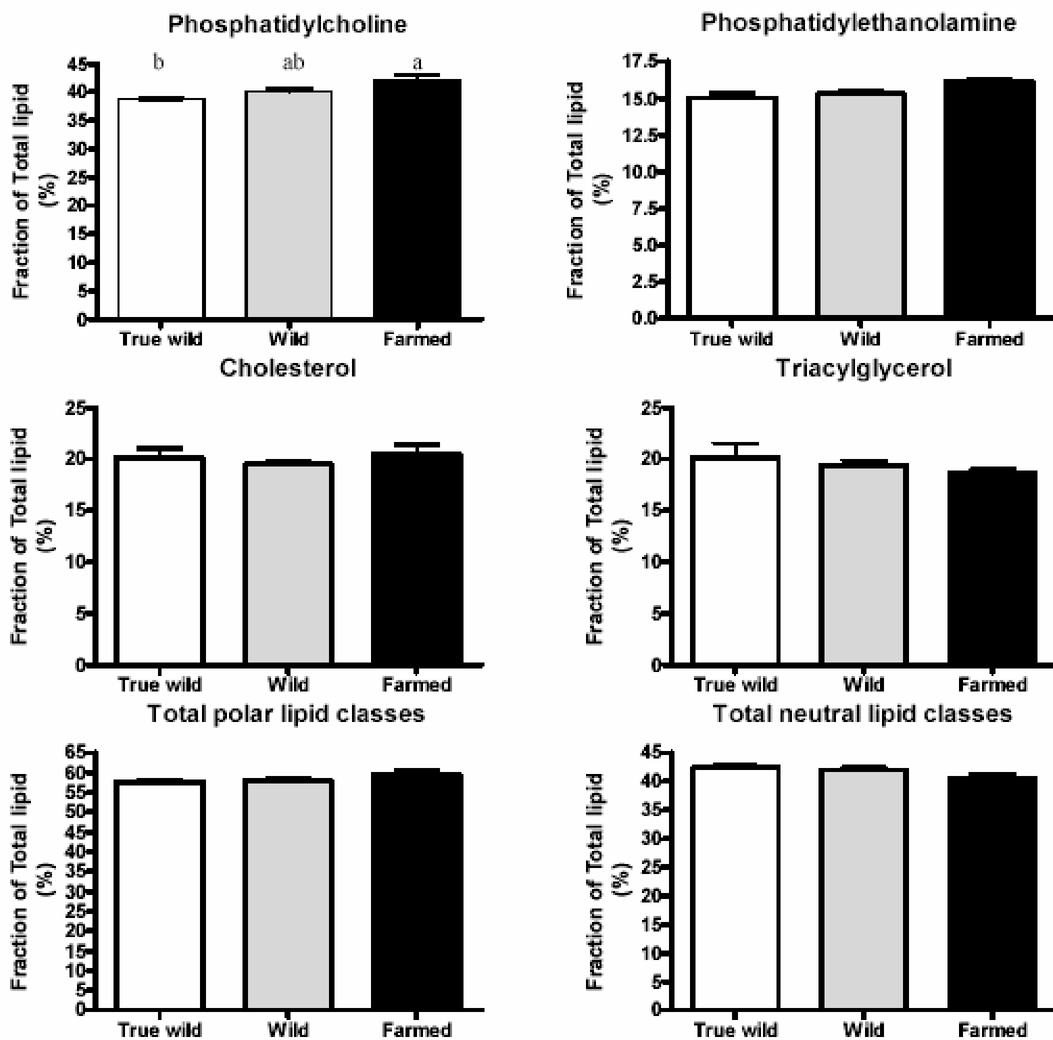


Figure 2: Lipid class analysis results in Day 0 eggs – Quantitatively major lipid classes and totals. Note: values with different superscript letters are significantly different, [a] shows the highest level.

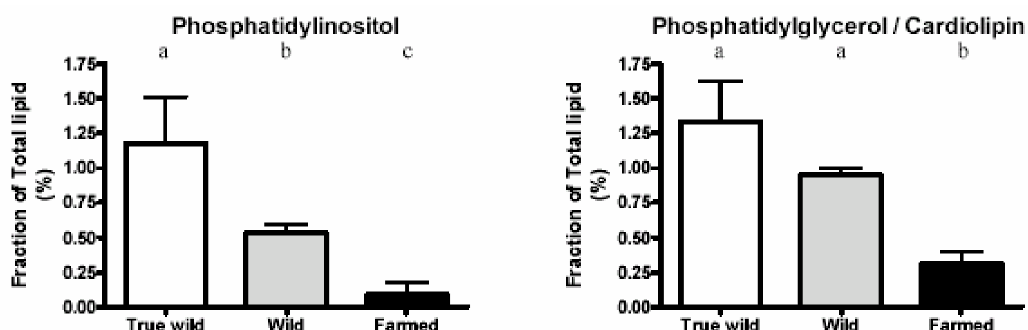


Figure 3: Lipid class analysis results in Day 0 eggs – Lipid classes of particular interest Note: values with different superscript letters are significantly different, [a] shows the highest level.

*Fatty acid analysis*. Figures 4 to 7 show data on fatty acid composition of eggs on day 0. There were no significant differences in DHA (22:6 n-3) concentrations in eggs from different origins (Figure 7). Significant differences between 'Wild' and 'Farmed' groups were detected in EPA (20:5 n-3) concentrations and DHA/EPA ratio ($P<0.001$) however the 'True wild' group showed similar levels to the 'Farmed' group. Significant differences were detected in arachidonic acid (ARA, 20:4n-6) concentrations which were present at higher concentrations in 'Wild' and 'True wild' eggs than in

'Farmed' eggs ($P<0.001$). Furthermore, although 'True wild' and 'Wild' eggs were not different, a clear decreasing trend was observed between the three origins of eggs. Another noticeable ratio is the EPA/ARA ratio. The results show that 'Farmed' eggs have a very significantly higher EPA/ARA ratio than 'Wild' and 'True wild', which are not significantly different from each other (Figure 4). Trends (either decreasing or increasing) can also be observed in some other fatty acids, eg. 22:4n-6 or 22:1n-11 (Figures 5 and 6).

Among the quantitatively important fatty acids (16:0, 18:1n-9, 20:5n-3 (EPA), and 22:6n-3 (DHA)), only EPA showed some significant differences between the three origins of eggs (Figure 7). However, more differences were found in the fatty acids present in lower amounts in the eggs (Figures 5 and 6). Some significant differences were found in every group of fatty acid (saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids), but most of the differences were found in the n-6 PUFA. The significant differences found in the values for Total n-6 PUFA clearly reflect this, as all other totals showed no significant differences.

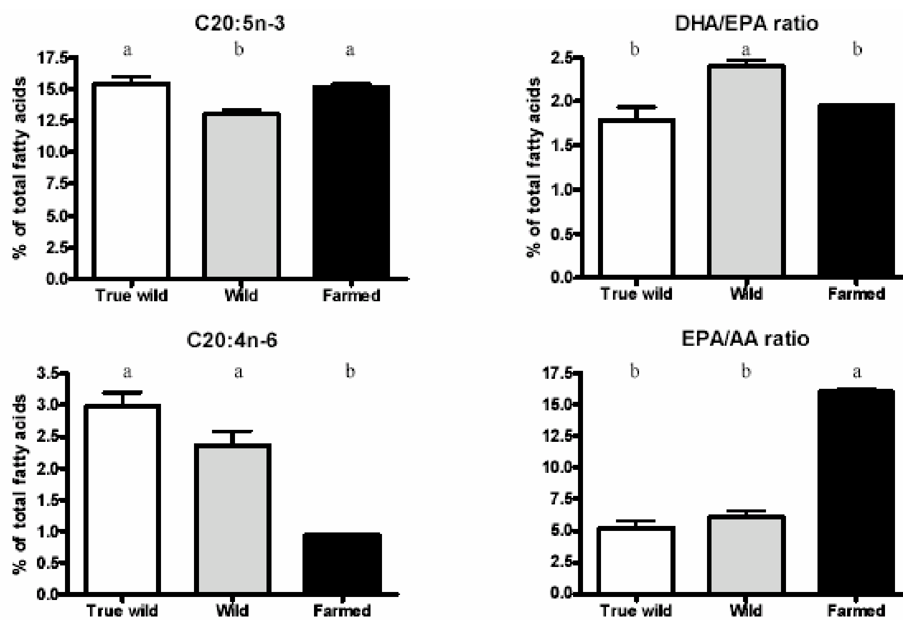


Figure 4: Fatty acid analysis in Day 0 eggs – Results of particular interest. Note: values with different superscript letters are significantly different, [a] shows the highest level.

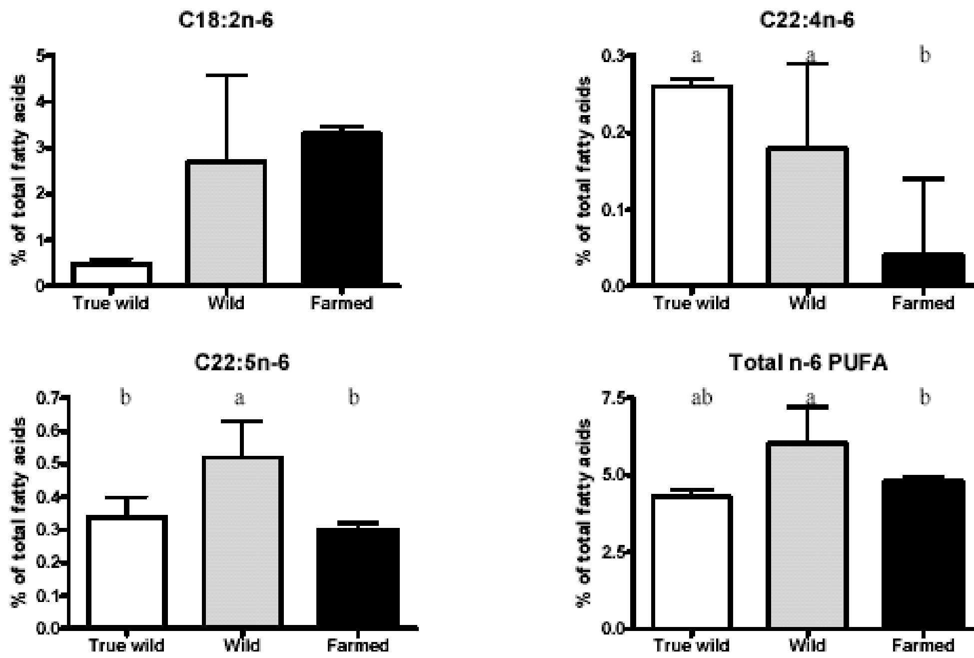


Figure 5. Fatty acid analysis in Day 0 eggs – Some of quantitatively minor fatty acids (a) Note: values with different superscript letters are significantly different, [a] shows the highest level.

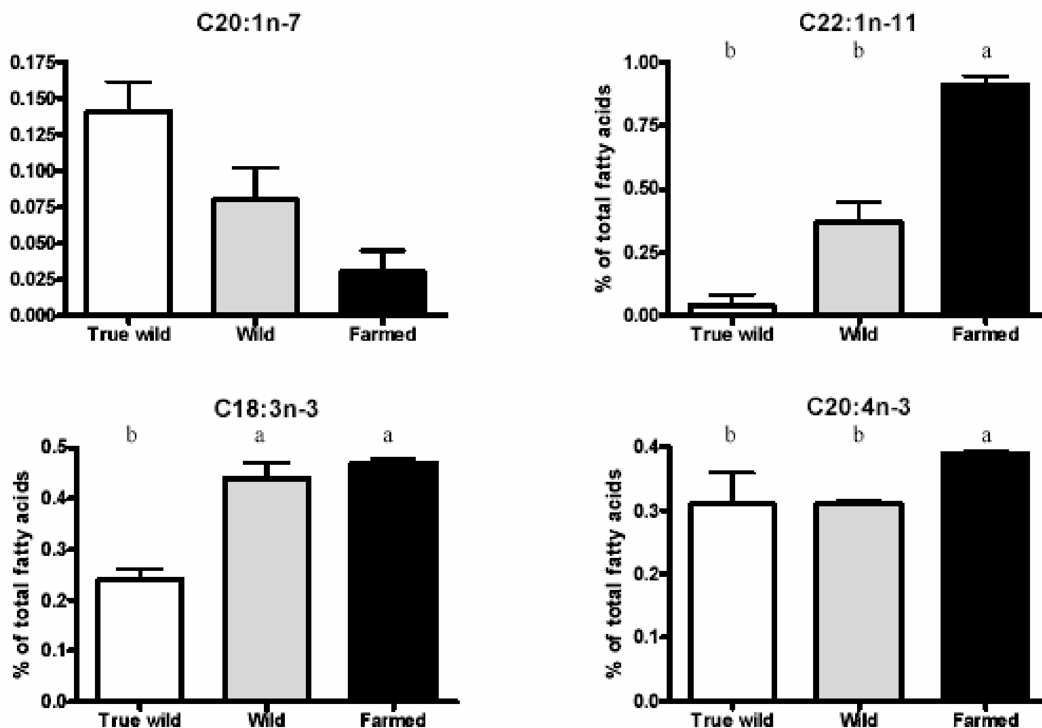


Figure 6: Fatty acid analysis in Day 0 eggs – Some of quantitatively minor fatty acids (b)
Note: values with different superscript letters are significantly different, [a] shows the highest level.

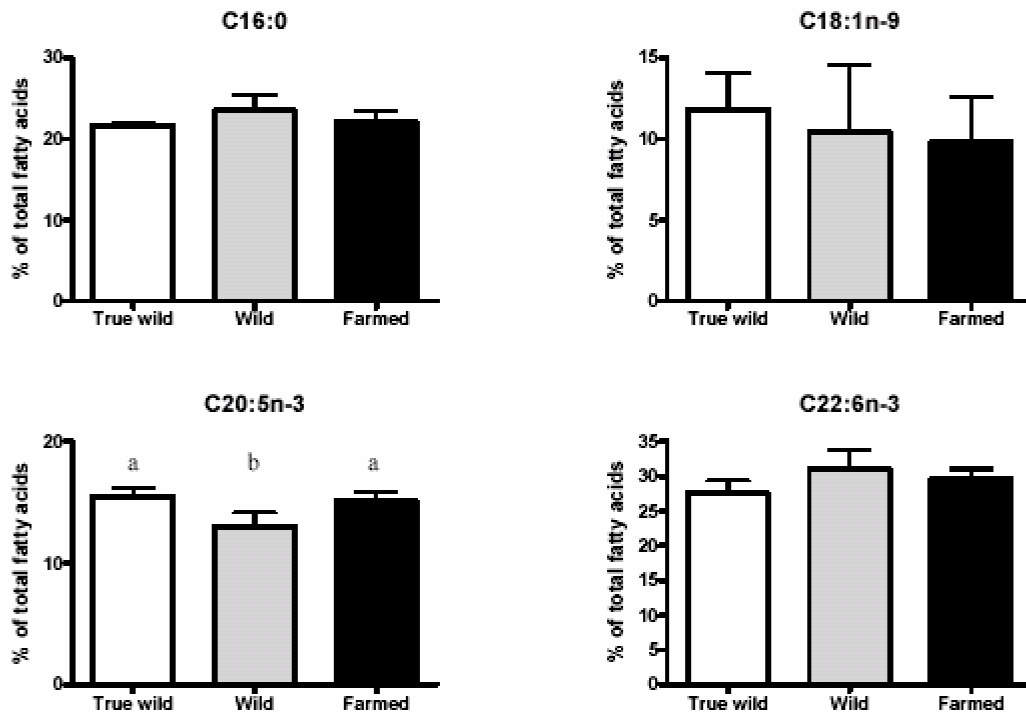


Figure 7: Fatty acid analysis in Day 0 eggs – Quantitatively major fatty acids Note: values with different superscript letters are significantly different, [a] shows the highest level.

3.1.4 Lipid analysis of eggs in incubation

Full details of the results of lipid analysis in incubation are described in Appendix 1. The analysis provided data on the complex relative changes in lipid class and fatty acid profiles during egg development.

3.1.5 Pigment content

Table 4 and Figure 8 present results of pigment analysis. Data from the two 'True wild' batches were significantly different (Figure 8). Significant differences were found in the total pigment content: 'Farmed' eggs contained significantly less pigment than 'Wild' eggs. Astaxanthin showed a similar pattern: the 'Wild' eggs contained more astaxanthin than the 'Farmed' eggs. Canthaxanthin was found in 'Wild' eggs only.

Table 4: Egg pigment content (µg/mg of total lipid)

| | Wild | Farmed |
|---|---|---|
| *Astaxanthin* | 0,033±0,0064 | 0,009±0,0012 |
| *Canthaxanthin* | 0,006±0,0042 | 0,000±0,0002 |
| *Total pigment* | 0,039±0,0073 a | 0,013±0,0010 b |

Note: values with different superscript are significantly different, [a] shows the highest level.

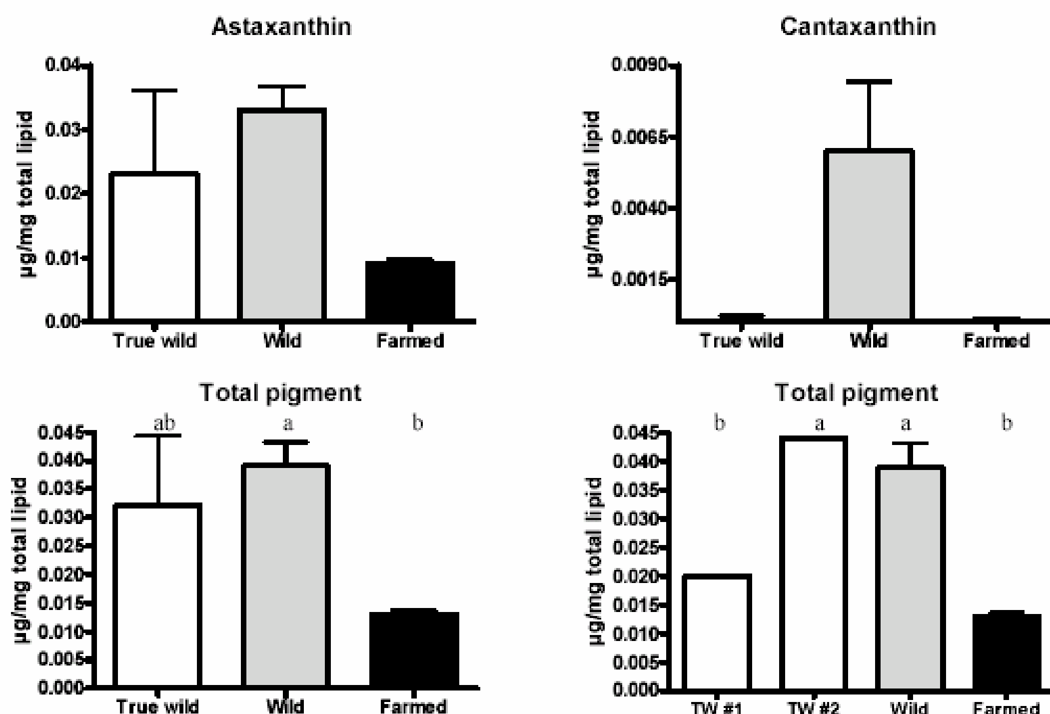


Figure 8: Egg pigment content. Values with different superscript letters are significantly different, [a] shows the highest level.

3.2. Study 2

3.2.1 Larval performance data

*Standard length*. There were no differences in standard length between the four treatment groups at 51.4 and 95.7 degree days. Larvae fed with rotifers enriched with 'SV12' were significantly longer than the ones fed with 'IoA enrichment' at 143.5 DD, and at 188.4, 244.9 and 285.6 DD, groups fed with 'SV12' and 'MBlowARA' were longer than than those reared using 'IoA enrichment' (Table 5 and Figure 9).

Table 5: Mean standard length of larvae ± SD for the four different treatments at 6 time points over feeding period. Significant differences ($P<0.05$) were identified using LSD's Post Test. Values with different superscript letters are significantly different, [a] shows the highest level.

| DD | 51.4 | | 95.7 | | 143.5 | | 188.4 | | 244.9 | | 285.6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MBlowARA* | 4.73 | ± 0.06 | 5.03 | ± 0.15 | 5.83 | ± 0.35ab | 6.83 | ± 0.06a | 7.60 | ± 0.26a | 7.90 | ± 0.53a |
| *IoA enrich* | 4.67 | ± 0.06 | 4.97 | ± 0.06 | 5.47 | ± 0.12b | 6.13 | ± 0.40b | 6.80 | ± 0.20b | 7.10 | ± 0.20b |
| *SV12* | 4.70 | ± 0.20 | 5.17 | ± 0.15 | 5.93 | ± 0.12a | 6.70 | ± 0.35a | 7.47 | ± 0.23a | 7.97 | ± 0.38a |
| *DHA PS* | 4.63 | ± 0.06 | 5.00 | ± 0.10 | 5.77 | ± 0.12ab | 6.47 | ± 0.21ab | 7.27 | ± 0.35ab | 7.73 | ± 0.50ab |

*Dry weight* showed a similar pattern to standard length: the larvae were significantly heavier when fed with 'SV12' and 'MBlowARA' than with 'IoA enrichment' at 275.6 DD (Table 6).

*Survival* was significantly higher for larvae fed on rotifers enriched with 'MBlowARA' and 'DHA-PS' than for the ones fed on rotifers enriched with 'SV12' at 123.9 DD (Table 7). Survival rates in different tanks were highly variable and no other treatment differences were identified.

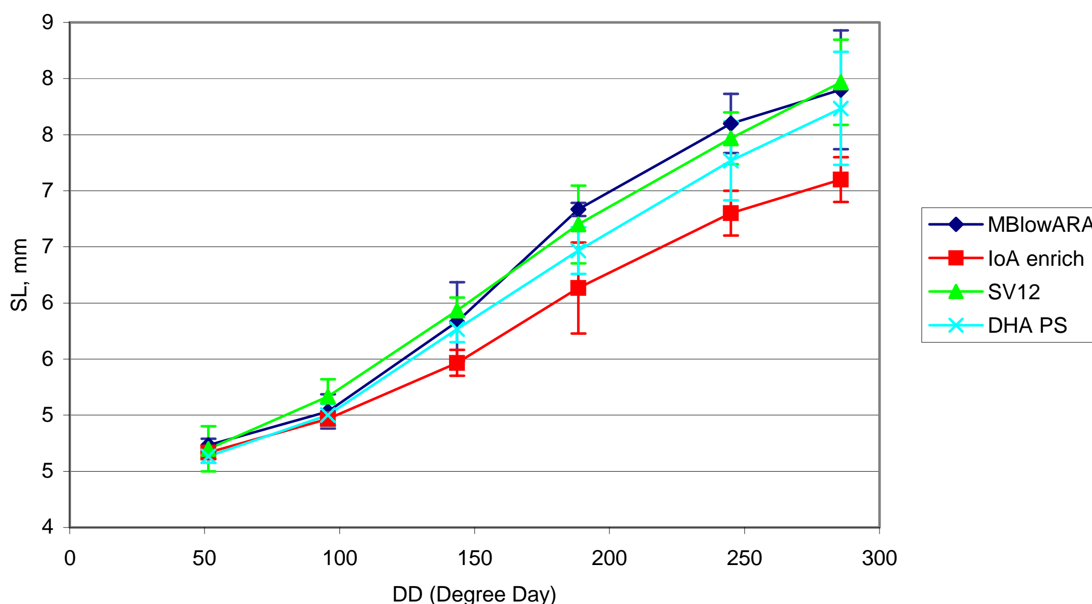


Figure 9: Mean standard length of larvae for the four different treatments at 6 time points over the feeding period

Table 6: Mean dry weight of larvae ± SD for the four different treatments at 4 time points over the feeding period. Significant differences (P<0.05) were identified using LSD's Post Test. Values with different superscript letters are significantly different, [a] shows the highest level.

| DD | 59.1 | | 105.1 | | 206.4 | | 275.6 | |
|---|---|---|---|---|---|---|---|---|
| *MBlowARA* | 0.10 | ± 0.10 | 0.70 | ± 0.96 | 3.03 | ± 0.29 | 6.10 | ± 1.15a |
| *IoA enrich* | 0.00 | ± 0.00 | 0.00 | ± 0.00 | 2.13 | ± 0.57 | 4.00 | ± 0.44b |
| *SV12* | 0.00 | ± 0.00 | 0.27 | ± 0.21 | 2.87 | ± 1.11 | 6.07 | ± 0.75a |
| *DHA PS* | 0.03 | ± 0.06 | 0.17 | ± 0.29 | 2.23 | ± 0.31 | 5.10 | ± 0.10ab |

Table 7: Mean survival of larvae ± SD for the four different treatments at 6 time points over the feeding period. Significant differences (P<0.05) were found using LSD's Post Test. Values with different superscript letters are significantly different, [a] shows the highest level.

| DD | 25.5 | | 77.1 | | 123.9 | | 171 | | 215.7 | | 265.1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MBlowARA* | 100 | ± 0 | 63.7 | ± 28.3 | 40.9 | ± 8.5a | 24.5 | ± 3.9 | 16.8 | ± 7.1 | 11.9 | ± 5.6 |
| *IoA enrich* | 100 | ± 0 | 57.9 | ± 15.1 | 34.5 | ± 4.5ab | 14.9 | ± 8.8 | 6.9 | ± 1.9 | 5.1 | ± 1.4 |
| *SV12* | 100 | ± 0 | 32.2 | ± 7.8 | 20.9 | ± 11.8b | 14.2 | ± 8.7 | 11.4 | ± 6.0 | 9.0 | ± 4.7 |
| *DHA PS* | 100 | ± 0 | 65.3 | ± 13.9 | 42.0 | ± 13.5a | 20.5 | ± 4.2 | 13.7 | ± 8.7 | 11.1 | ± 8.3 |

Table 8: Mean percentage of larvae ± SD feeding on the four different treatments at 6 time points over the trial period. Significant differences (P<0.05) were identified using LSD's Post Test. Values with different superscript letters are significantly different, [a] shows the highest level.

| DD | 51.4 | | 95.7 | | 143.5 | | 188.4 | | 244.9 | | 285.6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MBlowARA* | 50.0 | ± 17.3 | 73.3 | ± 15.3a | 96.7 | ± 5.8 | 100 | ± 0 | 100 | ± 0 | 100 | ± 0 |
| *IoA enrich* | 33.3 | ± 23.1 | 63.3 | ± 15.3ab | 93.3 | ± 5.8 | 96.7 | ± 5.8 | 100 | ± 0 | 100 | ± 0 |
| *SV12* | 43.3 | ± 20.8 | 83.3 | ± 5.8a | 100 | ± 0 | 93.3 | ± 11.5 | 100 | ± 0 | 100 | ± 0 |
| *DHA PS* | 60.0 | ± 26.5 | 46.7 | ± 15.3b | 100 | ± 0 | 96.7 | ± 5.8 | 100 | ± 0 | 100 | ± 0 |

*Feeding rate*. The feeding rate was significantly higher for the larvae fed rotifers enriched with 'SV12' and 'MBlowARA' than for the larvae fed 'DHA-PS' enriched rotifers at 95.7 DD. 100% of the larvae were feeding after 244.9 DD in all the four different treatments (Table 8).

*Swim bladder inflation.* There was no significant difference between the treatments. 100% of the larvae had an inflated swim bladder for 'DHA-PS', 'SV12' and 'IoA enrichment' treatments after 285.6 DD.

Table 9: Mean percentage of larvae possessing a swim bladder ± SD in the four different treatments at 6 time points over the trial. Significant differences (P<0.05) were identified using LSD's Post Test.

| DD | 51.4 | | 95.7 | | 143.5 | | 188.4 | | 244.9 | | 285.6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MBlowARA* | 0 | ± 0 | 70.0 | ± 0 | 80.0 | ± 26.5 | 93.3 | ± 5.8 | 96.7 | ± 5.8 | 96.7 | ± 5.8 |
| *IoA enrich* | 0 | ± 0 | 56.7 | ± 5.8 | 70.0 | ± 26.5 | 83.3 | ± 15.3 | 96.7 | ± 5.8 | 100 | ± 0 |
| *SV12* | 0 | ± 0 | 73.3 | ± 5.8 | 90.0 | ± 10.0 | 90.0 | ± 10.0 | 96.7 | ± 5.8 | 100 | ± 0 |
| *DHA PS* | 0 | ± 0 | 56.7 | ± 35.1 | 96.7 | ± 5.8 | 96.7 | ± 5.8 | 100 | ± 0 | 100 | ± 0 |

Table 10: Fatty acid composition (% of total fatty acids) of rotifers enriched for 15 h at 20-25°C. Significant differences (P<0.05) were identified using Tukey's Post Test. Values with different superscript letters are significantly different, $^{a}$ shows the highest level.

| % of total fatty acids | Unenriched rotifers | Rotifers + MBlowARA | Rotifers + IoA enrich | Rotifers + SV12 | Rotifers + DHA-PS | Rotifers + Nanno |
|---|---|---|---|---|---|---|
| 14:0 | 1.78±0.03$^{c}$ | 3.00±0.28$^{a}$ | 2.81±0.47$^{ab}$ | 1.71±0.16$^{c}$ | 2.23±0.22$^{bc}$ | 1.88±0.23$^{c}$ |
| 16:0 | 14.33±1.93$^{b}$ | 24.95±5.62$^{a}$ | 16.53±2.76$^{b}$ | 15.74±0.52$^{b}$ | 19.22±2.16$^{ab}$ | 17.18±0.71$^{b}$ |
| 18:0 | 3.91±0.29$^{ab}$ | 2.92±0.47$^{b}$ | 3.53±0.61$^{ab}$ | 3.86±0.16$^{ab}$ | 4.75±1.04$^{a}$ | 3.56±0.30$^{ab}$ |
| Total saturated | 21.06±2.46$^{b}$ | 31.55±6.54$^{a}$ | 23.94±4.19$^{ab}$ | 22.16±0.44$^{ab}$ | 27.82±4.19$^{ab}$ | 23.47±0.89$^{ab}$ |
| 16:1n-7 | 10.23±0.31$^{a}$ | 3.72±0.16$^{d}$ | 6.06±0.66$^{c}$ | 5.69±0.14$^{c}$ | 8.56±1.12$^{b}$ | 6.03±0.47$^{c}$ |
| 18:1n-9 | 27.37±1.92$^{a}$ | 11.37±0.34$^{d}$ | 17.36±1.58$^{c}$ | 16.57±0.26$^{c}$ | 23.05±2.58$^{b}$ | 16.41±0.40$^{c}$ |
| 18:1n-7 | 3.87±0.16$^{a}$ | 2.19±0.07$^{c}$ | 4.16±0.34$^{a}$ | 3.08±0.03$^{b}$ | 4.18±0.59$^{a}$ | 3.02±0.07$^{b}$ |
| 20:1n-9 | 4.53±0.40$^{a}$ | 2.15±0.07$^{c}$ | 4.06±0.56$^{ab}$ | 3.66±0.06$^{b}$ | 4.53±0.32$^{a}$ | 3.55±0.18$^{b}$ |
| Total monounsat. | 52.06±3.21$^{a}$ | 22.52±0.38$^{c}$ | 38.90±4.37$^{b}$ | 34.89±0.03$^{b}$ | 47.02±4.94$^{a}$ | 34.73±0.84$^{b}$ |
| 18:2n-6 (linoleic acid) | 9.55±0.75$^{b}$ | 6.54±0.15$^{b}$ | 7.83±1.05$^{b}$ | 14.33±0.52$^{a}$ | 8.14±0.92$^{b}$ | 16.12±1.19$^{a}$ |
| 20:2n-6 | 0.49±0.08$^{b}$ | 0.37±0.03$^{b}$ | 0.43±0.00$^{b}$ | 0.97±0.10$^{a}$ | 0.47±0.02$^{b}$ | 0.85±0.07$^{a}$ |
| 20:3n-6 | 0.23±0.05$^{b}$ | 0.41±0.05$^{a}$ | 0.15±0.01$^{b}$ | 0.35±0.02$^{a}$ | 0.20±0.03$^{b}$ | 0.36±0.04$^{a}$ |
| 20:4n-6 (ARA) | 0.60±0.13$^{b}$ | 2.45±0.50$^{a}$ | 0.94±0.15$^{b}$ | 0.98±0.03$^{b}$ | 0.60±0.11$^{b}$ | 0.61±0.04$^{b}$ |
| Total n-6 PUFA | 11.00±1.11$^{b}$ | 14.46±3.14$^{a}$ | 9.99±1.42$^{b}$ | 17.41±0.56$^{a}$ | 9.71±1.21$^{b}$ | 17.98±1.34$^{a}$ |
| 18:3n-3 (linolenic acid) | 1.59±0.18$^{c}$ | 0.99±0.08$^{c}$ | 1.60±0.21$^{c}$ | 6.24±0.31$^{b}$ | 1.33±0.20$^{c}$ | 10.49±0.98$^{a}$ |
| 18:4n-3 | 0.44±0.11$^{b}$ | 0.41±0.11$^{b}$ | 1.38±0.14$^{a}$ | 0.32±0.11$^{b}$ | 0.51±0.23$^{b}$ | 0.35±0.13$^{b}$ |
| 20:4n-3 | 1.36±0.35$^{ab}$ | 1.26±0.24$^{ab}$ | 1.02±0.24$^{b}$ | 1.63±0.06$^{a}$ | 1.01±0.23$^{b}$ | 1.76±0.14$^{a}$ |
| 20:5n-3 (EPA) | 4.40±1.43$^{b}$ | 4.35±0.91$^{b}$ | 8.78±2.13$^{a}$ | 5.31±0.34$^{b}$ | 4.42±1.61$^{b}$ | 3.71±0.44$^{b}$ |
| 22:5n-3 | 3.27±0.88 | 2.47±0.44 | 2.16±0.59 | 3.44±0.04 | 2.72±0.46 | 3.02±0.07 |
| 22:6n-3 (DHA) | 4.67±1.66$^{b}$ | 21.77±7.04$^{a}$ | 12.08±3.91$^{ab}$ | 7.98±0.82$^{b}$ | 5.35±4.86$^{b}$ | 3.79±0.78$^{b}$ |
| Total n-3 PUFA | 15.88±4.56$^{b}$ | 31.47±8.78$^{a}$ | 27.17±7.19$^{ab}$ | 25.55±0.98$^{ab}$ | 15.45±7.65$^{b}$ | 23.82±0.96$^{a}$ |
| Total PUFA | 26.88±5.65$^{bc}$ | 45.92±6.92$^{a}$ | 37.16±8.56$^{abc}$ | 42.95±0.42$^{ab}$ | 25.16±8.81$^{c}$ | 41.80±1.40$^{ab}$ |

3.2.2 Fatty acid content of rotifers

Rotifers enriched with algae (both 'SV12' and *Nannochloropsis* sp. ('Nanno')) had a significantly higher percentage of linoleic acid and linolenic acid than all the other enrichments. Rotifers enriched with 'MBlowARA' had a significantly higher percentage of ARA than all the other treatments, and a significantly higher percentage of DHA than rotifers unenriched and enriched with 'Nanno', 'SV12' and 'DHA-PS'. Rotifers enriched with 'IoA enrichment' had a significantly higher percentage of EPA than all the other treatments. Rotifers enriched with 'Nanno' were not fed to larvae.

The EPA/ARA ratio was significantly lower in rotifers enriched with 'MBlowARA' compared to all of the other treatments and significantly higher in the rotifers enriched with 'IoA enrichment' compared to rotifers enriched with 'Nanno', 'SV12' and 'MBlowARA'. The DHA/EPA ratio was significantly higher in rotifers enriched with 'MBlowARA' compared to all of the other treatments (Table 10).

3.2.3 Fatty acid content of larvae

Larvae fed rotifers enriched with 'SV12' had a significantly higher percentage of linoleic acid and linolenic acid than all the other enrichments. Larvae fed on rotifers enriched with 'MBlowARA' had a significantly higher percentage of ARA than all the other treatments. Larvae fed on rotifers enriched with 'IoA enrichment' had a significantly higher percentage of EPA than all the other treatments, whereas larvae fed on rotifers enriched with 'MBlowARA' had a significantly lower percentage of EPA than all the other treatments. Larvae fed on rotifers enriched with 'MBlowARA' had a significantly higher percentage of DHA than all the other treatments (Table 11 and Figure 10).

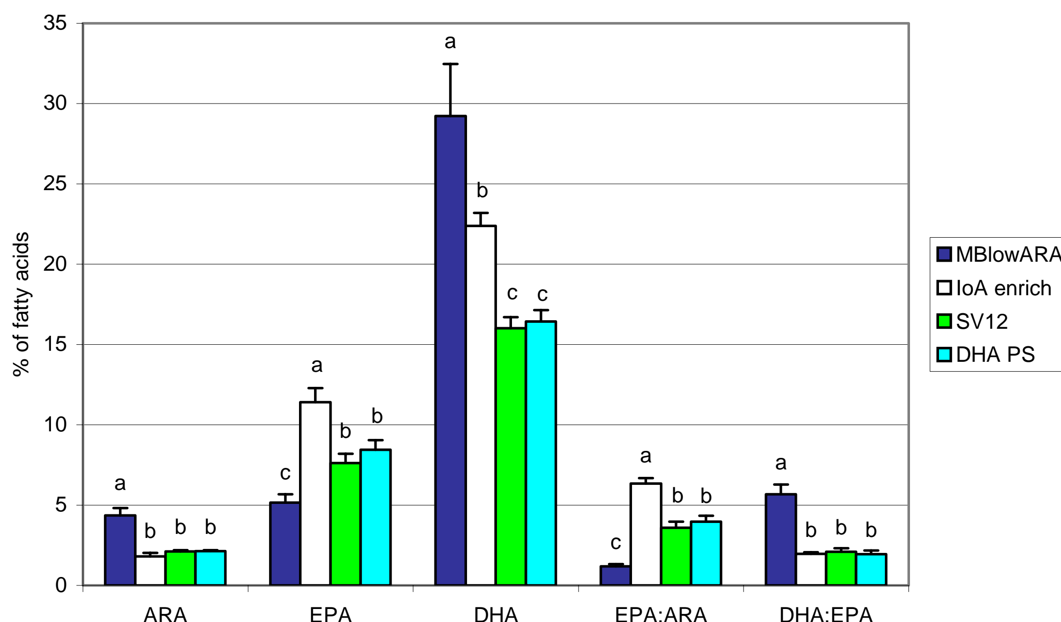


Figure 10: Relative concentration (% total fatty acids) of ARA, EPA and DHA in cod larvae on day 29 (275.6DD) reared using different rotifer enrichments. Values with different superscript letters are significantly different, [a] shows the highest level.

The EPA/ARA ratio was significantly lower in larvae fed on rotifers enriched with 'MBlowARA' and significantly higher in the larvae fed on rotifers enriched with 'IoA enrichment' compared to all of the other treatments. The DHA/EPA ratio was significantly higher in larvae fed on rotifers enriched with 'MBlowARA' compared to all of the other treatments (Table 12).

Changes in major fatty acids during larval rearing are described in Appendix 1.

Table 11: Fatty acid composition (% of total fatty acids) of larvae at the end of the trial (day 29; 275.6 dd). Significant differences (P<0.05) were found using Tukey's Post Test. Values with different superscript letters are significantly different, [a] shows the highest level.

| % of total fatty acids | Larvae + MBlowARA | Larvae + IoA enrich | Larvae + SV12 | Larvae + DHA-PS |
|---|---|---|---|---|
| 14:0 | 1.29 ± 0.05 | 1.34 ± 0.24 | 1.11 ± 0.17 | 1.08 ± 0.21 |
| 16:0 | 18.11 ± 1.32 | 15.61 ± 1.04 | 17.02 ± 0.93 | 16.69 ± 0.75 |
| 18:0 | 6.99 ± 0.46[bc] | 6.23 ± 0.47[c] | 7.39 ± 0.68[ab] | 8.40 ± 0.49[a] |
| Total saturated | 27.14 ± 1.98 | 23.98 ± 1.63 | 26.36 ± 1.98 | 26.85 ± 1.40 |
| | | | | |
| 16:1n-7 | 1.85 ± 0.69[c] | 3.27 ± 0.08[ab] | 2.89 ± 0.09[b] | 4.06 ± 0.27[a] |
| 18:1n-9 | 10.53 ± 0.42[b] | 13.40 ± 0.75[a] | 14.59 ± 1.68[a] | 15.03 ± 0.43[a] |
| 18:1n-7 | 2.66 ± 0.14[c] | 3.66 ± 0.05[a] | 3.34 ± 0.27[b] | 3.64 ± 0.16[a] |
| 20:1n-9 | 1.15 ± 0.76 | 2.16 ± 0.36 | 1.07 ± 1.02 | 2.03 ± 0.06 |
| Total monounsat. | 18.66 ± 2.06[b] | 26.28 ± 2.00[a] | 25.67 ± 0.41[a] | 28.55 ± 0.76[a] |
| | | | | |
| 18:2n-6 (linoleic acid) | 4.93 ± 0.40[c] | 6.96 ± 0.62[b] | 8.99 ± 0.77[a] | 7.15 ± 0.43[b] |
| 20:2n-6 | 0.73 ± 0.04[b] | 0.73 ± 0.03[b] | 1.10 ± 0.10[a] | 0.87 ± 0.04[b] |
| 20:3n-6 | 0.44 ± 0.03[a] | 0.17 ± 0.02[b] | 0.34 ± 0.09[a] | 0.38 ± 0.09[a] |
| 20:4n-6 (ARA) | 4.37 ± 0.45[a] | 1.80 ± 0.22[b] | 2.14 ± 0.20[b] | 2.14 ± 0.07[b] |
| Total n-6 PUFA | 15.42 ± 2.84[a] | 10.18 ± 0.85[b] | 13.89 ± 1.18[ab] | 11.66 ± 0.41[ab] |
| | | | | |
| 18:3n-3 (linolenic acid) | 0.90 ± 0.20[b] | 0.84 ± 0.57[b] | 3.28 ± 0.39[a] | 1.60 ± 0.21[b] |
| 18:4n-3 | 0.23 ± 0.02[b] | 0.67 ± 0.03[a] | 0.29 ± 0.11[b] | 0.25 ± 0.01[b] |
| 20:4n-3 | 0.87 ± 0.09[c] | 0.97 ± 0.09[bc] | 1.15 ± 0.09[ab] | 1.29 ± 0.09[a] |
| 20:5n-3 (EPA) | 5.17 ± 0.50[c] | 11.40 ± 0.88[a] | 7.65 ± 0.07[b] | 8.46 ± 0.58[b] |
| 22:5n-3 | 2.20 ± 0.27[c] | 3.24 ± 0.34[b] | 3.84 ± 0.43[ab] | 4.79 ± 0.21[a] |
| 22:6n-3 (DHA) | 29.23 ± 3.23[a] | 22.39 ± 0.81[b] | 17.37 ± 2.20[c] | 16.42 ± 0.71[c] |
| Total n-3 PUFA | 38.78 ± 4.01[ab] | 39.55 ± 2.17[a] | 34.08 ± 1.92[b] | 32.94 ± 0.42[b] |
| | | | | |
| Total PUFA | 54.20 ± 2.27[a] | 49.74 ± 2.93[ab] | 47.97 ± 2.18[b] | 44.60 ± 0.82[b] |

Table 12: Summary of approximated values for larval EPA/ARA and DHA/EPA ratios in the 4 treatments

| | MBlowARA | IoA enrich | SV12 | DHA-PS |
|---|---|---|---|---|
| *EPA/ARA* | 1:1 | 6:1 | 4:1 | 4:1 |
| *DHA/EPA* | 6:1 | 2:1 | 2:1 | 2:1 |

## 4. Discussion

4.1 Study 1: The influence of cod egg lipid composition on egg quality

Study 1 demonstrated differences in egg quality between eggs of wild and farmed origin. Such differences have been observed in commercial broodstock in UK and have resulted in a high volume

of cod eggs used being supplied from wild-caught fish. The underlying causes of poor egg quality in farm reared cod broodstock are unknown. This problem is not unique to cod, and a wide range of physiological, nutritional, environmental and social factors have been investigated in a variety of species (reviewed by Bromage 1988, Watanabe 1985 ).

The present study investigated the role lipid composition (total lipid content, lipid class composition, fatty acid composition and carotenoid pigment content) in relation to egg quality in cod. There was no difference in total lipid content of eggs and only minor differences in the major lipid classes and most fatty acids. Higher concentrations of carotenoid pigments found in 'Wild' eggs were as previously reported (Grung et al. 1993; Pavlov et al. 2004) and the value of these pigments in cod broodstock feed requires further investigation.

Interesting differences were observed in the phospholipids, phosphatidylinositol and phosphatidylglycerol/cardiolipin, which were higher in eggs from wild fish and lower in eggs from farmed fish. In fish, phosphatidylinositol is rich in ARA and is considered to be an important source of the ARA precursor of eicosanoids (Sargent *et al.* 1993). Fatty acid analysis showed that the ARA content of eggs from wild fish was higher and the EPA content lower than eggs from farmed broodstock. The EPA/ARA ratio is considered to be important in relation to eicosanoid activity (see section 1.1). The results of this study suggest a possible link between low levels of ARA and/or high EPA/ARA ratios and poor egg quality in eggs from farmed fish. As a result of this study, fatty acid analysis of commercial egg samples has become routine as we collect further data to evaluate the significance of ARA concentrations and EPA/ARA ratios in relation to cod egg quality.

In sea bass, use of trash fish diets containing relatively high levels of ARA resulted in lower EPA/ARA ratios in eggs and also in improved egg quality when compared with fish reared on a formulated diet (Thrush *et al.* 1993). Experimental use of tuna orbital oil in formulated broodstock diets improved the EPA/ARA ratio in sea bass eggs from 6.1 to 2.2 , compared with a ratio of 1.5 in high quality eggs from broodstock reared using trash fish (Bruce *et al.* 1999; Sargent *et al.* 1999). This demonstrates that with the use of speciality oils in broodstock feeds it is possible to manipulate the fatty acid profile of eggs to improve egg quality. The practical value of such manipulations on egg production and egg quality still remains to be demonstrated and experimental studies are planned to address this question in relation to cod.

4.2 Study 2: The role of lipid composition on nutritional value of live feeds for cod larvae

The problems associated with larval rearing of cod were demonstrated in Study 2. Mean survival from day 2 to day 29 was approximately 9% and there was considerable variation between tank replicates. Larval growth was slow and also rather variable, but some clear differences were identified. Fatty acid profiles of larvae reflected the fatty acid profiles of rotifers reared on the different enrichment products. Larvae reared using rotifers fed 'MBlowARA' showed relatively high levels of DHA and ARA, whilst those reared on rotifers fed 'IoA enrichment' showed high levels of EPA. DHA is considered essential for normal development in many species and must be supplied in the diet (Mourente *et al.* 1991; Sargent *et al.* 1999; Estevez *et al.* 1999). In previous studies with cod larvae, low DHA levels were correlated with poor growth during the initial rotifer feeding phase (Kjorsvik *et al*. 2004). Other studies in marine fish larvae have shown that the optimum dietary ratio of DHA/EPA in sea bass larvae is around 2 and the optimum dietary ratio of EPA/ARA is around 1. The optimal dietary ratio of DHA/EPA in turbot and halibut larvae is similarly around 2 but the optimum dietary ratio of EPA/ARA to avoid malpigmentation in these species is 4 or greater (Sargent *et al.* 1999). Further studies are necessary to determine optimum DHA/EPA and EPA/ARA ratios for cod larvae.

In terms of survival and growth, the 'IoA enrichment' performed relatively poorly in comparison with other enrichments. This suggests that the EPA/ARA in rotifers enriched with 'IoA enrichment' was probably too high. Larvae reared using 'MBlowARA' performed significantly better than those on 'IoA enrichment', but there were few differences between 'MBlowARA', 'DHA-PS' and 'SV12' making it difficult to draw conclusions in terms of larval performance. On the basis that high

DHA/EPA and low EPA/ARA ratios are nutritionally desirable for cod larvae, the 'MBlowARA' enrichment was most suitable but larvae reared using 'DHA-PS' and 'SV12' also performed adequately. No cost comparison of the enrichment products was carried out but this will probably be an important consideration for commercial farms.

4.3 Benefits of the project

- Reliable data on lipid profiles in cod eggs and larvae and changes during egg and larval development.
- Development of fatty acid analysis as a tool to measure egg quality
- Identification of significant fatty acids for further studies on supplementation of cod broodstock feeds.
- Comparative data on lipid profiles and performance of commercial enrichment products
- Further data on fatty acid requirements of cod larvae

**Acknowlegements**

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**APPENDIX TO FINAL REPORT Lipids, egg and Larval quality in Cod.**

A1. Study 1: Changes in lipid profiles during egg development

*Lipid class analysis*. Figures A1 and A2 show the changes in lipid classes during egg incubation. Two-way ANOVA revealed that there were significant differences between incubation stage (start, end, and larvae), but there were few significant differences in major lipid classes between batches (Figure A1).

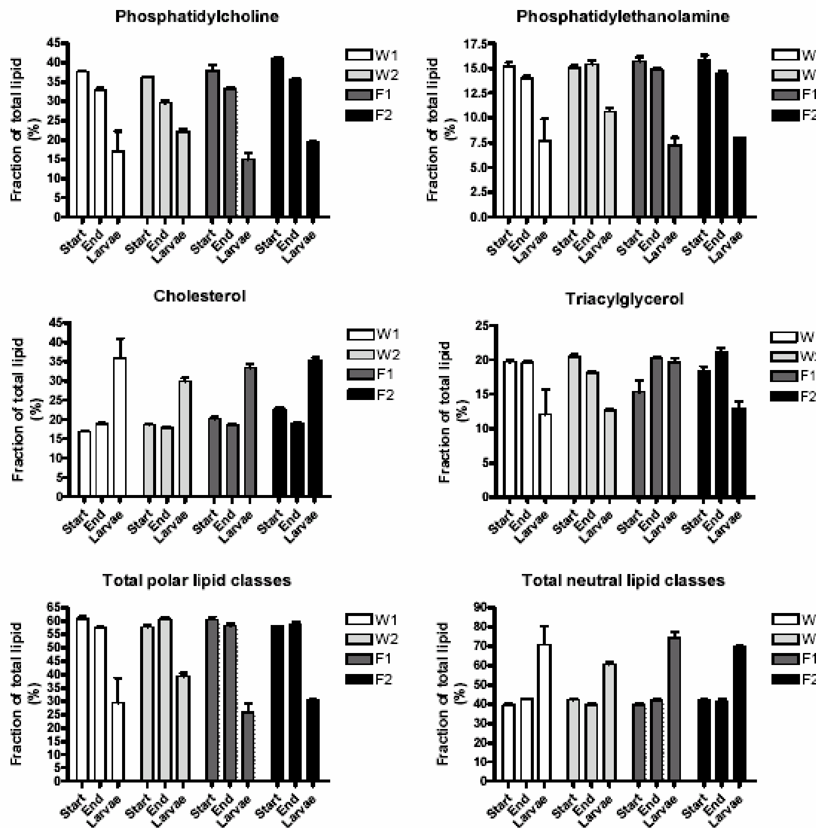


Figure A1: Lipid class analysis in Incubated eggs – Quantitatively major lipid classes

More significant differences were found in quantitatively minor lipid classes such as phosphatidylserine, phosphatidylinositol, phosphatidylglycerol/cardiolipin and sphingomyelin. For these lipid classes, differences between egg origins were found at the end and at the beginning of the incubation (Figure A2).

*Fatty acid analysis*. As with lipid class, many differences in fatty acid profiles were found in the larvae at the end of incubation stage (Figure A3). However, with the fatty acid composition, a substantial number of differences were also found at the beginning of incubation. Moreover, for some fatty acids such as 18:1n-9, 18:2n-6, or 22:4n-6, the degree of difference actually decreased with incubation.

A similar pattern to that for the major lipid classes is observed for the quantitatively major fatty acids: only a few significant differences were revealed, and these were all at the larval stage. This is with the exception of 18:1n-9, which showed very significant differences between batches at the beginning of the incubation. The first batch of ‘Wild’ eggs had more 18:1n-9 than the two ‘Farmed’ batches.

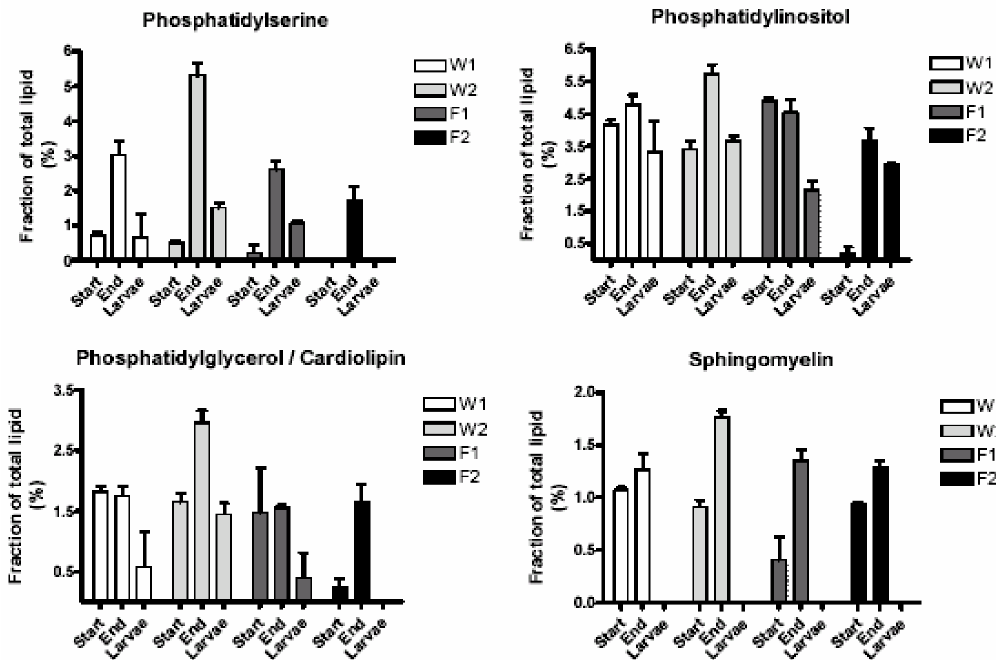


Figure A2: Lipid class analysis in Incubated eggs – Quantitatively minor lipid classes

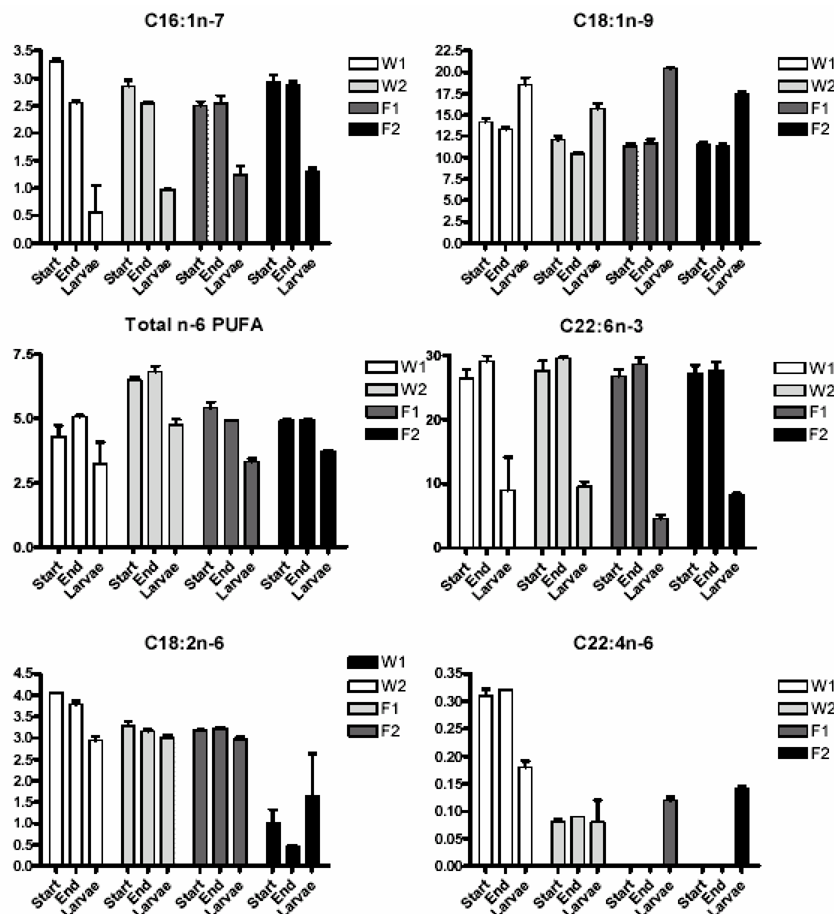


Figure A3: Fatty acid analysis in Incubated eggs

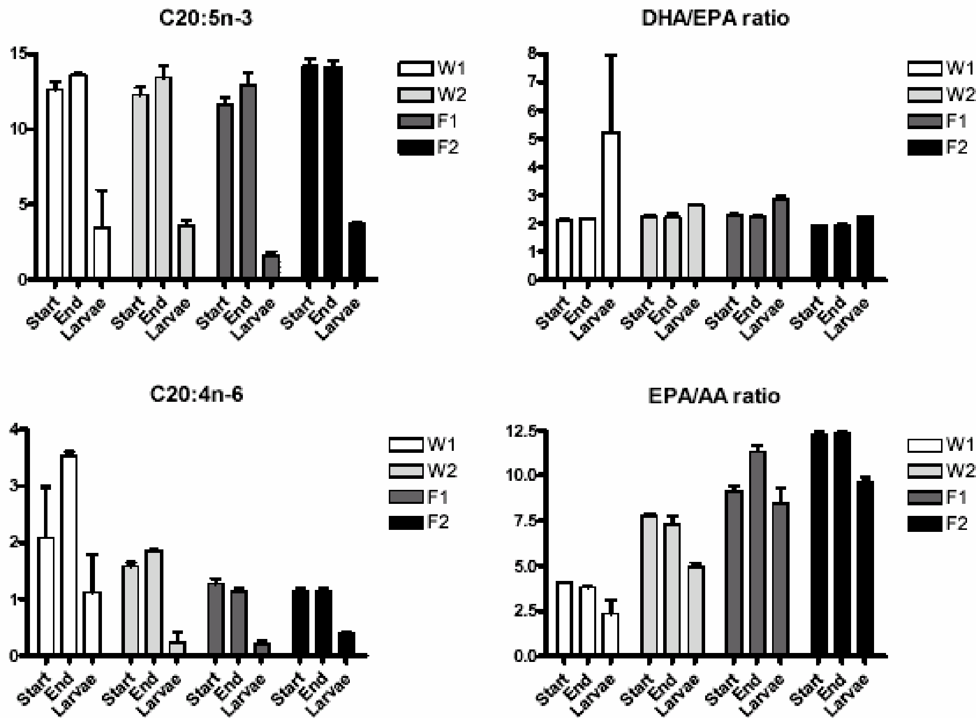


Figure A4: Fatty acid analysis in Incubated eggs – Fatty acids of particular interest

No significant differences between batches were found for either EPA content or DHA/EPA ratio. However, differences were exposed for arachidonic acid and the EPA:ARA ratio The latter is the most noticeable, with all batches being very significantly different from each other (except the two 'Farmed' batches) and with a very clear increasing trend from the 'True wild' to 'Farmed' incubated eggs (Figure A4).

A2: Study 2 Changes in fatty acid profiles during larval development

Larvae reared using 'MBlowARA; showed the same differences in fatty acid profiles at day 11 and at day 22 as observed at day 29 (275.6 DD). However, the ARA, EPA, DHA, EPA:ARA and DHA:EPA ratios remained constant for the larvae fed on rotifers enriched with IoA enrichment until day 11. Differences were observed only from day 22 (206.4 DD) (Figure A5).

**Figure A5: evolution of ARA, EPA, DHA, EPA:ARA and DHA:EPA in larvae at 4 time points over the feeding period, from before first feeding (day 2) until the end of the trial period (day 29).**