Atlantic Cod (Gadus morhua L.) Broodstock Nutrition: The Role Of Arachidonic Acid And Astaxanthin As Determinants Of Egg Quality

THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING
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By

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Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree. Except where specifically acknowledged the work described in this thesis is the result of my own investigations.

Jarin Sawanboonchun

Abstract

Cod hatcheries rely greatly on wild-caught broodstock as egg quality from farm-reared broodstock tends to be poor. Broodstock diet and levels of essential fatty acids have been linked to fecundity and egg quality in cod. Arachidonic acid (ARA) and astaxanthin (Ax) are important nutrients linked to fish egg quality and differences in levels have ben found between eggs from wild and farmed cod. The aim of this thesis was to investigate the impact of dietary supplementation with ARA and Ax on fecundity and egg quality in cod. The first experiment investigated the effect of feeding a diet supplemented with ARA, for 1, 2 or 3 months prior to peak spawning. Results showed that supplementation increased ARA in eggs and that Groups fed the supplement had improved fecundity and egg quality (though with no correlation between the duration of supplementation and number/quality of eggs). The second experiment investigated the effect of supplementation of Ax in broodstock diets on egg quality in farmed cod and showed that Ax was taken up into eggs and that fish fed supplemented diet had improved fecundity and egg quality. The third experiment compared the effect of diet supplementation with ARA and Ax on egg quality in wild and farmed cod and showed that despite the dietary supplementation, wild origin fish performed better on a number of egg quality and fecundity indices. However, it was not possible to take the greater ages and spawning experience of the wild broodstock into account, which may have influenced the results. The fourth experiment measured lipid and fatty acid profiles of eggs from two UK cod hatcheries. Variation was found between farms and across seasons. Results showed that supplementation of cod broodstock diet with ARA and Ax had a positive impact on egg quality and fecundity, although effects were not consistent across all egg quality parameters.

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Abbreviations and Acronyms

ANOVA	Analysis of Variance
ARA	Arachidonic acid (20:4n-6)
ave	Average
Ax	Astaxanthin
BHT	Butylated hydroxy toluene
CHOL	Cholesterol
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
F	Female
FA	Fatty acid(s)
FAME	Fatty acid methyl esters
FFA	Free fatty acids
FID	Flame Ionisation Detection
GC	Gas Chromatography
Kcal	Kilocalorie
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HUFA	Highly unsaturated fatty acid(s)
MDA	Malondialdehyde
M	Male
min	minute
MMERL	Machrihanish Marine Environmental Research Laboratory
MMF	Machrihanish Marine Farm Ltd
No.	Number
OFN	Oxygen- free nitrogen
PC	Phosphatidylcholine
PE	Phosphatidyl ethanolamine
PUFA	Poly unsaturated fatty acid(s)
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
VFF	Viking fish farm

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Chapter 1. General Introduction

1.1 Atlantic cod

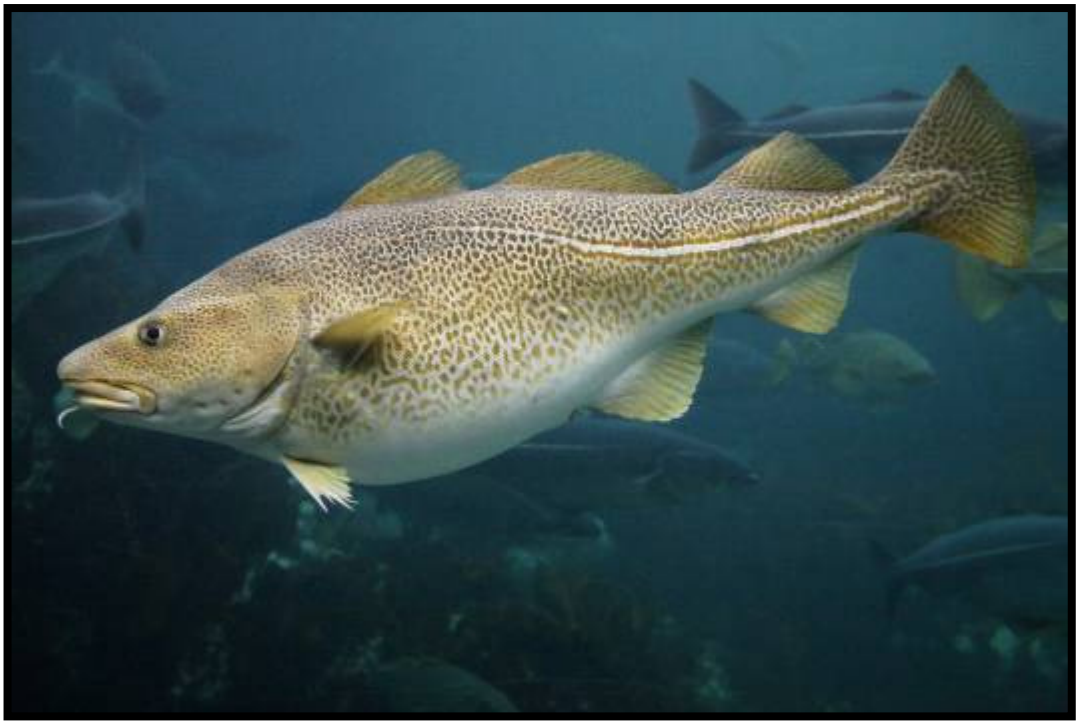

Figure 1.1 Atlantic cod picture by Dieter Craasmann.

1.1.1 Gadoid species

Gadoid species include cod (*Gadus morhua*, Linnaeus, 1758), haddock (*Melanogrammus aeglefinu*s), whiting (*Micromesistius poutassou*), hake (*Merluccius merluccius)* and pollock (*Pollachius virens*). The order Gadiformes contains the family Gadidae to which cod belong. There are 53 species in the family Gadidae. The genus *Gadus* has 3 species (Cohen et al. 1990). Adult fish range in size from small (15 cm) to very large (200 cm) and have a circumpolar to temperate range, mostly in the Northern hemisphere. They are found mainly in demersal or benthopelagic habitats although some can be pelagic. Most are marine but some species can live in low salinity conditions and some even in fresh water (Cohen et al. 1990).

1.1.2 The biology and life cycle of Atlantic cod

The scientific name of Atlantic cod, *Gadus morhua* (Linnaeus, 1758) is derived from the Greek word "gados" meaning fish. The species name "morhua" comes from the Latin word for cod. The Atlantic cod is a cold water marine finfish. Cod have three dorsal fins and a broad tail (Fig 1.1). A special characteristic of cod is a long thin organ protruding from the lower jaw known as a barbel (Fig 1.2) (Bigelow & Schroeder 2002). This organ is thought to be used to detect prey that may be buried in the sand (Bigelow & Schroeder 2002). Their colour can vary from grey to brick red to almost black. They have a well defined lateral line which runs along each side of the fish, this helps detect the presence of prey or predators and to orientate in the water current and avoid obstacles. Cod have separate sexes, males and females, and do not show hermaphroditism (Cohen et al. 1990). Cod can deal with high pressure when in deep waters because it can secrete gases into the swimbladder even when in deep water andat high external pressure. Failure of the swim bladder to develop in larvae leads to slow growth and skeletal deformities (Falk-Petersen 2005).

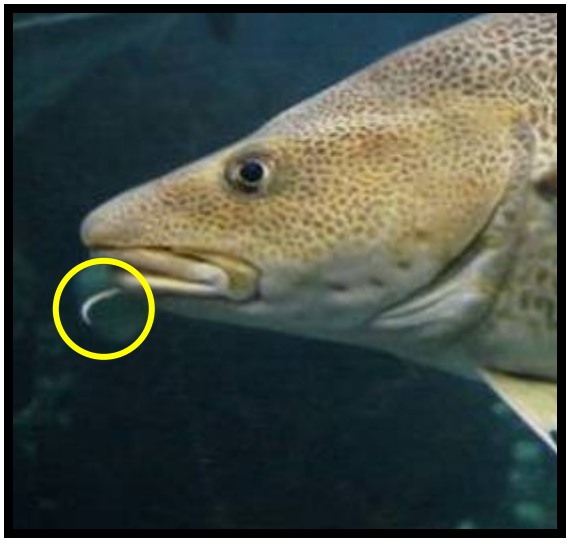

Figure 1.2 Atlantic cod showing the special characteristic, the barbel (circled).

The largest cod reported was 169 cm (Babayan 1978; Tretyak 1984), the age of this fish being about 30 years, according to ageing estimates (Tomlinson & Abramson 1961; Nikolsky 1974; Bromage 1995) .

Atlantic cod mature at between 2 and 10 years (Cohen et al. 1990). At first maturity, wild cod can vary in size between different stocks. For example, the age when fifty percent of the stock is mature was found to be between two and three years for cod from the Irish sea, and between eight and nine years from the south western parts of Greenland, reflecting the different growth rates of these stocks (Garrod 1977; Bromage 1995) However, maturation of farmed cod can occur in two years (Bromage 1995) which is earlier than wild cod from the same location (Braaten 1984; Godø & Moksness 1987; Bromage 1995).

Spawning occurs in deep water off the continental shelf, and inshore, generally during February though in many areas the reproductive season runs from December to June (Cohen et al. 1990). Spawning time varies according to location. For example, those in Norwegian waters spawn from February to May with peak spawning in March to April when temperatures are between 4 and 6 ^{o}C (Solemdal 1982; Pedersen 1984; Sundby & Bratland 1987; Bromage 1995; Pavlov et al. 2004). The development of gonads is affected by temperature which, thus, has an impact on the timing of spawning (Howell et al. 2004). In general, cod can bear temperatures from near freezing to 20^{o}C but usually are found below 10 ^{o}C, and larger fish prefer colder water (Cohen et al. 1990). While wild cod can live in areas of sub-zero to an upper limit of 23-24 ^{o}C, under farm conditions the temperature at which fish have been held will affect their future temperature tolerance (McKenzie 1934; Templeman & Fleming 1965; Bromage 1995).

Spawning occurs in open water where fish congregate (Rose 1993; Morgan et al. 1997). Eggs and sperm can remain viable for over one hour in sea water (Kjorsvik & Lonning 1983; Trippel & Morgan 1994) and the spawning takes place near to the seafloor. One female fish can produce over one million eggs and it is one of the world's most fecund fishes. For example, an average 2 kg female produces approximately 2.5 million eggs. The maximum production recorded was 9 million eggs from a 34 kg fish (Cohen et al. 1990). Generally speaking, the larger females produce more eggs and fertilization of the eggs is external. (Oosthuizen & Daan 1974; Kjesbu 1988). The average size of cod eggs ranges from 1.4 to 1.8 mm (Kjørsvik et al. 2004). In the water, egg density is highest in late winter and early spring but they can be found all year. Eggs hatch in two weeks to one month producing pelagic larvae.

Once the eggs have been fertilized they will float in the currents until they hatch. Development of larval cod is dependent on water temperature. The first stage recognised is the yolk sac stage but as larvae grow they change shape to look more like adult fish. After two to four months these larvae settle and become demersal (Fahay et al. 1999). Eggs and larvae have high mortality and this may be the reason for their very high fecundity. Larval cod are planktivorous and high densities of larvae can be found where there are high densities of plankton. The diet of the juvenile cod includes copepods, amphipods and other small crustaceans in the early life stages, changing to shrimp, spider crabs and squid in later stages. When cod are mature they eat a wide variety of fish and invertebrates and are considered as top predators within much of their range (Fahay et al. 1999).

1.1.3 The commercial importance of cod: exploitation and aquaculture

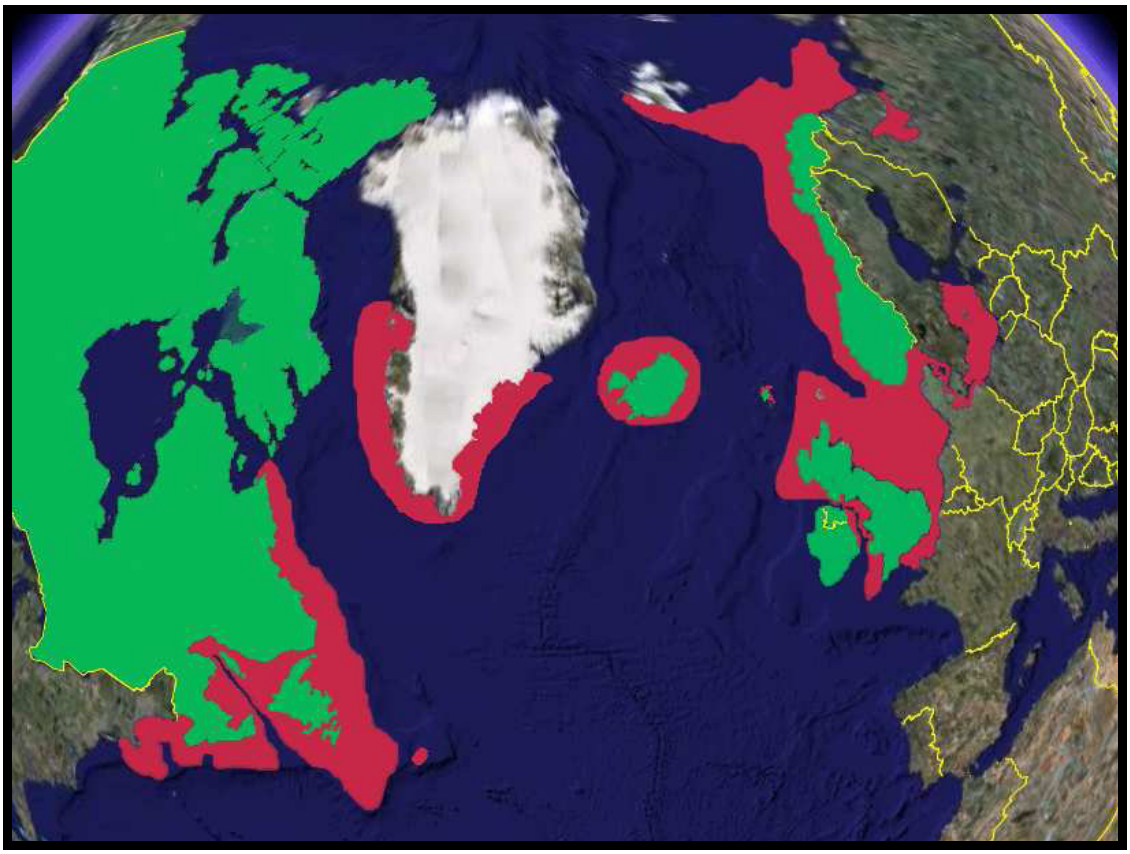

Figure 1.3 Distribution area of *Gadus morhua* wild populations (red) and countries with aquacultural production (green). (From Marteinsdottir et al., 2005).

Atlantic cod are found from the Bay of Biscay and the Baltic Sea to the Barents Sea, around Iceland, along the southern coast of Greenland and from Newfoundland to North Carolina in North America (Fig.1.3).

For many years cod has been a very important food fish in Europe and North America with a high economic value. However, in recent years the catch of cod has declined due to overfishing and environmental changes. In some areas the fisheries have collapsed resulting in closure of these fisheries (Fig.1.4) (FAO 2008). In Canada for example, cod peaked in 1968 at 1,866,000 ton and fell to 482,000 tonnes in 1978 and this fishery has been closed in recent times. The over-fishing has resulted in

changes to the age that cod mature (currently 1.7 to 2.3 years compared to 5.4 to 6.3 years in 1959) with reduced egg production.

Despite this fall in production the demand for cod has remained high with increased market value (Brown et al. 2003). In order to address this demand, aquaculture of cod has become a commercially viable option in recent years. Cod aquaculture has taken place for many years and production has been increasing steadily (Fig.1.5). Production in Scotland has grown from 80 tonnes in 2001 to 543 tonnes in 2006 (FRS 2006). The earliest experiments with raising cod were undertaken in the early 1980s with mass rearing of juveniles (Kvenseth & Øiested 1984).

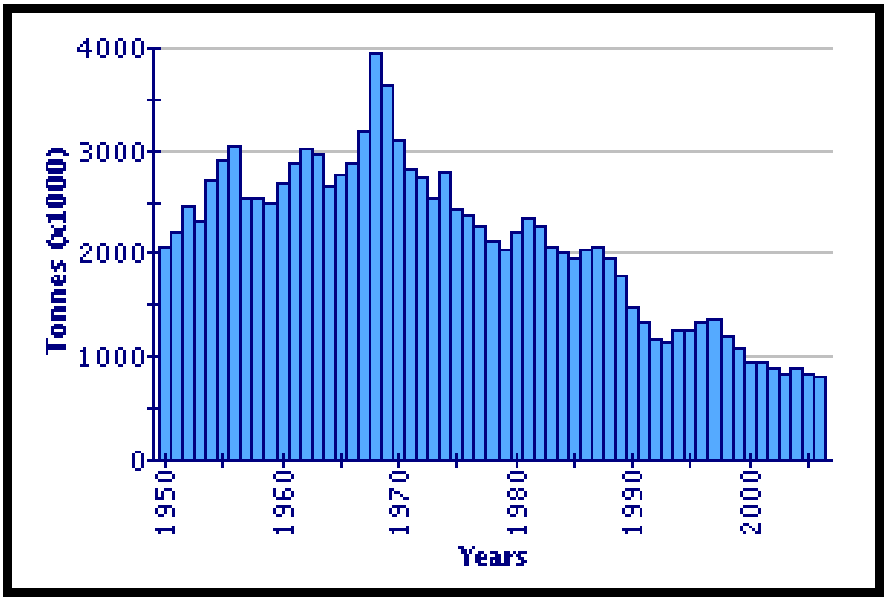

Figure 1.4 Global Capture production for cod from 1950 to 2006 (from FAO Fishery Statistics; FAO 2008).

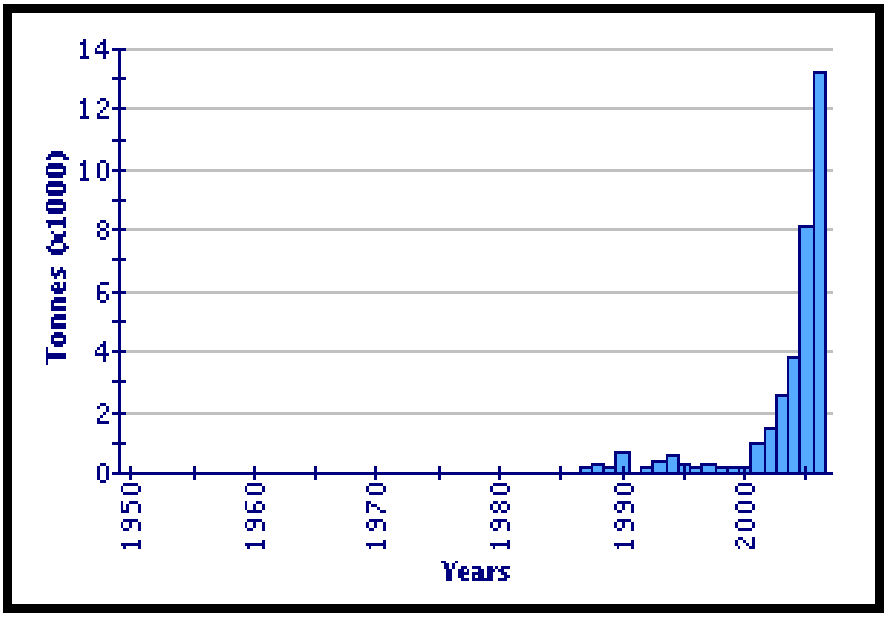

Figure 1.5 Global Aquaculture production for cod from 1950 to 2006 (FAO Fishery Statistic 2008).

Whilst a great deal of progress has been made with grow-out of fish, a major constraint to production remains the survival of eggs and larvae from hatcheries. Because the survival of eggs and larvae remain low there has been considerable focus

on the problems concerning the production of juveniles. Many factors affect survival of eggs and larvae such as the environmental, physical and microbial conditions of egg and larval rearing and nutritional requirements through phases of first feeding and weaning (Kvenseth & Øiested 1984).

1.2 Broodstock and nutrition

Of the factors that affect the quality of eggs produced by farmed fish, broodstock nutrition is one of the most important (Izquierdo et al. 2001). This is because all of the diet is generally provided by artificial feed. Broodstock may need to be reared for many years and if their diet is not optimal then levels of some essential nutrients may become depleted over time (Izquierdo et al. 2001). For example, some studies have shown that in cod, fecundity is affected by feed intake during gonad growth (Kjesbu 1991; Karlsen et al. 1995). However, feeds that are used for ongrowing are not always optimal for broodstock. This has been shown to be true for cod where broodstock need a different formulated feed compared to that needed for growth and survival of production fish (Izquierdo et al. 2001). It has been shown in species such as sea bass (*Dicentrarchus labrax*), sea bream (*Diplodus sargus*), yellowtail (*Seriola quinqueradiata*) and halibut (*Hippoglossus hippoglossus*) that modifying dietary fatty acids and fat-soluble micronutrients can lead to an increased level of these nutrients in the developing eggs with improved egg quality as a result (Watanabe & Miki 1993; Ashton H.J et al. 1993; Verakunpiriya et al. 1997; Czesny & Dabrowski 1998; Gallagher et al. 1998; Sargent et al. 2002).

During spawning, nutrients are taken from body reserves. The composition of the broodstock diet and the duration of feeding are important because in cod egg

development begins between eight to nine months before spawning (Sargent 1995; Bromage 1995). During spawning appetite is reduced, particularly in female fish, and both male and female can lose body weight (10-50%) (Kjesbu et al. 1991; Fordham & Trippel 1999; Lambert & Dutil 2000). It is not clear why female fish lose their appetite at this time but possible reasons include their swollen state and hormone levels (Thorsen et al. 2003). However, other research has shown that cod do continue to eat although appetite is limited during spawning (Kjesbu et al. 1991; Kjørsvik 1994; Karlsen et al. 1995). In practice we see that individual fish stop feeding, or reduce feeding during spawning, but others in the tank which have already spawned, or are still to spawn still need to be fed. Therefore, if cod broodstock do eat during this time it is worth ensuring that feed provided is of the highest quality to maintain health as feeding conditions have a big impact on the egg quality and fecundity of the fish. In gilthead seabream, *Sparus aurata*, it has been shown that feeding diet deficient in n - 3 highly unsaturated fatty acids (n - 3 HUFA) but rich in both oleic (18:1n - 9) and linolenic (18:3n - 3) acids for a short period before and throughout the 2 months during spawning has a positive effect. It has been recommended to feed seabream broodstock for at least 3 months before and during spawning (Almansa et al. 1999).

The main components of the diet are protein, lipids (including polyunsaturated fatty acids (PUFA)), vitamins, carotenoids and trace elements (Kjørsvik 1990; Bromage 1995).The impact of feed quality, including protein quality and digestibility, amino acid content, lipid composition and vitamins, on egg and larval viability and performance has been demonstrated in many species (Watanabe 1985; Kjørsvik et al. 1990). In nature, lipid levels in fish are linked to food abundance with the highest levels found in summer and lowest in winter (Bromage 1995). Spring spawning fish develop gonads over winter and larval hatching is timed to coincide with the highest levels of plankton

on which the newly hatched larvae feed (Lovern & Wood 1937; Bromage 1995). During winter, some lipid will be used to provide energy during periods of low feed supply and some will be used for gonad production (eggs & sperm). Lipids function in two ways during gonadal development, to provide energy and to form vitellogenin (as components of lipovitellin) during oocyte development (Mazorra 2000).

The fatty acids that fish require to be provided in their diet are known as essential fatty acids (EFAs) since they cannot be synthesised by the fish themselves (Gurr & Harwood 1991). Broodstock seem to have different specific requirements for fatty acids and vitamins compared to juvenile fish (Almansa et al. 1999; Bruce et al. 1999; Ishizaki et al. 2001; Dabrowski & Ciereszko 2001; Izquierdo et al. 2001). Many studies have shown that low levels of EFAs affect fecundity, egg quality, hatching success, and larval quality and survival rate (Watanabe 1985; Rainuzzo et al. 1997). The most important EFAs are arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These are essential for normal growth, reproduction and the development of eggs and larvae due to their important role in cell membrane synthesis and function, particularly in neural systems that are vital for early development (Watanabe 1985; Fernández-Palacios et al. 1997; Bell et al. 1997; Navas et al. 1997; Almansa et al. 1999).

1.3 Lipid requirements for marine fish including cod

Lipids can be described as "water-insoluble organic biomolecules that can be extracted from cells and tissues by nonpolar solvents, e.g., chloroform, ether or benzene (Gurr & Harwood 1991). Lipids are classified as either simple or complex. Complex lipids contain fatty acids and so can be saponified and include phospholipids, triacylglycerols, waxes and steryl esters. Simple lipids do not contain fatty acids and cannot be saponified and include for example cholesterol (Jobling 2004). Fatty acids all contain a long hydrocarbon chain and a terminal carboxyl group. They can be either saturated or unsaturated with one or more double bonds, those with more than one double bond are called polyunsaturated fatty acids (PUFA), and those with three or more double bonds and contain 20 or more carbons may be described as highly unsaturated fatty acids (HUFAs) (Michael et al. 1997; Jobling 2004). They are classified by the length of the carbon chain and number and position of double bonds.

The main C_{18} PUFAs of fish are EPA and DHA which are also HUFA. However, these are not generally abundant in the marine environment (Sargent et al. 2002). The main HUFA in fish are eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) of the n-3 series and arachidonic acid (20:4n-6; ARA) of the n-6 series. Palmitic (16:0) acid is one of the most common and major saturated fatty acids found in fish (Huynh & Kitts 2009). The structure of palmitic acid and oleic acid (generally the most common monounsaturated fatty acid) (18:1n-9), ARA, EPA and DHA are shown in Fig 1.6.

In general most marine species have limited ability to synthesise HUFA, such as DHA, EPA and ARA (Tocher et al. 1992; Mourente & Tocher 1993; Sargent et al. 1994; Bromage 1995; Bell 1998; Cunnane 2000) because they lack the enzymes to

convert C_{18} (Fig 1.6) fatty acids to C_{20} and C_{22} HUFA due to the absence or very low activity of Δ5-desaturase (Sargent et al. 2002). As a result of this and their key physiological role EPA, DHA and ARA are considered EFA in marine fish (Castell et al. 1994; Bell et al. 1995; Bell 1998; Furuita et al. 2000; Jobling 2004). In contrast, most fresh water fish can convert C_{18} PUFA, 18:3n-3 and 18:2n-6, to DHA and ARA although they also thrive when given HUFA (Sargent et al. 2002). ARA is a precursor of eicosanoids, a group of oxidised ARA derivatives/metabolites with a range of bioactive functions and is therefore an essential component of diets. The pathways for these conversions are shown in Fig 1.7.

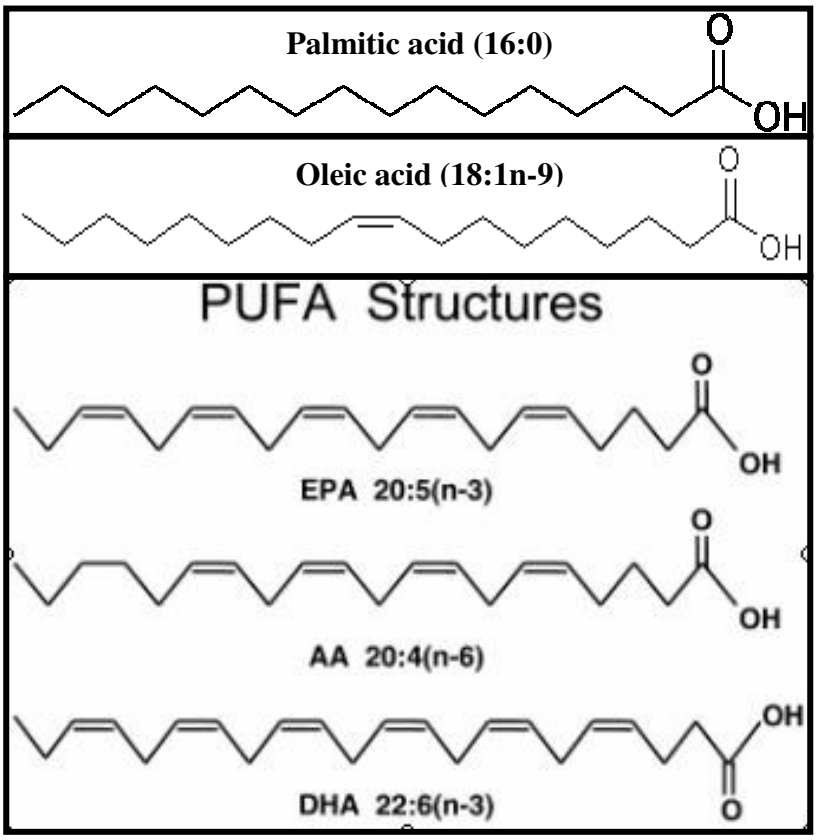

Figure 1.6 The structure of a saturated fatty acid palmitic acid 16:0, the monounsaturated oleic acid (18:1n-9) and the three key polyunsaturated fatty acids essential to marine animal nutrition.

20:5n-3 = eicosapentaenoic acid (EPA); 20:4n-6 = arachidonic acid (ARA); 22:6n-3 = docosahexaenoic acid (DHA).

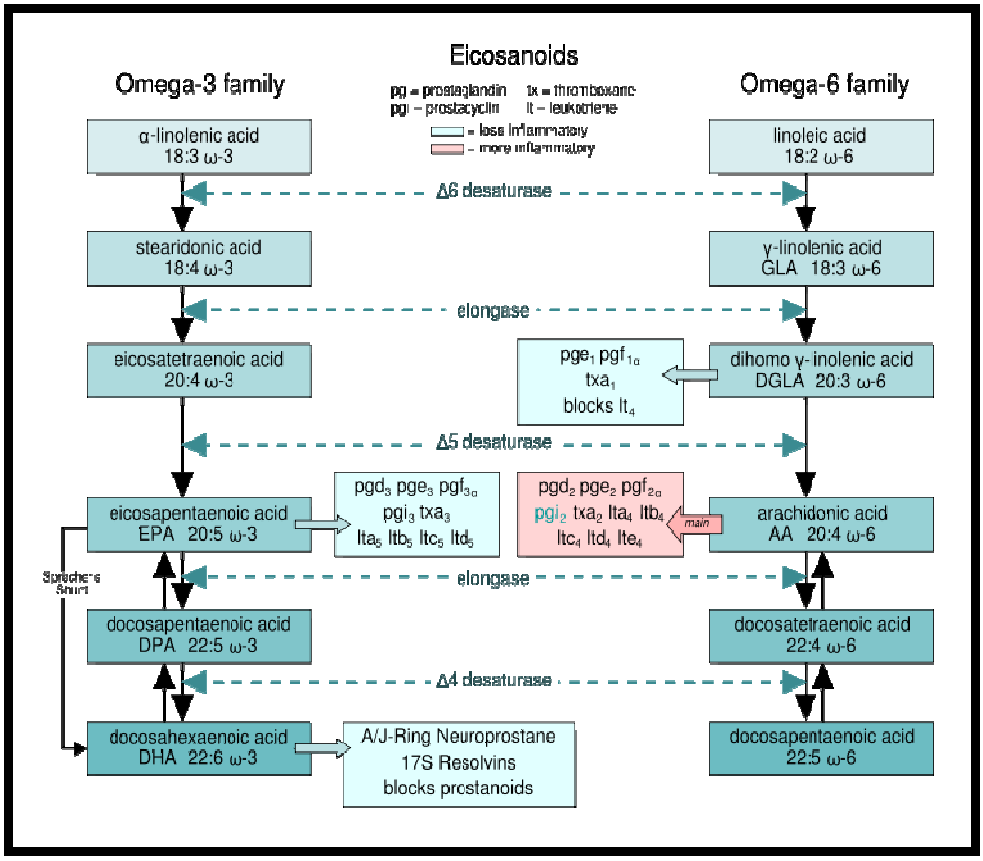

Figure 1.7 Diagram showing pathways in the production and metabolism of essential fatty acids to form eicosanoids.

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Broodstock diets low in HUFA have been shown to result in eggs of poorer quality when compared to those produced by fish receiving complete formulations (Watanabe 1985). Different levels of EFAs have been found to have a significant effect on hatching and fertilization rates, and survival, in sea bream (Fernández-Palacios et al. 1995; Rodriguez et al. 2004), and sea bass (Bruce et al. 1999). In addition to ARA alone, the EPA/ARA ratio has been correlated with differences in egg quality in cod (Salze et al. 2005). Hatching success and other egg quality parameters have been linked to ARA concentration in wild cod (Pickova et al. 1997). Moreover, DHA/EPA ratio and ARA levels have been linked to blastomere morphology, hatching rate (Pickova et al. 1997) and larval survival (Furuita et al. 2003). Broodstock fish therefore have an

absolute requirement for n-3 and n-6 HUFA and probably have an optimal DHA/EPA/ARA ratio which is likely species specific (Sargent et al. 1990; Sargent et al. 1993).

1.4 Lipid use in early development

1.4.1 Lipid composition in cod eggs

The source of fatty acids in fish eggs is primarily from the adipose tissue of the female fish via vitellogenin which enters the egg during gonadogenesis (Bromage 1995). The main types of fatty acid sources in fish eggs are phospholipids, TAG and cholesterol. In general, cod eggs contain around 13% of dry weight as lipid of which 13% is TAG and 72 % is phospholipid. The phospholipid is made up of PUFA (46%), DHA (16%) and EPA (11%) (Tocher & Sargent 1984). The fatty acid composition of cod eggs is shown below (Table 1.1). In cod, as well as most other fish species, phosphatidylcholine (PC) is the major egg lipid class (Fraser et al. 1988). ARA is found in small quantities in marine fish phospholipids with highest levels found in phosphatidylinositol (3% of total amount of cod egg) (Bell & Dick 1991) and is thought to be involved in eicosanoid formation (Bromage 1995).

Table 1.1 Typical lipid and fatty acid composition of Atlantic cod eggs.
(Data assembled from Tocher and Sargent 1984).

Egg diameter (mm)	1.35
Moisture content (%)	74
Lipid content (% dry weight)	13.2
Polar lipid (% total lipid)	71.7
Neutral lipid (% total lipid)	28.3
Phosphatidylcholine (% total lipid)	45.6
Triacylglycerol (% total lipid)	12.5
Cholesterol (% total lipid)	6.1
Fatty acids in polar lipid (% total)	
Saturates	28.1
Monounsaturates	20.3
20:4(n-6)	1.9
20:5(n-3)	15.3
22:6 (n-3)	28.6
Fatty acids in triacylgycerols (% total)	
Saturates	21.3
Monounsaturates	41.5
20:4(n-6)	1.2
20:5(n-3)	10.9
22:6 (n-3)	16.0

The role of lipids as an energy source during egg and larval development is important because they provide most metabolic energy. TAG are used by the embryo for metabolic energy while phospholipids are used mainly for formation of cell membranes in rapidly developing tissues (Bromage 1995). Eggs must contain all the energy required by the developing larvae prior to exogenous feeding (Terner 1979; Boulekbach 1981). It is also very important that eggs contain enough n-3 and n-6 HUFAs in the form of both TAGs and phospholipids to allow normal development and hatching of the larvae. In addition, there must be enough pre-formed phospholipid for the larvae to synthesise cell membranes (Bromage 1995). The level and ratio of the fatty acid composition changes as the embryo develops towards hatching (Bromage 1995).

1.4.2 Lipid functions in fish

Fatty acids have two main functions, the first role is to provide energy for egg and larval development, while phospholipids and HUFA of the n-3 and n-6 series, including ARA, also have an essential role in the formation of cell membranes and tissue (Bromage 1995). ARA is an essential HUFA required by all vertebrates. It is present largely in the phospholipids of cell membranes. DHA, is also required for normal cellular function and is important for neural tissue deveopment especially the brain and retina (Tocher & Harvie 1988; Mourente et al. 1991) and is found in high quantities in larvae. EPA also has a role in cell membranes and modulates formation of eicosanoids from ARA by competing for enzymes that synthesise eicosanoids (Sargent et al. 1993).

As described above, ARA is as a precursor and regulator of eicosanoid production including prostaglandins (PG) of the 2-series such as PGE_2 (Bell et al. 1995; Sargent et al. 1997; Sargent et al. 1999). Through modulation of the cortisol release response, prostaglandins including PGE_2 have a function in the stress response of fish (Koven et al. 2003). In addition, PGs are important in the final maturation of oocytes, as pheromones to stimulate male sexual behaviour, for synchronisation of male and female spawning and may act directly to influence sperm motility. Biochemical stress and malpigmentation in fish may result from excess PG production although the mechanism is not clearly understood (Sargent et al. 1999; Villalta et al. 2005; Bransden et al. 2005; Villalta et al. 2005). For example, in common sole (*Solea solea* L.*)* larvae, growth and survival was not found to be related to dietary ARA levels though malpigmentation levels were (Lund et al. 2007; Lund et al. 2008).

1.4.3 Astaxanthin

For cod eggs and larvae one of the most important carotenoids is astaxanthin (Ax). Ax is the major carotenoid pigment in the marine environment and cannot be synthesised by fish and must be obtained entirely through the diet (Davies 1985; Matsuno & Hirao 1989; Schiedt 1998). Carotenoids have a wide range of functions in fish including reproduction, egg respiration, cell growth and proliferation, as a precursor of vitamin A, in vision, as a source of pigment and as an antioxidant (Tacon 1981; Craik 1985; Torrissen 1990; Pavlov et al. 2004). Its role in reproduction may be as a substance which increases fertilization rates (Christiansen & Torrissen 1997). For example, in rainbow trout, high levels of Ax and canthaxanthin in eggs were found to improve fertilization rates (Craik 1985). In addition, (Ahmadi et al. 2006) showed that dietary supplementation with Ax in rainbow trout broodstock gave a significant improvement in egg quality. Mikulin & Soin (1975) found that Ax may help improve egg quality during embryonic development. It has also been suggested that Ax functions as an antioxidant and protects against light induced free radical production (Pavlov et al. 2004).

Many researchers have demonstrated the importance of dietary supplementation with Ax for a variety of species including yellowtail (*Seriola quinqueradiata*) (Verakunpiriya et al. 1997), striped jack (*Pseucocaranx dentex*) (Vassallo-Agius et al. 2001), sea urchin (*Lytechinus variegates*) (George et al. 2001) and red seabream eggs (Watanabe & Miki 1993; Verakunpiriya et al. 1997). A lack of carotenoids in the diet of broodstock has been shown to result in low fertility and deformities in marine fish larvae (Pavlov et al. 2004). However, it should be noted that no correlation was found between dietary carotenoid levels and egg mortalities, hatching rates and survival of

alevins (Torrissen 1984; Craik 1985; Tveranger 1986; Craik & Harvey 1986; Christiansen & Torrissen 1997; Choubert et al. 1998). The levels of carotenoids in eggs, and egg size and length of time for embryonic development have been shown to be correlated, such that species with large eggs require higher amounts of pigment than those with small eggs (Mikulin 2003).

In cod, it has been shown that carotenoid concentrations in wild fish eggs are higher than those of farm reared fish (Grung et al. 1993; Salze et al. 2005). In addition, it has been shown that dietary supplementation with Ax for cod broodstock can increase carotenoid concentrations in eggs (Grung et al. 1993). Differences between the astaxanthin content of eggs of different species have been seen in wild fish possibly related to diet and geographical location. In general, eggs from pelagic spawning species are colourless compared to demersal spawning species. Two reasons have been proposed for these differences. The first relates to the respiratory function of carotenoids, and suggest little pigment is needed by pelagic eggs due to the high oxygen content of the surrounding water. It is thought that carotenoids have an important role in oxidative metabolism when environmental oxygen levels are low. The second relates to the need of pelagic eggs to avoid predation and, as such, colouration may be a disadvantage (Pavlov et al. 2004).

1.4.4 Vitamin E

Vitamin E is a lipid soluble antioxidant which is important in the normal development of fish eggs and larvae (Hamre et al. 1998). Vitamin E activity is shown by at least two different types of molecule, tocopherols and tocotrienols, of which the former is more abundant (Palace & Werner 2006). The tocopherol with the highest vitamin E activity is α-tocopherol (NRC 1993). The main function of vitamin E is as an antioxidant that protects biological membranes, lipoproteins and lipid stores against oxidation (Hamre et al. 1998). Vitamin E has a range of other functions including the control of reproduction, testes function and macrophage function (Pavlov et al. 2004). Vitamin E has also been found to prevent potentially damaging oxidative by-products building up in tissues (Baker & Davies 1996). Lipid oxidation and rancidity can be measured using a TBARS (Thiobarbituric Acid Reactive Substances) assay which detects malondialdehyde (MDA; a product of lipid oxidation). MDA can build up in live fish as well as post-mortem and thefore the TBARS assay is also an indication of flesh quality (Frigg et al. 1990). Because vitamin E cannot be biosynthesised by fish it is an essential component of their diet especially so in broodstock. It has been found that the levels of vitamin E (measured as α-tocopherol) in fish eggs are correlated with that of broodstock feed. Diets with a high level of HUFAs also require an increased level of vitamin E to prevent oxidation (Pavlov et al. 2004). In addition, vitamin E has been found to have a strong and positive effect on egg hatching and larval success (Pavlov et al. 2004). Therefore, a reduced number of spawning fish, hatching success and low juvenile survival has been found when vitamin E level in the diet of broodstock is not sufficient to meet requirements (Watanabe 1985).

1.5 Egg quality, fecundity and their determinants

1.5.1 Importance of egg quality and fecundity

Egg quality and fecundity of broodstock are very important for hatchery operators. Problems with egg quality can affect the larvae and lead to later production problems such as slow growth, high mortality and deformities. This can affect the economics and profitability of production. Low fecundity of cod broodstock is also a problem for cod hatcheries because of the high mortality in eggs (Bromage et al. 1992). However it should be noted that poor survival through weaning can also be due to factors relating to environmental and feeding conditions as well as poor egg quality. Poor egg quality affects buoyancy, fertilization, development and hatch rates. Several methods have been developed to assess the egg quality of fish (Kjørsvik et al., 1990).

The fecundity of different species of fish varies significantly. Some produce many millions of eggs in one spawning whilst others produce only thousands, whereas some spawn in multiple batches over two to three months while others produce only a single batch per year (Bromage & Cumarunatunga 1988). Atlantic cod are very fecund and release their eggs in batches (Kjesbu 1989). The condition and age of cod females determines the number and quality of eggs that they produce with older more experienced fish producing more eggs (Kjesbu 1989; Kjesbu 1991; Kjesbu & Holm 1994; Chambers & Waiwood 1996; Kjesbu et al. 1996). The normal reproductive strategy for the cod use in the present study is show in Figure 1.8. Gonadal development and vittelogenisis occurs from the beginning of June or July to February the following year. Spawning occurs from March to May.

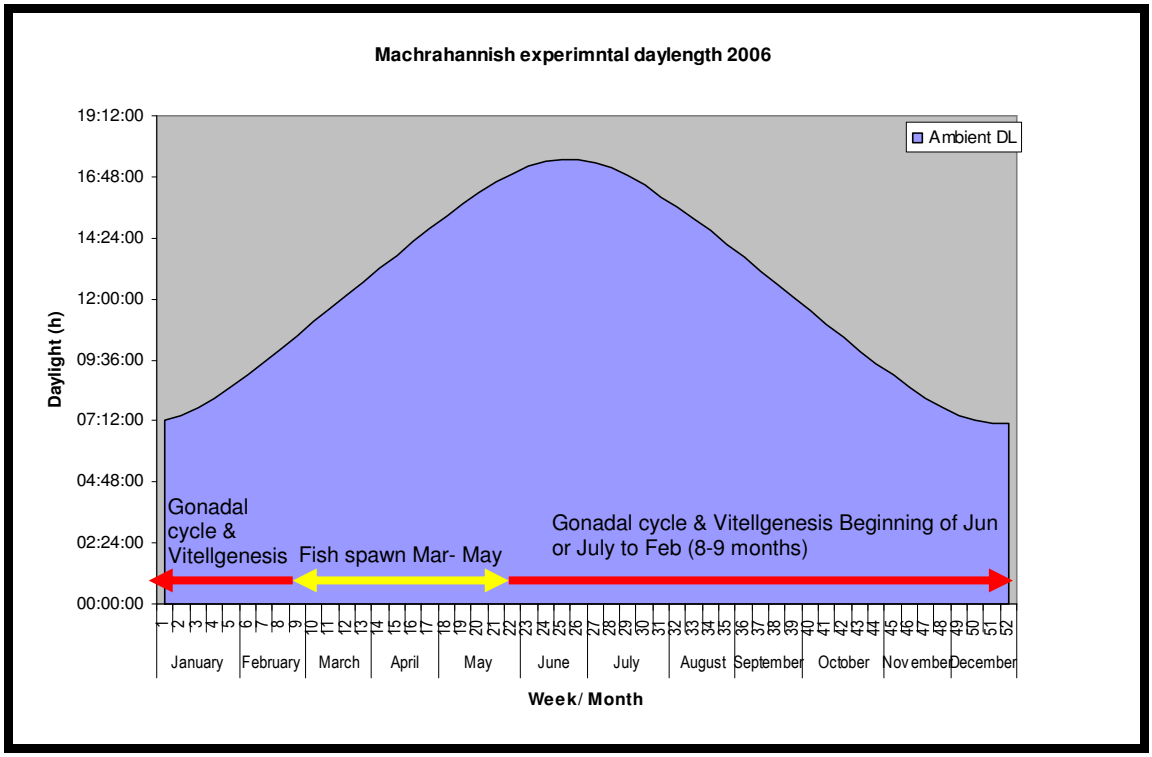

Figure 1.8 Cod spawning strategy and ambient daylength during 2006 at Machrihanish Marine Environmental Research Laboratory.

Yellow arrow indicates spawning period, red arrow indicates development of gonads and vittelogenesis.

Egg size is also considered to be an important factor in relation to fecundity. It is generally accepted that as fish size increases so does fecundity and the diameter of the eggs produced which means that the size of broodstock is a determinant of fecundity (Bromage 1995). Although older fish are larger they do not always produce more eggs per unit weight (Bromage & Cumaranatunga 1988). Fecundity is therefore affected by factors that influence fish size such as the daily and seasonal rates of feeding (Springate & Bromage 1985; Bromage & Cumarunatunga 1988; Bromage et al. 1992) and therefore, good quality broodstock diets are very important to ensure high fecundity in hatcheries. Fecundity can be measured as the total number of eggs produced per batch

or per unit weight of female broodstock. The batches or numbers of eggs can be further defined by their relative percentages of floating and sinking eggs.

1.5.2 The determinants of egg quality

Egg quality is described as "those characteristics of the egg which determine its capacity to survive" and is therefore very important for hatchery production efficiency (Bromage et al. 1992). If hatcheries can identify poor eggs, or batches of eggs at an early stage then they can be discarded and time and resources saved. Accurate methods for identifying poor quality eggs are therefore of high importance to hatcheries. Such egg quality assessment parameters have been developed for use in hatcheries (Kjørsvik et al. 1990; Fernández-Palacios et al. 1995). These include morphological (Kjørsvik et al. 1990; Thorsen et al. 2003) and biochemical parameters such as lipid (Sargent 1995; Bell & Sargent 2003; Tveiten et al. 2004) or vitamin concentrations (Ronnestad et al. 1997; Ronnestad et al. 1999; Maeland et al. 2003).

The ability of eggs to float or sink after fertilization (in sea water) is used by many hatcheries as a determinant of egg quality (McEvoy 1984; Carrillo et al. 1989; Kjørsvik et al. 1990). In cod, buoyant eggs are considered good quality though not always entirely reliable as not all floating eggs are fertilized. This determinant could be combined together with other factors such as rate. Floating eggs can be measured as the total weight of floating eggs produced each day, the number of batches with floating eggs or the total number of floating eggs per unit weight of female broodstock. However in Atlantic halibut the number of floating eggs has been found not to be a good indicator of egg quality and this may be the case for other marine species (Pavlov et al. 2004) .

In many species of marine fish, fertilization rate may be used as a measure of egg quality (Kjørsvik et al. 1990). However, the proportion of eggs which are spherical and with symmetrical cleavage is thought to be a better indicator of quality and has been link to the time elapsed between ovulation and fertilization of the eggs ("over-ripening") (McEvoy 1984; Bromage et al. 1994). For cod, fertilization can be measured by counting the percentage of eggs with normal blastomere development which is considered the most reliable method. Positive correlations have been found between normal blastomeres in the earliest cleavage stages (shown in appendix I), hatching rates and viability of hatching yolk sac larvae in cod. (Shields et al. 1997). In cod it has been suggested that combinations of egg quality indicators may give a better indication of overall egg quality (Newton 2003).

Other methods for estimating egg quality and survival include blastomere, cell symmetry, the appearance of the chorion, the shape of the egg, its transparency and the distribution of oil globules (Kjørsvik et al. 1990; Bromage et al. 1994). Egg size has also been considered as a good indicator of egg quality in some species (Bromage et al. 1992), although, egg size is not generally accepted as an indicator of quality in all species. While larger eggs generally produce larger fry this is not consistently linked to better survival and growth of larvae (Kjørsvik et al. 1990). For example, in rainbow trout, egg size was not found to be the main factor indicating egg and fry quality (Springate & Bromage 1985). For cod, which have small eggs, size may not be a practical measure of egg quality as it depends on broodstock size and age (Kjørsvik 1994). The average size of cod eggs ranges from 1.4 to 1.8 mm (Kjørsvik et al. 2004). However, changes in the size of cod eggs have been reported with a reduction in dry weight of between 20 – 30% as spawning progresses (Kjørsvik 1994).

Perhaps the most important indicator of egg quality is the number of eggs that survive and hatch. Two methods are used to measure hatching rate. In the first, a known number of eggs are incubated and the number of dead eggs removed and counted each day until hatching is complete. Alternatively, a batch of eggs can be incubated until hatched and all hatched larvae and dead eggs counted. The second method uses single eggs cultured in microtitre well plates and the number that hatch recorded (Vallin & Nissling 1998; Panini et al. 2001). The hatching rate can be expressed as the total number of hatched larvae per unit weight of female broodstock.

In addition to morphological methods of assessing egg quality, biochemical analyses such as lipid or pigment composition can also be used. A number of biochemical parameters have been used in this context including lipid,and fatty acid concentrations (Salze et al. 2005) and free amino acid concentrations as they are the main sources of energy, membrane constituents and protein growth for embryos (Ronnestad & Fyhn 1993; Sargent 1995). The ideal levels of EFA in broodstock diets are not precisely known though a ratio of 2:1 for DHA/EPA is recommended as this is the ratio found in most fish eggs, but varies according to species and between marine and fresh water fish, with generally lower values in the latter (Tocher & Sargent 1984; Cowey et al. 1985; Bromage 1995). A (n-3)/(n-6) PUFA ratio of between 5:1 and 10:1 has also been suggested as beneficial (Bromage 1995). Because of the importance of Ax in embryo growth and development and in increasing fertilization rates (Hubbs & Strawn 1957; Hubbs & Stavenhagen 1958; Georgiev 1971; Mikulin & Soin 1975; Craik 1985) the egg concentrations of this compound might also be an indicator of egg quality (Hartmann et al. 1947).

Other factors that affect egg quality include parental factors (age, spawning, parental stress, genetics), environmental conditions (husbandry practices, temperature, light, water quality, disease), and use of induced spawning as well as nutrition (Pavlov et al. 2004). Other factors that have been suggested as important include the size, chemical composition, microbial colonisation, overipening state of the egg and the amount of stress experienced by the eggs (Bromage & Cumarunatunga 1988; Barker et al. 1989; Hansen & Olafsen 1989; Kjørsvik et al. 1990; Barker et al. 1991; Bromage et al. 1992). However, when freshly ovulated eggs of different individuals are compared, the nutritional status of the broodstock is generally considered to be the single most important factor affecting egg quality (Watanabe 1985; Kjørsvik et al. 1990; Watanabe & Kiron 1995).

Over-ripening of eggs is a very important factor of egg quality – even in naturally spawning broodstocks, because if females are stressed their behaviour may be affected and they may not spawn their eggs at the optimal time in terms of freshness and viability i.e. they mayretain them in the lumen of the ovary too long. If males are stressed, they may not court the females to give them the stimulus to spawn eggs – or be able to fertilize the eggs when they are spawned. Stress can be caused by environmental factors and wrong sex ratios, aswell as nutritional stress. Even when spawning behaviour is good, there are always someeggs left behind in the ovary lumen after spawning and these ten to be retained and are only shed when the next fresh batch is spawned – so there will always tend to be a small number of over-ripe eggs in amongst the fresh eggs no matter how good the fish are (McEvoy & McEvoy 1992).

1.6 Current position

The decline in wild cod capture fisheries has meant an increased interest in cod farming. However, problems with hatcheries such as the quality of eggs and poor survival have limited production potential.

Despite many advances in broodstock nutrition (Hardy 1985; Watanabe 1985; Bromage 1995; Pavlov et al. 2004) and significant research effort on cod in Canada, Norway and Scotland, larval quality and survival rates remain poor and unpredictable (Brown et al. 2003). For example, in 2003 a high incidence of deformities in hatchery-reared juveniles of cod was recorded in Norway with nutrition being suggested as a contributing factor (Cahu et al. 2003). Many of these problems can be linked to a limited knowledge of broodstock nutrition, (Vassallo-Agius et al. 2001; Izquierdo et al. 2001) including for example, the conditioning of broodstock outwith the spawning season (Bromage et al. 2001). It is clear that EFAs, including ARA and also carotenoid pigments are very important for growth and development of cod eggs. However, there has been limited research on the role of EFAs in cod diets.

In other species a significant amount of research has focussed on the role of HUFA, vitamins, and the carotenoid pigment Ax. HUFA are important in reproduction and development of eggs and larvae and low levels in broodstock diets have been shown to influence fecundity, egg quality, hatching success, numbers of normal larvae and incidence of deformity (Watanabe 1985). Because marine fish can synthesise only limited amounts of fatty acids they must receive their HUFA requirements from the diet and in the correct concentrations and ratios (Watanabe 1985: Sargent 1995; Rainuzzo et al., 1997). The most important HUFA for successful reproduction are ARA, EPA and DHA (Sargent et al., 2002). Supplementation of broodstock diets with EFA and

carotenoids has the potential to greatly improve egg and larval quality as has been shown in sea bass, sea bream, yellowtail and halibut (Czesny and Dabrowski 1998; Ashton et al., 1993; Watanabe and Miki 1993; Verakunpiriya et al., 1997; Gallagher et al., 1998; Sargent et al., 2002).

Some hatcheries find that cod broodstock of wild origin produce better quality eggs in hatcheries than broodstock of farm origin. These differences may be due to diet, with farm reared broodstock being negatively impacted through long term feeding of sub-optimal diets. Evidence for this comes from the findings of Salze et al. (2005), who found differences in ARA levels, EPA/ARA ratios and carotenoid pigment concentration between wild and farmed cod broodstock, which were correlated with egg quality. The benefits of using farmed broodstock are that they can be produced on site, they are of known age and origin and there are opportunities to use selective breeding to improve the stock. Such selection criteria could include suitability for farming, disease resistance and improved growth rate. In addition the farm-bred fish are adapted to farm conditions and their health status is known. However, the poor egg quality from farm reared broodstock can result in low survival of larvae and problems with their health and welfare. Use of wild origin broodstock can cause some problems such as the risk of introducing disease, and the inability to use artificial selection to improve stocks will be viewed by the industry as unsustainable in the longer term. Commercial hatcheries now find it difficult to procure wild broodstock in the United Kingdon now because the fishing quota has to be used.

In different stocks of wild cod ARA level has been shown to be correlated with hatching success and improved egg quality parameters (Pickova et al. 1997). However, no differences were found in DHA levels or DHA/EPA ratio in egg between wild and

farmed broodstock (Salze et al. 2005). This finding suggests that low levels of ARA and HUFA could be, in part, responsible for dietary related egg quality problems in farm reared broodstock (Salze et al. 2005). In other species, such as halibut, sea bass and Japanese flounder (*Paralichthys olivaceus*) egg quality parameters were found to be improved by adding ARA to the diet of broodstock (Bruce et al. 1999; Furuita et al. 2003; Mazorra et al. 2003). However, when farm-bred cod broodstock were fed a diet containing 0.8% or 3.2% ARA supplementation for 5 months before they spawned no significant differences in egg quality indicators were found (Bell et al. 2005; Blanco 2005).

Carotenoid pigments are also important for egg quality and have been found to be lower in cod eggs from broodstock of farm origin compared to those of wild origin (Salze et al. 2005). Grung (1993), found similar results but also showed that supplementation of broodstock diets with carotenoid resulted in an increased concentration in the eggs. The positive effect of supplementation of broodstock diets with carotenoids has been demonstrated in other species such as salmonids, red sea bream and yellowtail (Watanabe & Miki 1993; Verakunpiriya et al. 1997) but as yet not for cod.

As discussed above DHA is important in larval development whilst EPA and ARA modulate eicosanoid activity. This is especially important for cod that have long lived broodstock which will receive artificial feed over many years which could result in dietary deficiencies. Supplementation of diets with DHA, EPA, ARA and Ax to improve larval quality have been used before with some success in species such as halibut, sea bass and Japanese flounder (Bruce et al. 1999; Furuita et al. 2003; Mazorra et al. 2003).

1.7 Objectives

Objective 1. To determine the optimum level of ARA in relation to egg quality in cod broodstock of wild origin by comparing egg quality in fish fed a diet containing supplementary ARA for 0, 1, 2 or 3 months prior to peak spawning.

Objective 2. To evaluate the effect of carotenoid supplementation on egg quality in hatchery reared cod broodstock by comparing egg quality in groups of fish fed diets with or without supplementary Ax.

Objective 3. To compare the relative effect of diets supplemented with optimum levels of ARA and Ax on egg quality in wild and farmed cod broodstock.

Objective 4. To determine egg lipid content and fatty acid composition, especially ARA, DHA, EPA/ARA ratio and carotenoid in relation to egg quality in UK cod hatcheries over a whole spawning season.

Chapter 2. Materials and methods

2.1 The origin of broodstock

The broodstock of wild origin were captured during the winter of 2003-4 from the Firth of Clyde, UK. Fish were quarantined and weaned onto artificial diets at the Machrihanish Marine Environmental Research Laboratory (MMERL), then transferred to broodstock tanks at Machrihanish Marine Farm Ltd. (MMF). Fish were returned to MMERL in July 2005. All these fish would probably have spawned in captivity in spring 2005 for the first time.

The broodstock of farm origin came from the reared broodstock (F1 generation) hatched at MMF during November 2003. They were transferred to MMERL in February 2004. These were first time spawners in spring 2006.

2.2 Experimental diet and feeding regime

The basal diet was based on a commercial moist feed formulation (Vitalis® Marine Broodstock Mix) specially prepared to contain no supplementary Ax or added arachidonic acid and supplied by Skretting Ltd, (Longridge, UK) (formulation in Appendix II). The feed was supplemented with additional nutrients depending on the requirement of each experiment. The feed was supplied as a dry mix which was constituted for feeding by the addition of water (0.7 L/kg dry mix).

Feed supplements included Ax as Carophyll Pink® (10% astaxanthin w/w) and ARA as Vevodar® oil containing 373 mg/kg ARA). Both were supplied by DSM Nutrition Products (Basel, Switzerland).

The feed was prepared by first weighing out 5 kg dry meal and transferring to the mixer (Fig 2.1.). Before adding to the feed mix the supplements were weighed and mixed with water. Vevodar® is an oil and was sprayed directly on the feed. Astaxanthin is a powder and was mixed directly with water used to prepare the feed. After adding supplements the feed was mixed well. The mixer was stopped when necessary to loosen and break up any compacted feed. Once mixed, the feed was removed by hand, rolled flat and chopped into pellets of approximately 2.5 cm^3. (Fig2.2). Prepared feed was kept for 1 to 2 days in a feed bin, or for up to 1 month in the freezer.

Figure 2.1 Preparation of feed, the mixer. The arrow indicates the mouth of the feed mixer.

Figure 2.2 Final preparation of feed, manually chopped into 2.5 cm^3 pellets.

2.3 Experimental tanks

Fish were held in Glass Reinforced Plastic (GRP) experimental tanks at MMERL. Each tank was covered and individually equipped with four (36 W) fluorescent tube lights and two (50 W) halogen lamps at about 70 cm above the water surface. The wild broodstock were kept in 4 metre diameter tanks of 12 m^3 volume (Fig.2.3) and the farm broodstock kept in 3 metre diameter tanks of 7 m^3 (Fig.2.4) and a water depth of 1.2 metres for each tank. A flow of seawater was maintained at all times but during summer when the water temperature rose the flow was reduced to allow a water cooler to operate and maintain temperature. Environmental conditions for each experiment are outlined in the relevant chapters.

Figure 2.3 Experimental tank layout of 4 metre diameter and 12 m^3 volume, water depth of 1.2 metres.

Figure 2.4 Experimental tank layout of 3 metre diameter and 7 m^3 volume tanks, water depth of 1.2 metres.

2.4 Environmental conditions

A drum filter (100 μm) was used to filter the seawater supplying the fish tanks used for the experiments. The water inflow allowed a circular flow of water to be maintained in the tanks. The water flowed out through a central drain. Tanks were supplied with seawater at 40 L/min in a flow-though system. The average water temperature and salinity were recorded every day during the experimental period and are described in the relevant chapters. Oxygen probes connected to a computer were used to monitor oxygen levels in each tank. The oxygen concentration was maintained at above 6.5 ppm using oxygen diffusers in the experimental tanks. All fish were held under simulated natural photoperiod (SNP) provided by fluorescent lights located on tank covers with simulated dawn/dusk using an automatic dimming system.

2.5 Broodstock husbandry

The wild-caught fish were acclimatised to farm conditions and held in tanks for at least 12 months before the start of trials. The broodstock were allocated to four 12m^3 tanks in November 2005.

The farm broodstock fish were held in four tanks of 3 metre diameter and 7 m^3 volume. These fish were F1 generation hatched at MMF in November 2003 and transferred to MMERL in February 2004. They were spawning for the first time in spring 2006.

In January 2006, the experimental fish were individually weighed, screened by ultrasound to determine sex and state of maturation, and then reallocated so that each

tank contained a similar number and biomass of males and females. Fish were fed to satiation twice daily. The food that remained at the bottom of the tank was observed to determine the next day feeding rate. The fish were fed at approximately 1.5 % of body weight. The bottom of the tanks were flushed daily except during spawning season, to remove waste feed and faeces to maintain the water quality.

2.6 Egg collection

The cod usually spawned at night so the egg collection was conducted in the morning around 9.30 am. The 500 µm plankton mesh egg collector was put under the drain pipe from the tank (Fig 2.5 & 2.6).

Figure 2.5 Collection point for eggs. The arrow indicates the collection apparutus.

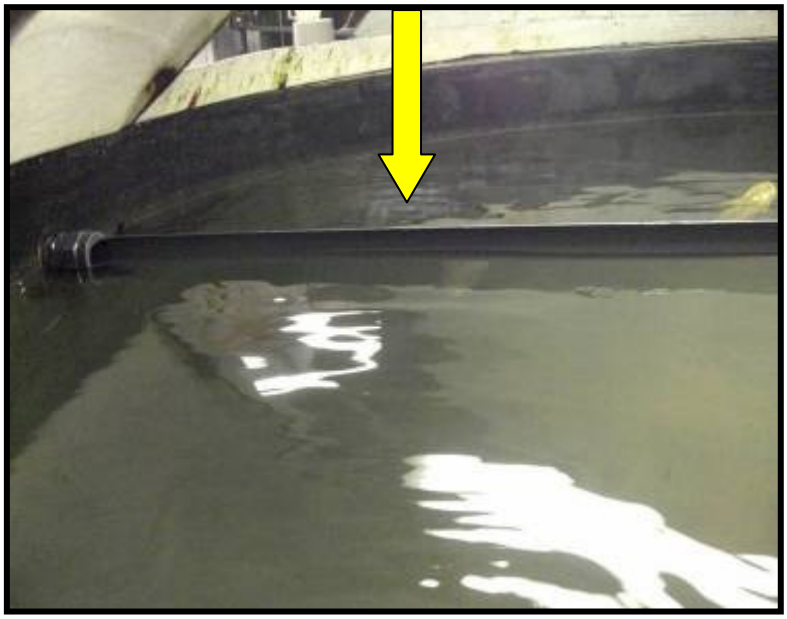

Figure 2.6 Collection of floating eggs, the half PVC pipe is used to catch the floating eggs from the surface and deposit them in the collection outlet.

The arrow indicates the collector on the water surface.

The outlet was opened at the surface of the tank from the egg collection pipe. The floating eggs came out through the overflowing outlet and were directed into the 500 μm mesh egg collectors to allow egg collection (Fig 2.6).

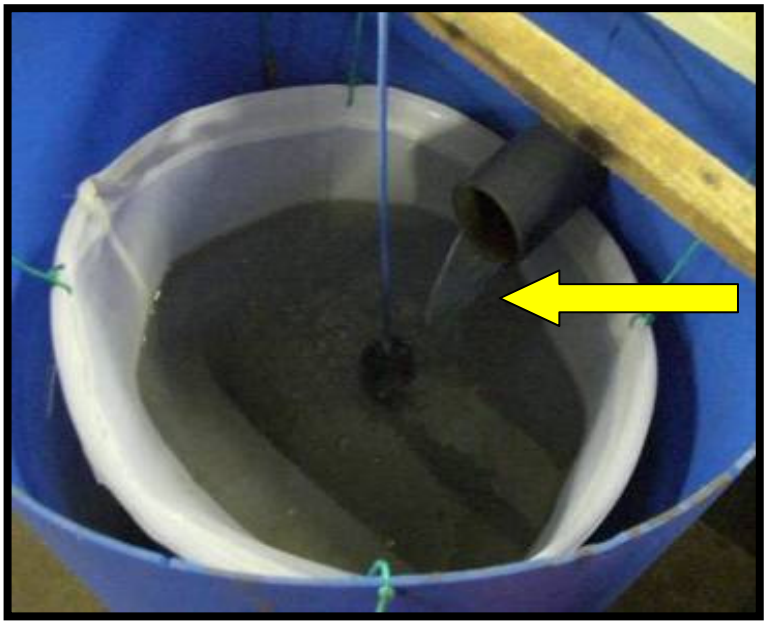

Figure 2.7 The collection of floating eggs at the tank outlet.
The arrow indicates the water flow from the broodstock tank into the collection net.

After collection the eggs were washed with clean seawater until free of debris (Fig 2.8). Then the eggs were placed in a 5 litre bucket with a 500 µm mesh to allow the water to overflow. From this point the eggs were weighed as a total batch wet weight on an electronic balance (precision +/- 1 g).

Figure 2.8 The egg collectors were washed until cleaned of debris.
The arrow indicates the egg collection net which is washed into the white container.

The eggs were then transferred into a 5 litre bucket filled with seawater. After approximately 15-30 minutes the floating eggs were separated from the sinking eggs. The floating eggs from the surface of the bucket were then siphoned into a separate container (Fig 2.9). The sinking eggs were then weighed as wet weight using the balance as above.

Figure 2.9 Separation of floating and sinking eggs. The floating eggs are collected from the surface of the bucket and siphoned into a separate container.

Egg quality of the floating eggs was determined using a random sample collected using a small aquarium net scoop and transferred to a small plastic 250 ml jar (Fig 2.10).

Figure 2.10 Floating eggs transferred to a small jar before determining egg quality.

A random sample of 100 eggs was then taken for counting using a Bogorov tray (Fig 2.11).

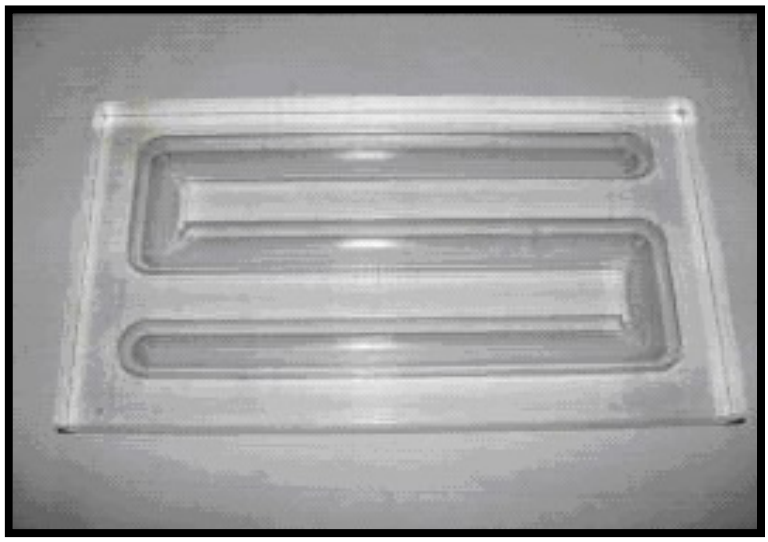

Figure 2.11 Bogorov tray use for counting eggs and examining their developmet stage.

The number of non fertilised eggs and fertilised eggs was determined by examining blastomere morphology using a dissecting microscope (Olympus SZ3060) Figure (2.12).

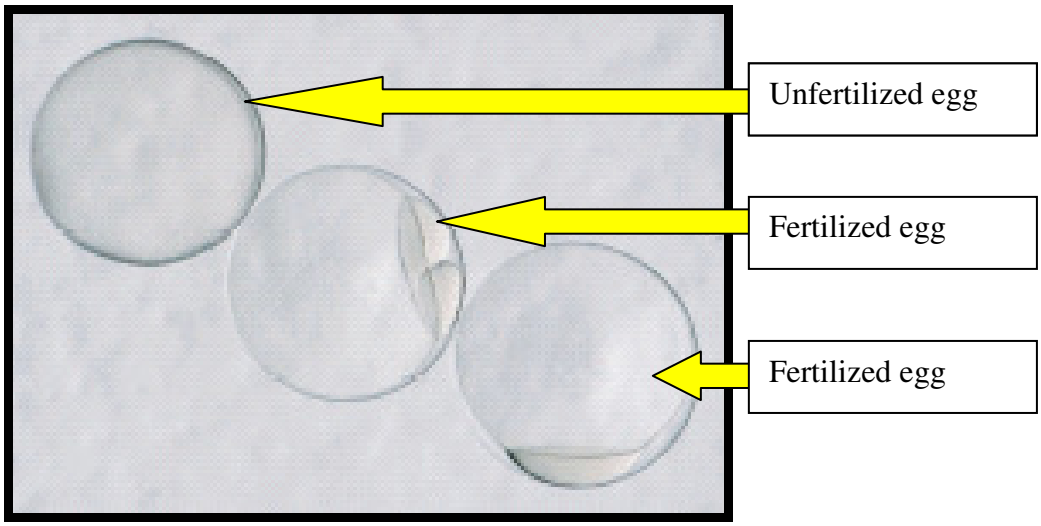

Figure 2.12 Blastomere morphology picture by Thorsen, et al 2003. Unfertilized (left) and fertilized (middle and right) cod eggs.

The net was then cleaned and replaced for collection the following day (Fig 2.13).

Figure 2.13 The cleaned egg collection nets.

2.7 Egg incubation

Survival and mortality rates were determined from a random sample of 2 g (approximately 1000) of eggs taken from each broodstock tank, each week. These were incubated separately in 500 ml beakers of UV sterilized, temperature (6-7 ^{o}C) controlled water that was kept oxygenated with an air stone (Fig.2.14). Survival and mortality were measured by counting the number of live and dead larvae after 15 days.

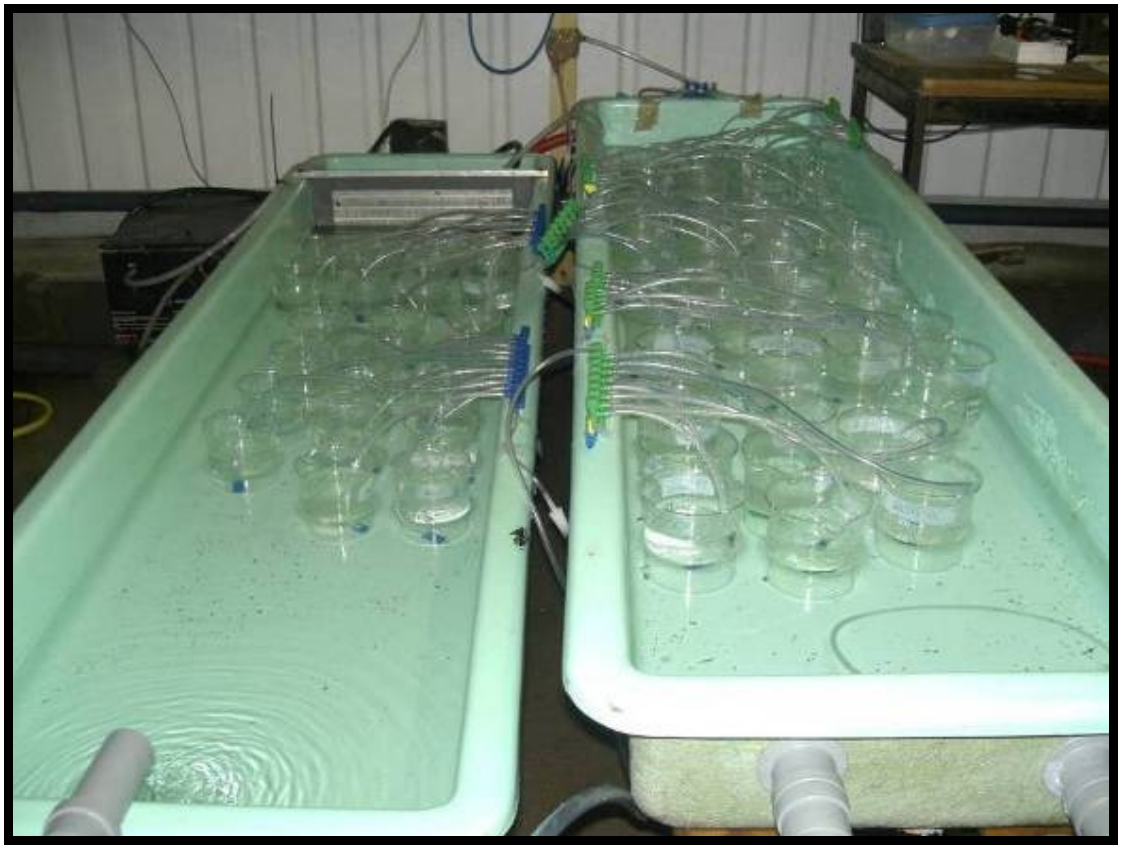

Figure 2.14 Incubation of eggs using 500 ml beakers. The beakers contain 2 grams of eggs (approximately 1,000 eggs), temperature is controlled using the water bath.

Hatching rates of individual eggs were measured by taking a random sample of 36 eggs from each broodstock tank every week, of the same developmental stage, (blastomere) (selected using an Olympus dissecting microscope). These eggs were placed in a 96-well plastic microtitre plate with seawater and placed in an incubator at 6 ^{o}C for 15 days. The total number of hatched eggs after 15 days per plate were counted. Russell (1976), showed that 15 days was the average time to hatch for cod eggs incubated at 6 ^{o}C. Numbers were calculated from egg batch weight measurements assuming 500 eggs/g.

2.8 Egg quality assessment

Each day during the 92 day spawning period, egg batches were collected and egg quality was assessed using standard techniques, as described above, to measure total egg production, floating egg production and fertilization rate. Dropout of eggs within each broodstock tank was estimated over a 24 hr period on five different dates. Samples of floating eggs from all four tanks (11-13 batches per tank) were collected for fatty acid analysis on different dates. Fourteen batches of floating eggs from each tank were incubated in order to attempt to determine hatching rates.

Egg quality and production parameters are summerised and described in Table 2.1. Eggs collected, refers to the actual weight of eggs collected in gram (g). Eggs produced refers to the calculated number of eggs in gram multiplied by female biomass (using the estimate that 1 g of eggs = 500 eggs).

Table 2.1 Summary and description of egg quality and production parameters used during experiments, including these for total, floating, fertilized and hatching eggs.

Egg quality parameter	Description
Total eggs	
1.Total no. of eggs produced/kg female	= Total number of sinking plus floating eggs produced per day divided by average weight of female per tank.
2.Mean no. per batch of eggs produced/kg female	= Total no of eggs produced/kg female divided by the number of batches produced.
3.Total weight of eggs collected (g)	= The total weight of eggs collected during spawning (sum of floating, sinking and drop out eggs)
4.No. of batches collected	= The total no of days on which batches were collected.
5. Mean wt of collected egg batches (g)	= The average of weight divided by batches.
Floating eggs	
6. Total no of floating eggs/kg female	= The total no of floating eggs divided by the total weight of female fish
7. Mean no of floating eggs per batch / kg female	= The mean wt of floating egg batches (g) divided by total no of floating eggs/kg female.
8. Total wt of floating eggs (g)	= The total weight of floating eggs each day
9. Mean wt per batch of floating eggs (g)	= Total wt of floating eggs (g) divided by number of batches with floating eggs
10. Total no of floating eggs/kg female	= Total number of floating eggs collected each day divided by the weight of female fish.
11. Mean no per batch of floating eggs/kg female	= The mean of the total no of floating eggs/kg female divided by number of batches with floating eggs.
Fertilized eggs	
12. Mean fertilization rate (% floating eggs)	= The mean of the rate as a percentage of floating eggs.
13. Total no of fertilised eggs/kg female	= Number of eggs with blastomere divided by weight of females
14. No of batches with fertilised eggs	= no of batches produced minus the number of batches with no fertilized eggs.
15. Mean no per batch of fertilised eggs/kg female	= no of batches with fertilised eggs divided by the weight of females.
Hatched eggs	
16. Mean hatch rate (% floating eggs)	= The mean of the hatching rate as a percentage of floating egg
17. Total no. of hatched larvae/kg female	= Total number of larvae hatching after incubation divided by weight of females
18. Mean no. per batch of hatched eggs/kg female	= Mean hatch rate divided by no. of batches with floating eggs.

2.9 Collection and storage of samples for lipid analysis

Random samples of 20 floating eggs were taken for lipid analysis for experiment 1 and 2 whilst in Experiment 3, 100 eggs were collected (as it was found that more lipid was required to improve analysis than was present in 20 eggs). Samples for biochemical analysis were collected from each tank. Wet egg samples were stored in approximately 5 ml of a mixture of chloroform and methanol (HPLC grade) at a ratio of 2:1 (v/v) in a sealed glass 7 ml vial. Dry egg samples were collected in plastic sample vials (without chloroform and methanal) and stored in a freezer at a temperature of -20 ^{o}C until required for analysis in the laboratory.

2.10 Biochemical analysis

2.10.1 Lipid extraction

The fatty acid composition and total lipid content of feed and egg samples was measured using the method of (Folch et al. 1957). Firstly samples were placed in 15 ml labelled glass test tubes, then 5 ml of chloroform/methanol (C:M) (2:1,v/v) was added to the tubes. The samples were then homogenised using an Ultraturrax^{TM} (the probe was rinsed in C:M (2:1 v/v) between samples). The tubes were stoppered and left on ice for approximately 1 hour. After that, 0.25 vulume of 0.88% (w/v) KCl was added to 2.5 ml of the homogenised sample. These samples were then mixed on a vortex mixer and left to stand on ice for 5 minutes. Samples were then centrifuged at 400 x g average (1500 rpm, C 412 bench centrifuge) for 2-3 minutes. After this the top layer of the aqueous material was removed by aspiration. The bottom layer was then filtered into tubes

through pre-washed with C:M (2:1 v/v) 11 cm Whatman no. 1 filter papers. The solvent was then evaporated to dryness under a stream of oxygen-free nitrogen (OFN). Then 1.5 ml of C:M with butylated hydroxy toluene (BHT) was added and the sample transferred to a 2 ml glass vial. The solvent was then evaporated to dryness under a stream of OFN and desiccated *in vacuo* overnight. The samples and tubes were re-weighed and lipid re-dissolved in C/M (2:1 v/v) + 0.01% (w/v) butylated hydroxyl toluene (BHT).

2.10.2 Preparation and purification of fatty acid methyl esters (FAME)

The total lipid prepared as above was used to prepare fatty acid methyl esters. The lipid sample, normally ~1 mg, was transferred into a small (15 ml) labelled test tube and 17:0 free fatty acid added, if required, at 10 % of the total lipid mass to act as an internal standard. The organic solvent was then evaporated to dryness under nitrogen. Then 1 ml of toluene and 2 ml of the methylating reagent, 1 % (v/v) sulphuric acid in methanol, were added. The tubes were mixed on a vortex mixer and flushed with nitrogen and stoppered with a piece of tissue or paper to prevent the stopper blowing out upon heating. The tube was incubated overnight (16 hr) at 50^{o}C in a hot-block. The tubes were removed from the hot-block and cooled. The tissue was removed and 2 ml 2% potassium hydrogen carbonate ($KHCO_3$) added to neutralise the acid. Five ml iso-hexane/diethyl ether (1:1, v/v) + 0.01% (w/v) BHT was added and the tubes were vortexed and centrifuged at 350-400 x g average (1500 rpm C312 or C412 bench centrifuge) for 2 min. The upper organic layer was transferred to another clean test tube and a further 5 ml iso-hexane/diethyl ether (1:1, v/v) (no BHT) added to the original tube before mixing and centrifuging as before. The upper layer was added to the other

tube as above. The solvent was evaporated on the nitrogen evaporator and the FAME re-dissolved in 100 µl iso-hexane. FAME were purified by TLC on 20 x 20 cm plates at a lipid loading of < 1.5 mg/cm. Sample origins were 1.5 cm from the bottom of the plate and samples separated by at least 1.2 cm with a 2 cm margin at the edge of the plate. The samples were loaded using a 100 µl glass Hamilton micro-syringe (the syringe was repeatedly rinsed with iso-hexane between samples). The plate was then chromatographed in iso-hexane/ diethyl ether/acetic acid (90:10:1, v:v) to 1 – 1.5 cm from the top of the plate. The plate was then removed from the tank and the solvent allowed to evaporate in the fume cupboard before spraying with 1% (w/v) iodine in chloroform to visualise the FAMEs. The plate was masked off with blank glass plates so that only the very outer edge of the origins, were exposed. This section was then sprayed lightly with iodine. The FAME band was clearly visible and was marked with pencil. The FAMEs chromatograph as a doublet with saturated and monounsaturated fatty acids forming the upper band and PUFA the lower band (both bands were removed). 0.5 cm above and below the iodine stain were marked in pencil to ensure all the FAME were included. The sample FAME bands were all marked accordingly and then scraped from the TLC plate into test tubes using a straight edged scalpel blade. Since the FAME band is heterogeneous it was vital to ensure that all the silica was removed. FAMEs were eluted from the silica with 10 ml iso-hexane/diethyl ether (1:1, v:v), followed by mixing and centrifuging as described above, to sediment the silica. The solvent was removed to a clean 15 ml test tube. The solvent was evaporated on the nitrogen evaporator and the samples transferred to 2 ml glass vials in 1 ml of iso-hexane, evaporated to dryness and re-dissolved in iso-hexane to a concentration of 1 mg/ml. The FAMEs were flushed with nitrogen or argon and stored at -20^{o}C for gas liquid chomatograpy (GLC) analysis.

2.10.3 Separation and identification of FAME

FAME were separated and quantified by GLC using a Carlo Erba Vega 8160 (Milan, Italy) chromatograph equipped with cold on-column injection and using a 30 m x 0.32 mm id, 0.25 µm film thickness, capillary column (CP wax 52CB; Chrompak Ltd., London UK.). Hydrogen was used as the carrier gas at a flow rate of 2.0 ml/min (constant flow mode). The temperature programming was from 50°C to 150°C at 40°C/min and then to 225°C at 2°C/min. The fatty acid peaks were detected by flame ionisation detection (FID) at 250°C. The volume of the sample injected was 1 µl of 1 mg/ml FAME in iso-hexane. Individual methy1esters were identified by comparison to known standards and by reference to published data (Ackman et al. 1980). Peak data were processed using the Chromcard for Windows (version 1.19) computer package (Thermoquest Italia S.p.A., Milan, Italy).

2.10.4 Extraction of carotenoids

The carotenoid Ax, was determined in cod eggs from samples prepared following extraction of lipids using the protocol described in section 2.10.1. Solvent extraction followed by analysis by high performance liquid chromatography (HPLC) was used to quantify dietary pigment concentrations. The sum of the peak areas was calculated with reference to an Ax external standard. The method was as follows.

Extracted lipid samples were re-weighed after desiccation then dissolved in iso-hexane (1.5 ml) and mixed. This was then transferred to a plastic tube using a Pasteur pipette and centrifuged at 7,000 x g (10,000 rpm) for 5 min.

Then 1 ml was transferred to a 2 ml glass vial and the samples were read in a spectrophotometer at 470 nm against an iso-hexane blank. The Ax standard was measured at 470 nm, using quartz cuvettes against an iso-hexane blank.

The HPLC apparatus comprised of a pump and a Waters 486 Tunable Absorbance Detector with Millenium 32 Chromatography Manager (Waters Inc. Milford, USA) for data analysis. The column was a Lichrosorb 5µ Silica 60 (150 x 4.6mm, Phenomenex, Macclesfield, UK) and the mobile phase was iso-hexane/acetone (86:14, v/v) at a flow rate of 1 ml/min and maximum pressure of 1000 psi. The injection volume was 50 µl and detection was by absorbance at 470 nm. The total run time was 25 minutes.

To calculate the concentration of standard from the spectrophotometer:

$$[C] = \frac{1}{wt(g)} \times [Abs\ @470\ nm] \times \frac{10{,}000}{2{,}100} \times DF$$

Where 2100 is the extinction co-efficient of Ax in iso-hexane

DF = dilution factor, C = concentration (µg/ml)

To calculate the concentration f standard from the spectophotometer:

$$SC = [Abs@470\ nm] \times \frac{10{,}000}{2{,}100}$$

where SC = standard concentration (µg/ml)

To calculate total and individual pigment concentrations from the HPLC:

$$\text{Concentration (mg/kg)} = \frac{SC \times y \times DF}{\text{Std area} \times \text{wt of sample (g)}}$$

Where y = the total carotenoid or individual peak area.

2.10.4 Analysis of lipid classes by HPTLC

Lipid classes in samples of total lipid were determined by high performance TLC (HPTLC) using double development chromatography to separate polar and neutral classes. Lipids were detected by charring and quantified using a CAMAG 3 TLC scanning densitometer (CAMAG, Muttenz, Switzerland) with identification of the classes against known polar and neutral lipid standards.

Samples were prepared using the lipid extraction protocol in 2.10.1 and the following method applied. First, the top 2 corners of the 10 x 10 cm HPTLC plate were marked and pre-washed in a tank containing 20 ml chloroform/methanol (2:1, v/v) to the top of the plate. After pre-washing, the plate was dried in a fume cupboard and then in a vacuum desiccator to remove the solvent. The plate was activated in a drying oven at 110 ^{o}C for approx 20 min. A 5 mm band of silica was removed from the top of the plate with the straight edge of a scalpel. With a soft pencil, the position of the origin was marked 1 cm up from the bottom of the plate and the planned positions of the first (5.5 cm up) and second (9.0 cm up) solvent fronts also marked. Using a 10 µl glass Hamilton syringe, each sample was loaded on the origin as a 2 mm streak, using 1 to 1.5 µl of a 10 mg/ml total lipid stock solution. The syringe was rinsed with C/M (2:1) between samples. A known lipid class standard was used as reference. Ideally this should have a similar class profile to the samples being analysed. The plate was first chromatographed in a polar solvent to the 5.5 cm mark. The freshly made solvent comprised methyl acetate/ propan-2-ol/ chloroform/ methanol/ 0.25 % (w/v) KCL (25:25:25:10:9 by vol). The plate was removed from the tank, air dried in a fume cupboard and then in a vacuum desiccator for at least 15 min. The plate was run in the second neutral lipid solvent to the 9 cm mark. This was also freshly prepared and

comprised iso-hexane/diethyl ether/glacial acetic acid (80:20:2 v/v/v). The plate was removed from the tank and air dried. The plate was sprayed with 3 % aqueous copper acetate (w/v) containing 8 % (v/v) phosphoric acid. Excess solution was allowed to run off and any excess removed at the bottom edge of the plate. The plates were charred at 160 ^{o}C in an oven for 15-20 min. The lipid classes were then quantified by scanning densitomet.using a CAMAG TLC scanner (tungsten lamp – wavelength 370 nm) and using CAMAG " WinCATS" Planar Chromatography manager software, version 1.2.0.

2.10.5 Determination of vitamin E in cod eggs

For vitamin E analysis, the relevant number of large test tubes were labelled and 5 ml 2 % (w/v) ethanolic pyrogallol added to each. The wet weight of egg samples was measured using a balance (approximately 1 g to two decimal places) and added to the corresponding tubes. The samples were homogenised with the Ultraturrax, and the stoppered test tubes incubated for 5 min in a water bath at 70 ^{o}C. The tubes were removed from the water bath and 1 ml 60 % KOH added and the contents mixed. The tubes were flushed with nitrogen, then stoppered and shaken before returning to the water bath for a further 20 min. The tubes were shaken at 5 minute intervals. Then removed and cooled on ice. Four ml distilled water and 6 ml iso-hexane + BHT were added and the tubes mixed by vortexing for exactly 1 minute to extract the vitamin E into the iso-hexane layer. The layers were allowed to separate. If two distinct layers did not appear, the tubes were placed in a freezer (-20 ^{o}C) for 10 min which should facilitate the separation. The corresponding number of 15 ml test tubes were labelled and 4 ml of the iso-hexane (top) layer was removed into these tubes using 5 ml glass pipettes. The solvent was evaporated to dryness under nitrogen and 1 ml of methanol

added to each of the tubes to re-dissolve the samples before transfering to 2 ml glass vials prior to analysis by HPLC. The vials were flushed with nitrogen and removed to the freezer for storage.

The HPLC analysis was conducted using a Thermo Finnegan HPLC and a UV 3000 absorbance Detector with ChromQuest for Thermo LC software. The HPLC column was a LUNA 5µ C18 (4.6 x 150 mm; Phenomenex, Macclesfied, UK) and the mobile phase was methanol/ultrapure water (98:2 v/v). The system used a constaMetric 4100 pump to supply the solvent at a flow rate of 1.2 ml/min at ambient temperature and maximum pressure of 1000 psi. The injection volume was 50 µl and detection was at 293 nm. The total run time was 25 min and the elution order was:

δ-tocopherol, γ-tocopherol, β-tocopherol, α-tocopherol.

The following formula was used to calculate the concentration of Vitamin E in the fish eggs:

$$\text{Std conc ng / 50 µl} \times \frac{\text{peak area}}{\text{Average std.area}} \times \frac{\text{Vol. iso-hexane}}{\text{Vol.layer removed}} \times \frac{\text{Vol. MeOH}}{\text{Vol.injected}} \times \frac{1}{\text{wt}}$$

Where (std) is the concentration of the standard used in ng / 50 µl injection.

2.10.6 Measurement of thiobarbituric acid reactive substances (TBARS) in cod eggs

The required number of large tubes were labelled and 9 ml of 50 mM potassium phosphate buffer pH 7.4 added. Cod eggs were weighed (to 2 decimal places) into the corresponding tubes and homogenised using the Ultraturrax. A similar number of 15 ml tubes were labelled, plus one for a reagent blank. To each tube 2 ml of 10 % (w/v)

trichloacetic acid, 2 ml of 1 % (w/v) thiobarbituric acid, and 50 µl of 0.2 % (w/v) BHT in ethanol were added. One ml of the cod egg homogenate was added to each tube and mixed gently making sure the stoppers were loose. The tubes were placed in a pan of boiling water on a hot plate for 20 minutes and shaken every 5 min. The tubes were removed and cooled on ice. The micro centifuge tubes were labelled and centrifuged at 10,000 rpm (7,700 g) in the micro centrifuge for 10 minutes. The supernatants were transferred to plastic cuvettes and the absorbance recorded against a reagent blank at 532 nm. The concentration of malondialdehyde (as TBA reactive substances; TBARS) can be calculated using the following equation:

µmole MDA/g tissue= (sample abs/0.156) x (50/wt of tissue (g))

2.10.7 Extraction of eicosanoids from cod eggs– Prostaglandin E2 (PGE2)

The determination of PGE_2 in cod egg samples was carried out using an enzyme immunoassay kit (Prostaglandin E_2 – Monoclonal; Catalog No. 514010; Cayman Chemical Co., Ann Arbor, Michigan, USA). The assay was carried out as described by the manufacturer's protocol. The assay is a Monoclonal Competitive Enzyme Immunoassay and is based on competition between PGE2 and a PGE2–acetylcholinesterase (AChe) conjugate (PGE2 tracer) for PGE2 monoclonal antibody. During the assay, the PGE2 tracer levels are kept fixed. The amount that is combined with the PGE2 monoclonal antibody is the inverse of the concentration of PGE2 in the assay wells. Once the enzyme reaction has been completed the amount of PGE2 in the wells is measured using a spectrophotometer (at 412 nm).

Samples for the enzyme immunoassay were homogenised in at least 4 volumes of Hank's balanced salt solution (HBSS) containing 15% (v/v) ethanol and 50 µl/ml of 0.2M formic acid. Samples were then stored in a freezer at -20^{o} C until extracted. The purification of the sample was conducted as follows: for cod egg homogenates and cell media, samples were centrifuged in a bench centrifuge for 5 min, as above, to remove debris. The supernatant was extracted as follows;

The supernatant was loaded onto a C18 "Sep-Pak" cartridge which had been washed first with 5 ml methanol followed by 10 ml distilled water (drip). Samples were washed by slowly eluting with 10 ml distilled water followed by 5 ml 15 % ethanol (v/v) followed by 5 ml hexane/ chloroform 65:35 (v/v).

The eicosanoids were then eluted into a stoppered test tube with 10 ml ethyl acetate. The Sep-Pak should then be regenerated by washing with 10 ml methanol and 10 ml distilled water prior to loading the next sample. The extract was then dried under nitrogen, redissolved in 1 ml methanol and stored in a small glass vial at -20 ^{o}C before analyis.

2.11 Statistical analysis

Egg quality indices used for group comparisons included batch weights of eggs collected, batch weights of floating eggs, fertilization rate and hatch rate, and estimates of mean numbers per batch of eggs spawned, eggs collected, floating eggs, viable (ie floating, fertilised eggs) and hatched eggs. Numbers were calculated in terms of the biomass of female fish to compensate for small differences in broodstock biomass and

allow comparison with other stocks. Numbers were calculated from egg batch weight measurements averaging 500 eggs/g.

Analysis of variance (ANOVA), or Kruskal-Wallis tests, were used to identify differences in egg quality or biochemical parameters between individual groups. Where differences were identified, appropriate multiple comparison tests were used to identify differences between the group averages. Differences were regarded as significant when $P < 0.05$ (Zar 1999). Spearman's rank test was used to detect any correlation between fatty acid composition and egg quality.

Chapter 3. Determination of the optimum level of dietary arachidonic acid (ARA) in relation to egg quality in wild origin cod

3.1 Introduction

As previously discussed in the general introduction, nutrition of broodstock has a major impact on gonadal growth, fecundity, egg quality and larval growth (Watanabe 1985; Mourente & Odriozola 1990; Harel et al. 1994). Of all the nutritional factors in broodstock diet that affect spawning quality and egg composition, dietary essential fatty acid content is considered to be the most important (Watanabe et al. 1984; Watanabe 1985). Fatty acids function in eggs and larvae as a source of metabolic energy and also as components of phospholipids in form cell and tissue membranes (Sargent 1995). ARA is a precursor of eicosanoids, an important group of compounds with a wide range of biological functions which including a role in final maturation of oocytes (Bell et al. 1994; Sargent 1995; Sargent et al. 2002). However, the EFA include ARA, EPA and DHA which, in fish, cannot be synthesised from n-3 PUFA (Tocher et al. 1992; Mourente & Tocher 1993; Sargent et al. 1994; Bell 1998; Cunnane 2000).

Therefore, it is important to make sure that the broodstock fish used in aquaculture obtain all of these nutrients in their diet through appropriate supplementation. Supplementation of broodstock diet with lipids during gonadogenesis has been shown to have effects on egg quality and spawning success. (Watanabe et al. 1984; Watanabe et al. 1984; Mourente & Odriozola 1990; Harel et al. 1994; Watanabe & Kiron 1995). For example, gilthead sea bream broodstock fed a diet of 1 % n-3 HUFA for a short period before spawning had increased levels of EFA in their gonad and produced higher quality eggs (Harel et al. 1992). In European sea bass, supplementation of broodstock diet with ARA has been shown to increase egg and larval quality (Bell et al. 1997; Bruce et al. 1999) Supplementation is of special

relevance for cod broodstock operations because they may be given artificial feed and conditioned for spawning in tanks over many years. If the diet is not nutritionally complete this could lead to long term reduction in fecundity and egg quality.

The nutrient content and time of feeding for broodstock is important because during spawning nutrients are transferred from the body to the eggs (Sargent 1995; Bransden et al. 2007). In Atlantic cod, egg development starts about eight to nine months before spawning which would suggest that fish should be fed supplements from this time to give best performance (Sargent 1995). However, for hatcheries, it would not be economical to feed them supplements for such a long period. Blanco (2004), for example, showed that supplementation of cod broodstock diet with ARA at 0.8 % or 3.2 % for five months before spawning had no effect on fecundity or egg quality, although hatch rate and larval survival was low in both groups. In contrast, Fernández-Palacios et al. (1995) showed that dietary supplementation with fatty acids for just three weeks before spawning can increase their levels in eggs of gilthead sea bream. These findings suggest that supplementation of cod broodstock could be carried out over shorter periods or during the spawning period itself.

However, over-supplementation of diet with ARA has been shown to make gilthead seabream larvae more prone to stress through increased PGE_2 production and could result in increased larval mortality (Koven et al. 2001). Wild cod broodstock eggs ARA levels have been found to be higher while the EPA/ARA ratio was lower compared to the farmed broodstock (Salze et al. 2005). Wild cod also had better egg quality and fecundity than the farm-reared broodstock. Therefore, in this experiment captive, wild-caught broodstock were used as a standard to study dietary ARA supplementation. The first experiment investigated the effect of feeding a diet

supplemented with ARA, for 1, 2 or 3 months prior to peak spawning, on egg quality from wild cod broodstock in order to determine the optimum period of supplementation for best reproductive performance. The principal measures of egg quality used in the study were total number of eggs produced per kg female, total number of floating eggs per kg female, mean fertilization rate, total numbers of viable eggs produced per kg female, and numbers of larvae produced per kg female. Biochemical measurements focussed on fatty acid composition in eggs.

3.2 Materials and methods

3.2.1 Fish and husbandry

The experiment was carried out at MMERL. The experimental design used four treatment groups of wild-caught Atlantic cod broodstock each housed in identical tanks. The wild origin fish were caught during winter of 2003/4, in the Firth of Clyde (as described in chapter 2) and had been quarantined, acclimatised to farm conditions for two years and weaned onto artificial feed. For the experiments, the fish were allocated to four $12m^3$ tanks in November 2005. The number of fish in each tank is shown in Table 3.1. It is likely that these fish were not first time spawners and would have spawned in spring 2005. At the start of the experiment, the fish were weighed and scanned by ultrasound (on 5th January 2006).

The environmental conditions for all fish were SNP from fluorescent lights. Dawn and dusk were simulated by automatic dimming Figure 3.1.

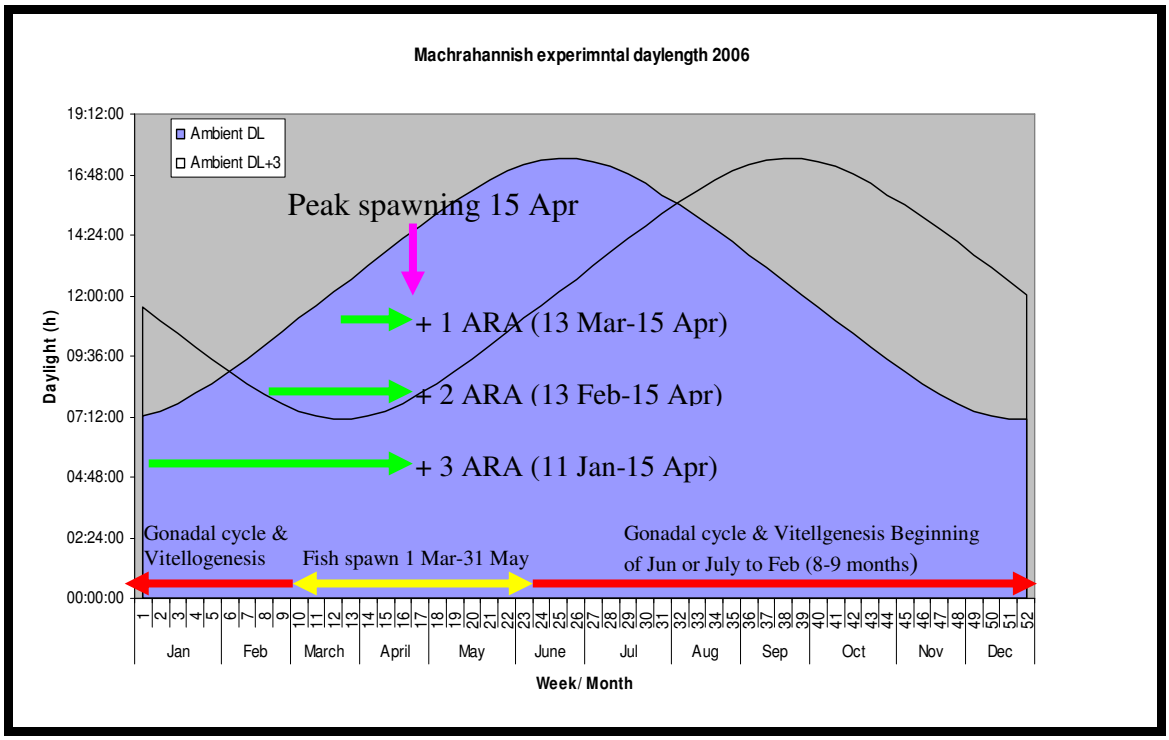

Figure 3.1 Daylength for fish at MERL during 2006 experimental period, reproductive strategy and feeding supplementation regime.

Red arrow indicates gonadal development and vitellogenesis. Yellow arrow indicates fish spawning period (peak spawning shown by the pink arrow). Green arrows indicates feed supplementation (1, 2 and 3 months).

A flow of seawater was maintained at all times but during summer when the water temperature rose the flow was reduced to allow a water cooler to operate and maintain temperature. The average water temperature during the experimental period was 8 ^{o}C (n=151, SD ± 1.3) and ranged between 5 ^{o}C - 10 ^{o}C. The average salinity was 28 ppt (n=151, SD ± 1.1) and ranged between 23 ppt – 33 ppt (Figure 3.2). The sharp reduction in salinity during March 2006 was most likely due to heavy rain at that time.

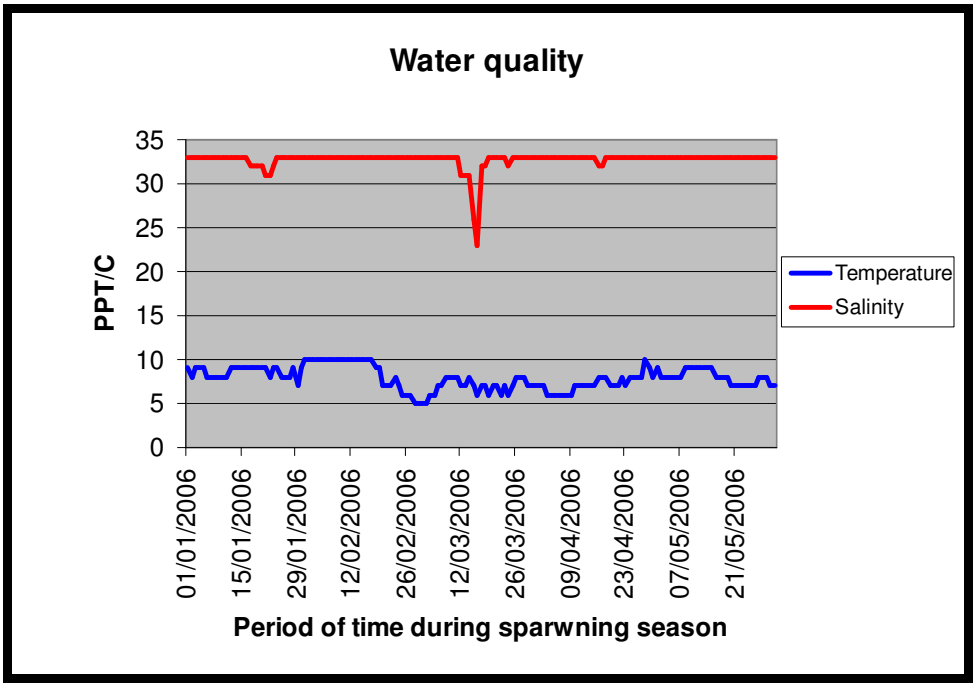

Figure 3.2 Water quality during cod spawning. Temperature (^{o}C) in blue and salinity (ppt) in red.

3.2.2 Experimental design

In January 2006 fish were reallocated, weighed and screened individually by ultrasound to determine gender and state of maturation so that there were sixteen males and eight or nine females in each tank. The biomass in each tank was; Group A (control) 78.6 kg, Group B (ARA supplement for 1 month) 75.6 kg, Group C (ARA supplement for 2 months) 86.4 kg, Group D (ARA supplement for 3 months) 78.9 kg. (Table 3.1). The average individual fish weight overall in each tank was 3.26 kg. The experiment used four treatment groups with one tank per treatment. Group A (control) was fed an unsupplemented control diet throughout the spawning period and Groups B, C and D were fed the ARA-supplemented diet for 1, 2 and 3 months prior to the peak-spawning date, respectively. During the experiment there were three mortalities of fish and, results were adjusted to account for this (Table 3.1).

Table 3.1 Experimental design: distribution and biomass of broodstock fish by treatment group (M = males, F = females & Kg = kilogram).

Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months, before peak spawning. Mortality of fish during experiment indicated.

Factor	Group A	Group B	Group C	Group D
Ratio of males to females	16 M: 9 F	16 M:8 F	16 M: 9 F	16 M: 8 F
Total biomass of male fish (Kg)	45.8	44.9	54.1	47.5
Total biomass of female fish (Kg)	32.8	30.8	32.4	31.4
Mean weight female (Kg)	3.64	3.85	3.60	3.92
Total biomass of fish (Kg)	78.6	75.6	86.4	78.9
Mortality	2 Females	No mortality	No mortality	1 Female

3.2.3 Feed

The basal feed was prepared as described in section 2.2. Vevodar® oil (DSM, Switzerland), containing 373 mg/kg ARA, was added to the mixture at a rate of 12 g/kg dry mix to prepare the ARA-supplemented feed. The concentration of Ax in the dry mix was 0.05 g/kg or 50 ppm.

Feed formula for Control group

The control group diet had no added ARA. Five kg dry meal was weighed and transferred to the mixer. Then 2.5 g of Carophyll Pink was weighed out along with 3.5 kg water. The mixer was switched on then the water added along with the Carophyll Pink. Mixing was continued until homogenous. The mixer was stopped when necessary

to loosen and crumble the compacted feed. When well-mixed the mixer was stopped and the feed removed by hand.

Feed preparation for the ARA supplemented group

Basal feed was prepared as described above. Vevodar oil was transferred into a spray bottle and the spray bottle was tared to zero on the balance. When the feed mix had the appearance of gravel, the mixer was stopped and the compacted mixture loosened and crumbled by hand. The mixer was re-started and 60 g Vevodar oil sprayed onto the mix. The weight of the spray bottle was checked frequently to avoid adding too much. The diet was mixed until homogenous. The mixer was stopped as necessary to loosen the compacted feed. When well mixed, the mixer was stopped and the feed removed by hand. Before feeding to the fish, the feed was chopped into small pieces (approx 15 x 15 x 15 mm) as shown in Figure 2.2 in Chapter 2.

Feed proximate compositions were; protein 38.35%, lipid 9.15%, ash 9.54%, moisture 39.40% and energy 20.89 Kcal (Table 3.2). Proximate composition of unsupplemented and supplemented diet were the same. Fish were fed to satiation twice daily. The concentrations of ARA in the feed, as measured by GC, were elevated from 0.65% of total fatty acids (approximately 0.56 g/kg) in the basal diet to 3.0% of total fatty acids (or approximately 2.58 g/kg finished feed) in the ARA supplemented feed (Table 3.3).

Table 3.2 Average of feed proximate compositions (n=3) for supplemented and unsupplemented diet.

Proximate compositions	Protein	Lipid	Ash	Energy	Moisture
Wet weight	38.35	9.15	9.54	20.89	39.40
Dry weight	63.29	15.10	15.74	20.89	

Values are % for protein, lipid, ash and moisture and Kcal/g for energy values.

Table 3.3 Fatty acid compositions of cod broodstock feeds with and without arachidonic acid. Values are % weight of total fatty acids.

Fatty acids	**Unsupplemented**	**Supplemented**
14:0	5.2	4.8
16:0	16.2	15.7
18:0	2.8	3.1
Total saturated	24.9	24.0
16:1n-9	0.0	0.0
16:1n-7	4.8	4.4
18:1n-9	12.8	13.2
18:1n-7	3.0	2.8
20:1n-9	9.6	9.4
22:1n-11	11.8	11.4
24:1n-9	1.3	1.2
Total monounsaturated	45.1	44.6
18:2n-6	4.8	5.1
20:3n-6	0.0	0.3
20:4n-6	0.6	3.2
22:5n-6	0.2	0.2
Total n-6 PUFA	6.0	9.3
18:3n-3	1.2	1.2
18:4n-3	2.6	1.3
20:4n-3	0.7	0.7
20:5n-3	6.6	6.3
22:4n-3	0.0	0.0
22:5n-3	0.9	0.9
22:6n-3	11.1	10.4
Total n-3 PUFA	23.4	21.1
Total 16 PUFA	0.5	1.0
Total PUFA	29.9	31.4
(n-3) / (n-6)	3.9	2.3
DHA/EPA	1.7	1.7
EPA/ARA	10.7	2.0

Values are mean, (n=4). Total saturated includes 15:0, 20:0 and 22:0.

The ARA-supplemented tanks were started on test diet with added ARA at three months before peak spawning on the 11th January 2006 (Group D), two months before peak spawning on the 13th February 2006 (Group C) and one month before peak

spawning on the 13th March 2006 (Group B). All tanks were fed this diet until spawning was completed (Figure 3.1).

3.2.4 Egg quality assessment

Fish spawned from 1st March to 31st May 2006 with peak spawning on 15th April 2006. Eggs were collected each day during the 92 day spawning period. The egg quality was measured for all batches collected. Egg quality was assessed using the standard methods described in Chapter 2. The parameters measured included total egg production, floating egg production and fertilization rate. The number of dead eggs remaining in each broodstock tank (dropout) was also measured over 24 h periods on five different days. To do this the broodstock tank was flushed by dropping the drain stand pipe and collecting the eggs in a net. The wet weight of eggs was measured using a balance.

Analysis of fatty acids was carried out on a sample of floating eggs from eleven to thirteen batches collected from all four tanks during the experimental period.

Hatching rates were measured by incubating samples of floating eggs from fourteen batches collected during the experiment. Hatching rates were measured for the same eggs batches as used for biochemical analysis and according to the protocol described in Chapter 2.

3.2.5 Biochemical analysis

The fatty acid composition, lipid classes and total lipid content of feed and egg samples were measured using standard laboratory procedures as explained in Chapter 2.

3.2.6 Data analysis

A number of egg quality indices were calculated to allow comparisons between groups. These included batch weights of eggs collected, batch weights of floating eggs, fertilization rate and hatch rate, and estimates of mean numbers per batch of eggs spawned, eggs collected, floating eggs, viable (ie floating, fertilised eggs) and hatched eggs. In order to compensate for small differences in broodstock biomass and allow comparison with other stocks these indices were calculated in terms of the biomass of female fish. A full description of the indices used is given in Chapter 2.

Statistical tests used to identify differences between groups included ANOVA or Kruskal-Wallis tests. Multiple comparison tests were used to identify differences between the group averages. The Mann -Whitney test was used to compare differences between control and pooled groups and ANOVA for comparing eggs produced / batch. Correlation between egg fatty acid composition and egg quality was measured using Spearman's rank test. Statistical analyses were carried out using Minitab 14.0 (Minitab Inc, 2004).

Data from lipid analyses were analysed for homogeneity using Levene's test. Those sets that did not have normal distribution were transformed using arcsine, square

root or log 10 functions before further analysis. Statistical analyses were carried out using SPSS 16.0 (SPSS Inc, 2007).

3.3 Results

3.3.1 Egg quality and fecundity

Table 3.4 and Figures 3.2 and 3.3 summarise the principal egg quality indices in the four treatment groups in this experiment. The data for all three ARA treatment tanks were also pooled for comparison with the control group.

In Group A (control), the total number of eggs produced per kg female was 590,185 the mean number per batch of eggs produced per kg female was 9,675. The total weight of eggs collected was 24660 g. The number of batches collected was 61 (out of the total 92 days of spawning). Spawning started on the 1st March 2006 and continued until the 31st May 2006. The cumulative egg production was less than from each of the groups fed ARA i.e. diets B, C or D (Fig.3.3). The total number of collected eggs per kg female was 376,339 and the mean number per batch of eggs collected per kg female was 6169. Approximately 47 % of the eggs sank and were discarded and the remaining eggs floated.

There was a total weight of 13,118 g of floating eggs which gave a mean weight per batch of floating eggs of 215 g. and a total number of floating eggs per kg female of 200,195 eggs. The mean number per batch of floating eggs per kg female was 3282 (Table 3.4).

Mean percentage fertilization (as % floating eggs) was 55 % and the total number of fertilised eggs per kg female 125,222. The total number of batches with fertilised eggs was 59. The mean number per batch of fertilised eggs per kg female was 2,122 and the mean percent hatch was 24 % equivalent to a total of 48462 hatched larvae per kg female (12.8 % of the total eggs collected).

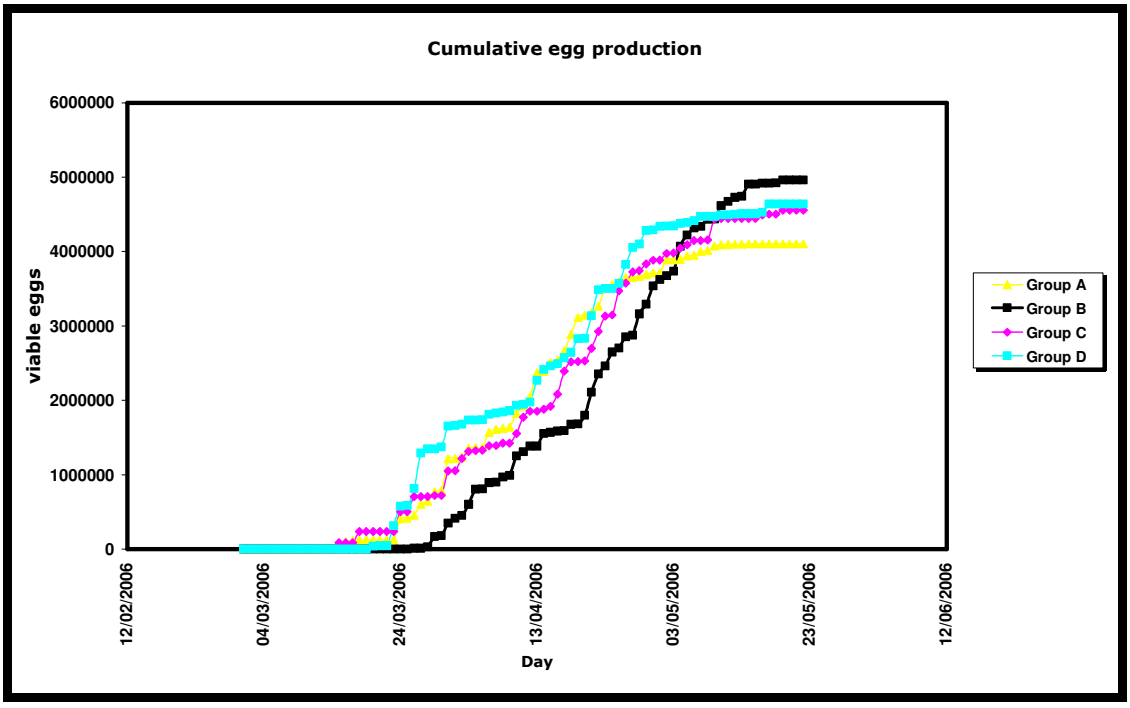

Figure 3.3 Cumulative egg production in the four treatment groups during spawning season.

Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months before peak spawning.

In Group B (Fed ARA 1 month), the total no. of eggs produced per kg female was 738,132 the mean number per batch of eggs produced per kg female was 11,905. The total weight of eggs collected was 34,220g. The number of batches collected was 62 (out of the total 92 days of spawning). Spawning started on the 13th March until the

31st May. This group started spawning last when compared to other groups (Fig 3.2). The mean weight of collected egg batches was 552 the total number of collected eggs per kg female was 556,097 and the mean number per batch of eggs collected per kg female was 8,969 (Table 3.4).

Approximately 49 % of the eggs sank and were discarded and the remaining eggs floated. There was a total weight of 17,352g of floating eggs which gave a mean weight per batch of floating eggs of 284 g. and a total number of floating eggs per kg female of 281,981. The mean number per batch of floating eggs per kg female was 4,623 (Table 3.4).

In terms of fertilization, the mean rate (as % floating eggs) was 56 % and the total number of fertilised eggs per kg female 171,736. The total number of batches with fertilised eggs was 57. The mean number per batch of fertilised eggs per kg female was 3,013 and the mean percent hatch was 18 % of floating eggs i.e. 9.4 % of total eggs collected. The total number of hatched larvae per kg female was 52,005 (Table 3.4).

In Group C (Fed ARA 2 months), the total number of eggs produced per kg female was 356,707 the mean number per batch of eggs produced per kg female was 7,134. The total weight of eggs collected was 20,729 g. The number of batches collected was 50 (out of the total 92 days of spawning). The mean weight of collected egg batches was 415 g, the total number of collected eggs per kg female was 320,337 and the mean number per batch of eggs collected per kg female was 6,407.

Approximately 41 % of the eggs sank and were discarded and the remaining eggs floated. There was a total weight of 12,161 g of floating eggs which gave a mean weight per batch of floating eggs of 248 g and a total number of floating eggs per kg

female of 187,931 eggs. The mean number per batch of floating eggs per kg female was 3,835.

Mean percentage fertilization (as % floating eggs) was 70 % and the total number of fertilised eggs per kg female 140,817. The total number of batches with fertilised eggs was 48. The mean number per batch of fertilised eggs per kg female was 2,934 and the mean % hatch was 20 % (as percent of floating eggs). This equated to 12 % hatch of total eggs collected.. The total number of hatched larvae per kg female was 38,526 (Table 3.4).

In Group D (fed ARA for 3 months), the total number of eggs produced per kg female was 572,813 the mean number per batch of eggs produced per kg female was 10,415. The total weight of eggs collected was 26,537. The number of batches collected was 55 (out of the total 92 days of spawning). The mean weight of collected egg batches was 482 g, the total number of collected eggs per kg female was 442,727 and the mean number per batch of eggs collected per kg female was 8,045 (Table 3.4).

Approximately 48 % of the eggs sank and were discarded and the remaining eggs floated. There was a total weight of 13,877g of floating eggs which gave a mean weight per batch of floating eggs of 262 g and a total number of floating eggs per kg female of 227,011eggs. The mean number per batch of floating eggs per kg female was 4,283 (Table 3.4).

Mean percentage fertilization (as % floating eggs) was 60 % and the total number of fertilised eggs per kg female 152,231. The total number of batches with fertilised eggs was 50. The mean number per batch of fertilised eggs per kg female was 3,045 and the mean percent hatch was 16 % (expressed as % floating eggs). This

equated to 8.2 % hatch of total eggs collected. The total number of hatched larvae per kg female was 36,225 and the mean no. per batch of hatched larvae per kg female 683 (Table 3.4).

The differences in the total numbers of eggs produced were because of the differences in the number and size of batches. For example, Group A and Group B produced more batches of eggs than Groups C and D. In addition Groups B and D, fed ARA for 1 month and 3 months respectively, produced the highest number of eggs per batch (Table 3.4). Egg production was therefore greatest in the Group B and lowest in Group C. Statistically significant differences in the numbers of eggs produced per batch were found between group B and the group C. However, there was no statistically significant difference in the number of eggs collected per batch between groups.

There was also a statistically significant difference found between the mean number per batch of eggs collected per kg female (Parameter No 7 in Table 3.4). This was significantly higher in group B than group A (Kruskal-Wallis, $p<0.05$). However, there was no other consistent increase in egg production in the groups fed ARA (B-D). In addition there was no statistically significant correlation between the total number of eggs produced by each group and the duration of ARA supplementation.

Egg quality was better in the groups which received the ARA-supplemented diet for 1, 2, 3 months when compared to the control group. The mean weight per batch of floating eggs per kg per female (Parameter No. 11, Table 3.4) was significantly greater in Group C and D when compared to group A and group B. In addition, the mean number per batch of floating eggs per kg female (Parameter No. 15, Table 3.4) was

significantly higher in the pooled ARA-treated groups when compared to Group A (Mann Whitney U = 5914.0, P< 0.05).

The mean number of floating eggs from the pooled Group fed ARA was 1.29 times greater than Group A. The number of floating eggs per batch was also greater in the groups that received the ARA diet (Fig 3.4).

The mean number per batch of viable (fertilized) eggs per kg female was significantly higher in the ARA-treated Groups (B-D) than in Group A (Fig 3.4). The mean number of viable eggs (measured as totally number of floating eggs) in the pooled ARA group was 1.41 times higher than in Group A. In addition the number of viable eggs per batch was higher in the pooled ARA group when compared to Group A.

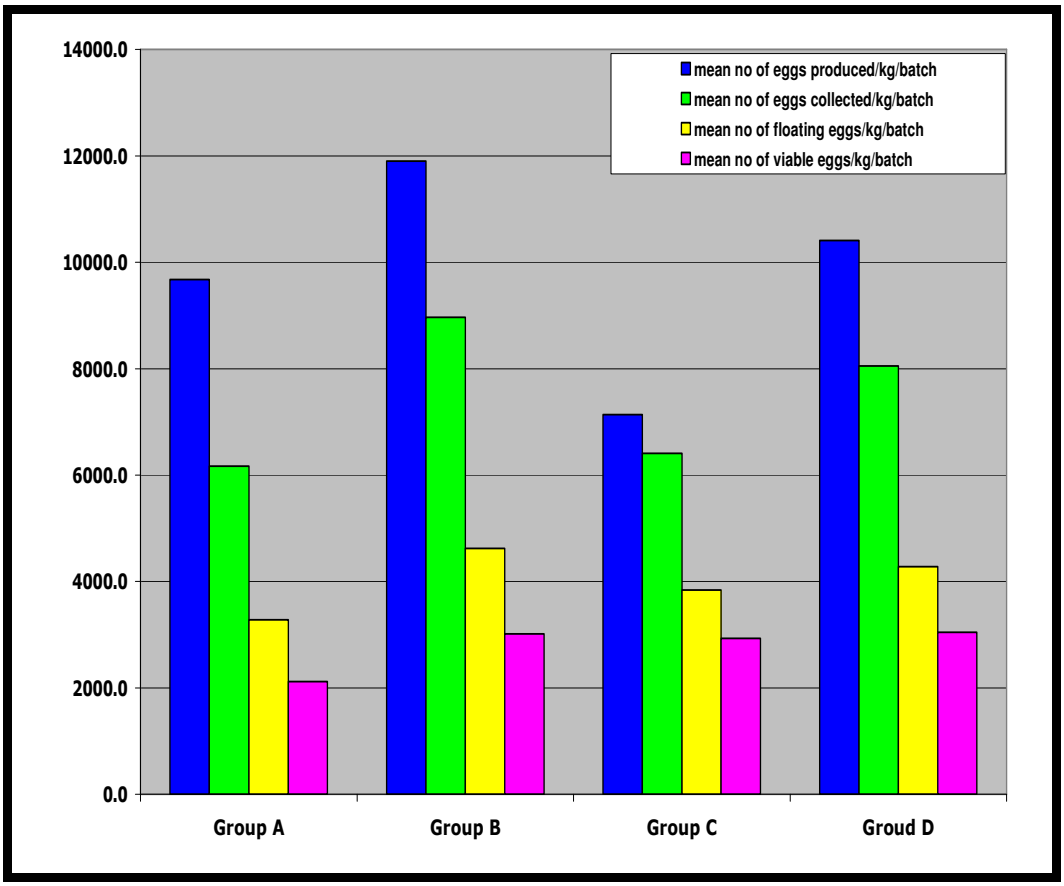

Figure 3.4 Egg production and egg quality parameters in cod broodstock fed an ARA supplement.

Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months before peak spawning.

Table 3.4 Egg production and egg quality indicators in experiment 1.

Egg numbers are expressed as numbers per kg female. Values within a row with a different superscript letter are significantly different ($p < 0.05$). Values within a row with * are significantly different ($p<0.05$), comparison only between Group A and all ARA treatment tanks pooled (B-D). Comparison made using Krusk Wallace test. Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months before peak spawning.

Parameter	Group A		Group B		Group C		Group D		+ ARA pooled	
	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N
1.Total no. of eggs produced/kg female	590185	61	738132	62	356707	50	572813	55	555884 ± 191275	167
2.Mean no. per batch of eggs produced/kg female	9675 ± 8065 ^{ab}	61	11905 ± 8869 ^{a}	62	7134 ± 6026 ^{b}	50	10415 ± 8103 ^{ab}	55	9818 ± 2441	167
3.Total weight of eggs collected (g)	24660	61	34220	62	20729	50	26537	55	27162 ± 6767	167
4.No. of batches collected	61	61	62	62	50	50	55	55	56 ± 6.0	167
5.Mean wt. of collected egg batches	404 ± 337	61	552 ± 411	62	415 ± 350	50	482 ± 384	55	483 ± 69	167
6.Total no. of collected eggs/kg female	376339	61	556097	62	320337	50	442727	55	439720	167
7.Mean no. per batch of eggs collected/kg female	6169± 5143 ^{b}	61	8969 ± 6682 ^{a}	62	6407 ± 5412 ^{ab}	50	8045 ± 6263 ^{ab}	55	7809 ± 1298	167
8.Total weight of floating eggs (g)	13118	61	17352	61	12161	49	13877	53	14463 ± 2645	163
9.Mean wt. per batch of floating eggs (g)	215 ± 245	61	284 ± 226	61	248 ± 247	49	262 ±249	53	265 ± 18	163
10.Total no. of floating eggs/kg female	200195	61	281981	61	187931	49	227011	53	232308	163
11.Mean no. per batch of floating eggs/kg female	3282* ± 3736	61	4623 ± 3671	61	3835 ± 3824	49	4283 ± 3954	53	4247* ± 395	163
12Mean fertilization rate (% floating eggs)	55% ^{b}	61	56% ^{ab}	61	70% ^{a}	49	60% ^{ab}	53	62 ± 7.2	163
13.Total no. of fertilised eggs/kg female	125222	59	171736	57	140817	48	152231	50	154928 ± 15635	155
14.No. of batches with fertilised eggs	59	59	57	57	48	48	50	50	52 ± 4.7	155
15.Mean no. per batch of fertilised eggs/kg female	2122* ± 2181	59	3013 ± 2853	57	2934 ± 3006	48	3045 ± 3656	50	2997* ± 57	155
16.Mean percent hatch (% floating eggs)	24	61	18	61	20	49	16	53	19 ± 1.2	163
17.Total no. of hatched larvae/kg female	48462	61	52005	61	38526	49	36225	53	42252 ± 8525	163
18.Mean no. per batch of hatched larvae/kg female	794 ± 904	61	853 ± 681	61	786 ± 784	49	683 ± 631	53	774 ± 86	163

Table 3.5 Fatty acid composition (% by weight of total fatty acids) of Atlantic cod eggs.

Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months, before peak spawning. Data from Group B, C and D were pooled in + ARA pooled column. Data analysed using ANOVA.

Fatty Acid	Group A	Group B	Group C	Group D	ARA Pooled
14:0	2.2 ± 0.6	2.1 ± 1.0	2.3 ± 0.9	1.8 ± 0.7	2.0 ± 0.3
16:0	24.9 ± 4.1	24.5 ± 2.7	27.0± 2.5	23.1 ± 3.2	24.8 ± 2.0
18:0	3.9 ± 0.8 [a]	4.1 ± 0.6 [ab]	4.6 ± 0.7 [a]	3.5 ± 0.5 [b]	4.0 ± 0.6
Total saturated	32.2 ± 5.2[ab]	32.3 ± 4.1[ab]	36.1± 5.5 [a]	29.4 ± 4.3[b]	32.6 ± 3.4
16:1n-9	1.6 ± 0.5	1.4 ± 0.4	1.6 ± 0.8	1.4 ± 0.3	1.5 ± 0.1
16:1n-7	2.3 ± 1.0	2.1 ± 0.8	2.8 ± 3.1	2.0 ± 0.7	2.3 ± 0.4
18:1n-9	9.7 ± 4.7	9.3 ± 3.3	9.7 ± 3.7	9.9 ± 3.7	9.5 ± 0.3
18:1n-7	2.4 ± 1.6 *	2.3 ± 1.1	2.3 ± 0.9	2.5 ± 1.3	2.3 ± 0.4 *
20:1n-9	3.0 ± 1.4	2.2 ± 1.5	2.1 ± 0.9	2.3 ± 1.5	2.4 ± 0.1
22:1n-11	1.6 ± 1.3	1.4 ± 1.5	1.0 ± 0.6	1.5 ± 1.2	1.3 ± 0.3
24:1n-9	0.5 ± 0.2	0.4 ± 0.2	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.0
Total monounsaturated	21.2 ± 8.3	19.4 ± 6.7	20.5 ± 6.3	20.3 ± 6.5	20.1 ± 0.6
18:2n-6	3.6 ± 0.6	3.1 ± 0.4	3.3 ± 0.1	2.9 ± 1.0	3.1 ± 0.2
20:3n-6	0.2 ± 0.1 *	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0 *
20:4n-6	1.8 ± 0.4 [b]	2.1 ± 0.7 [bc]	3.0 ± 1.2 [a]	2.2 ± 0.5 [ab]	2.4 ± 0.5
22:5n-6	0.3 ± 0.0 [a]	0.2 ± 0.0 [ab]	0.2 ± 0.1 [b]	0.2 ± 0.1 [ab]	0.2 ± 0.0
Total n-6 PUFA	6.2 ± 1.1	5.9 ± 1.0	7.1 ± 2.0	5.8 ± 0.8	6.3 ± 0.7
18:3n-3	0.6 ± 0.4	0.6 ± 0.3	0.8 ± 0.7	0.5 ± 0.1	0.6 ± 0.2
18:4n-3	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0
20:4n-3	0.5 ± 0.1 [a]	0.4 ± 0.1 [b]	0.4 ± 0.1 [ab]	0.5 ± 0.1 [a]	0.4 ± 0.1
20:5n-3	14.5 ± 2.9	12.1 ± 1.7	12.1 ± 2.5	13.2 ± 1.7	12.5 ± 0.7
22:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	2.0 ± 0.6* [a]	1.5 ± 0.2 [b]	1.6 ± 0.2 [ab]	1.7 ± 0.2 [ab]	1.6 ± 0.1 *
22:6n-3	31.5 ± 5.9 [a]	26.8 ± 3.8 [ab]	25.3 ± 5.0 [b]	29.1 ± 4.3 [ab]	27.1 ± 1.9
Total n-3 PUFA	44.9 ± 7.1 [a]	41.9 ± 5.8 [ab]	40.8± 8.1 [b]	45.6 ± 6.0 [ab]	42.7 ± 2.5
Total 16 PUFA	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.5 ± 0.1
Total PUFA	51.6 ± 7.8	48.3 ± 6.4	48.5 ± 10.0	51.8 ± 6.4	49.5 ± 2.0
(n-3) / (n-6)	7.4 ± 1.1* [a]	7.2 ± 1.0 [a]	5.8 ± 0.7 [b]	7.8 ± 1.5 [a]	7.0 ± 1.0 *
DHA/EPA	2.2 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.2 ± 0.13
EPA/ARA	8.4 ± 1.2 [a]	6.3 ± 1.7 [b]	4.4 ± 1.1 [c]	6.1 ± 0.8 [b]	5.6 ± 1.5

Values are mean ± S.D. Group A (n=11), Group B (n= 13), Group C (n=11), Group D (n=12) and Pooled (n= 36). Values within a row with the different superscript letter are significantly different p <0.05. Total saturated includes 15:0, 20:0 and 22:0.
Values within a row with * are significantly different p<0.05, comparison only between Group A(n=11) and all ARA treatment tanks pooled (B-D)(n=36)

3.3.2 Fatty acid composition of eggs

The results of selected fatty acids in eggs are also summarised in Table 3.5. There was no statistically significant difference between levels of EPA or the ratio of DHA/EPA between control and those fed ARA. There was a significant difference in DHA levels between Group C (25.3 %) and Group A (31.5.1 %) but not between other groups. There was a statistically significant difference in ARA levels between Groups A and C, and between Groups B and C. The lowest mean ARA levels (%) were found in the control group and highest in Group C (Fig 3.5). In addition, there was a significant difference in the EPA/ARA ratio between the control group and all diets with ARA. The highest ratio of EPA/ARA was in the control group and the lowest in the group C.

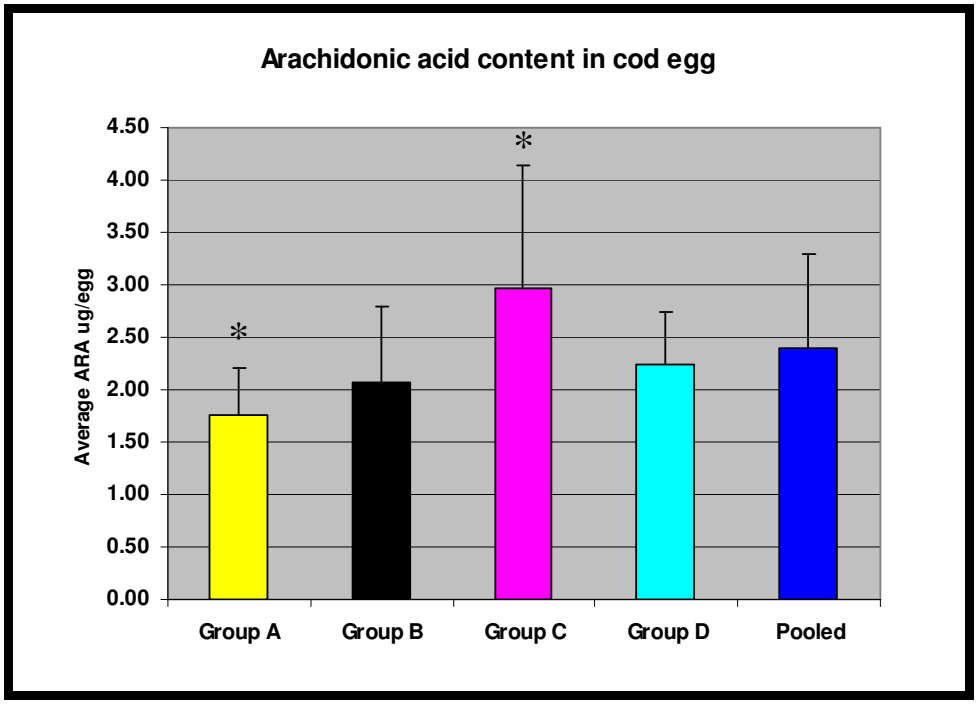

Figure 3.5 Egg ARA content and feeding treatment**.**

Values in the figure with * are significantly different $p<0.05$. Data analyed using ANOVA. Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2

months, Group C fed ARA for 3 months, before peak spawning. Data from Group B,C and D were pooled.

Significant differences in egg EPA/ARA ratio were also found between the control group (A) and all other groups, and between the group B and C ($p<0.001$, r = 19.5 %).

There was a significant correlation found between egg ARA level and the date of collection for group B ($p<0.01$, r = 42 %,). In addition, multiple regression analysis of overall data showed a significant correlation between eggs ARA levels and EPA/ARA ratio ($p<0.001$ r = 87.2 %.). EPA and DHA levels and DHA/EPA ratio were also correlated ($p<0.001$, r =98.7 %). However, there was no other correlation found.

There was a significant difference between levels of specific fatty acids in group A and the ARA pooled group including; Total saturated ($F=6.620$, $p < 0.05$), 18:1n-7 ($F= 4.641$, $p < 0.05$), 20:3n-6 ($F= 5.509$, $p < 0.05$), 22:5n-3 ($F=12.738$, $p < 0.05$) and (n-3) / (n-6) ratio ($F= 8.868$, $p < 0.05$) (Table 3.5).

The results of lipid class analysis of eggs are summarised in Table 3.6 and Fig 3.4. The main lipid class found in Atlantic cod eggs was phosphatidylcholine (PC), the second largest class was TAG, followed by cholesterol (CHOL), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and free fatty acids (FFA) respectively. There were no significant differences in the percentages of PI, PE, CHOL, FFA and TAG between groups.

PC percentage was significantly higher in group D (36.2%) compared to group C (30.7 %). Polar lipids percentage was significantly higher in Group D (56.6 %) compared to Group C (48.6%). Neutral lipids percentage was significantly higher in Group C (51.4 %) compared to Group D (43.4 %). However, there was no significant

difference in lipid class percentage between Group A and the pooled groups (B-D) results for all ARA-supplemented treatments.

Table 3.6 Egg lipid classes for the four individual dietary treatments and for the pooled ARA supplemented treatments.

(Group A n= 11, Group B n= 13 Group C n= 11 Group D n= 12).

FA (%)	Group A	Group B	Group C	Group D	ARA Pooled
	Value ± SD	Value ± SD	Value ± SD	Value ± SD	Value ± SD
Phosphatidylcholine	33.6 ± 3.1 ^{ab}	33.7 ± 4.7 ^{ab}	30.7 ± 4.4 ^{b}	36.2 ± 4.0 ^{a}	33.6 ± 4.8
Phosphatidyethanolamine	2.4 ± 1.7	2.5 ± 2.1	1.3 ± 1.5	2.7 ± 1.5	2.2 ± 1.8
Phosphatidylinositol	13.1 ± 1.8	13.9 ± 1.3	13.6 ± 2.4	14.8 ± 1.3	14.1 ± 1.8
Cholesterol	18.3 ± 2.2	17.6 ± 2.2	16.8 ± 2.0	18.1 ± 2.1	17.5± 2.1
Free fatty acids	1.7 ± 1.7	2.5 ± 2.9	3.8 ± 4.0	2.2 ± 1.6	2.8 ± 3.0
Triacylglycerol	22.3 ± 4.5	21.5 ± 3.6	21.9 ± 3.4	20.5 ± 2.0	21.3 ± 3.1
Total polar	54.0 ± 4.7 ^{ab}	53.2 ± 5.3 ^{ab}	48.6 ± 7.5 ^{b}	56.6 ± 3.4 ^{a}	52.8 ± 6.4
Total neutral	46.0 ± 4.7 ^{ab}	46.8 ± 5.3 ^{ab}	51.3 ± 7.5 ^{a}	43.4 ± 3.4 ^{b}	47.2 ± 6.4

Values within a row with a different superscript letter are significantly different $p < 0.05$. Data analysed using ANOVA. Pooled values are tanks B-D combined. Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months, before peak spawning.

When lipid class percentages were correlated with ARA levels for all tanks there were significant correlations detected between: PE and EPA/ARA ratio ($p<0.05$, r = 29.4 %). There was no correlation between TAG and other lipid classes.

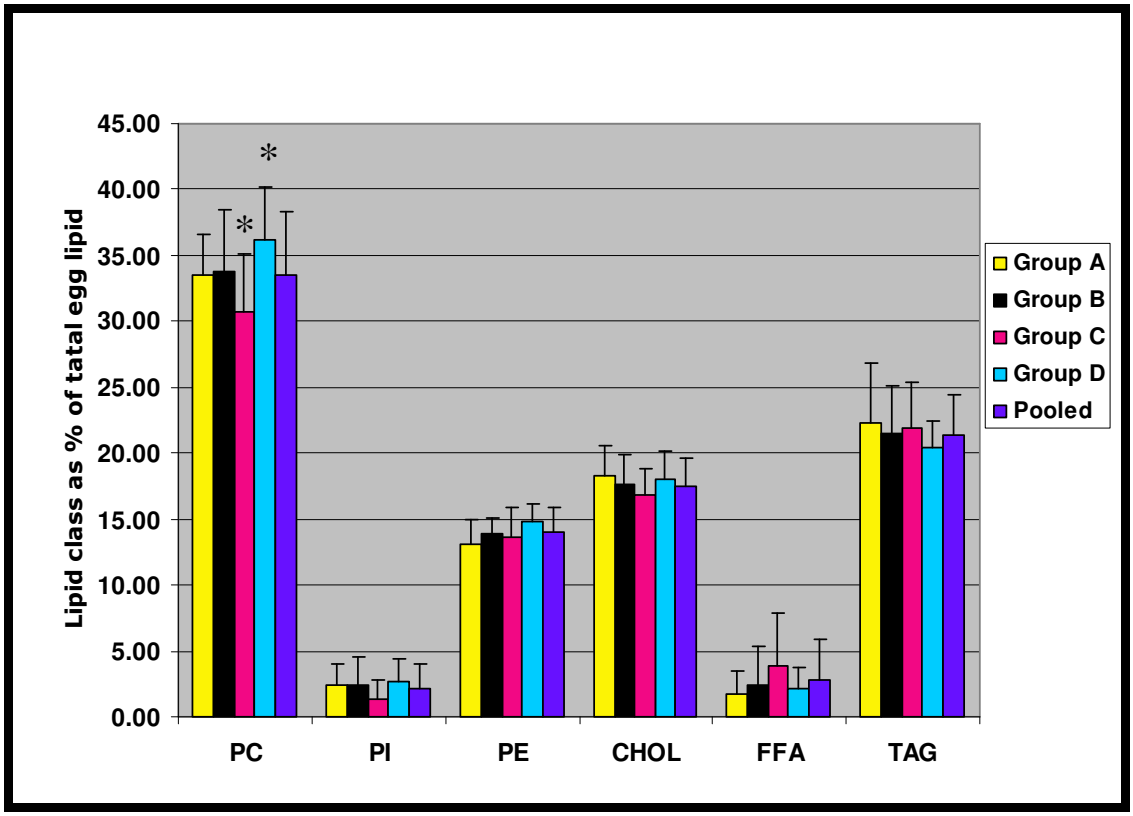

Figure 3.6 Lipid class compositions of cod eggs.

Values in the figure with * are significantly different $p<0.05$. Data analysed using ANOVA. Group A (control), Group B fed ARA for 1 month, Group C fed ARA for 2 months, Group C fed ARA for 3 months, before peak spawning. Data from Group B,C and D were pooled.

3.4 Discussion

3.4.1 Egg quality and fecundity

Previous studies have shown the importance of ARA in the diet of cod and other species. ARA has been linked to hatching success and other egg quality parameters in wild cod (Pickova et al. 1997; Salze et al. 2005). ARA plays a role as a precursor of

eicosanoids and in the control of ovulation, embryogenesis, development of the immune system, hatching and early larval performance (Tocher & Sargent 1987; Mustafa & Srivastava 1989; Bell et al. 1994). It would be expected that supplementation of broodstock diets with ARA should lead to improved fecundity and egg quality as has been shown in other species, for example European sea bass, Japanese flounder and halibut (Bruce et al. 1999; Furuita et al. 2003; Mazorra et al. 2003). The results of this experiment show that ARA supplementation of diet for 1 month, 2 months or 3 months before peak spawning did not significantly increase the overall number of eggs produced. However, greater numbers of eggs per batch were produced by fish receiving the ARA supplement and this parameter was 1.29 times higher when compared with the control group. In addition, the ARA-supplemented batches of eggs had greater numbers of floating fertilized eggs. For hatcheries, fewer and larger batches of eggs are better for incubating than more, smaller batches. When data from all the groups receiving an ARA supplement were pooled they were found to have 29 % more mean number of floating eggs/kg female and 41% more mean number per batch of fertilised eggs than the control group.

These results were not consistent between groups. The reasons for this finding could be due to natural variability in condition between broodstock populations in the tanks. However, it should be noted that many other factors can influence egg quality including genetics, broodstock age (Evans et al. 1996), differences between spawning seasons, stage in spawning cycle and other environmental factors. Evans et al (1996) for example, showed that some egg quality parameters do increase with the age of the broodstock of Atlantic halibut.

Other studies have also shown similar inconsistent results with ARA supplementation of farmed cod broodstock. For example, when cod were fed diets containing 0.5, 1, 2 and 4% ARA, it had no effect on gonadosomatic index. Fecundity was higher in the group fed 1% ARA, whilst there were no differences in egg quality between groups (Rosenlund 2006). In addition, long term supplementation of cod diet with ARA for 5 months had no significant impact on egg quality and fecundity (Blanco 2005). These results contrast with the finding of studies on other species which have shown that ARA supplementation improved egg quality and fecundity, such as in halibut (Mazorra 2000; Bromage et al. 2001) and haddock (Castell et al. 2001).

3.4.2 Fatty acid composition of eggs

The results of this experiment show that ARA was successfully transferred from diet to broodstock to eggs which confirms the findings of other authors (Blanco 2005). The control group had the lowest concentration of ARA, with the highest concentration in eggs from the group fed ARA for 2 months before peak spawning. The eggs from the group fed ARA for 3 months before peak spawning had a level of ARA between those fed ARA for 1 month. In practical terms for hatcheries, this would indicate that supplementation for 2 months before peak spawning is enough time to increase egg ARA concentration during spawning.

There was no correlation between time of supplementation of ARA and EPA, DHA levels or EPA/ARA ratio and DHA/EPA ratios in eggs. This would suggest that increasing ARA levels in the diet does not impact on the level of these other fatty acids in eggs.

In eggs from the group fed ARA for 1 month before peak spawning there was a positive correlation between egg ARA content and date of sample collection (Spearman's $r=0.687$, $p<0.01$). This would suggest that ARA was being accumulated in these eggs as the season progressed. This correlation was not found in the other groups suggesting that ARA had accumulated in these eggs and may already have attained a maximum level of incorporation. The mechanism for this is not known but may be related to individual fish in each tank accumulating different levels of ARA based on feed intake.

The results showed no significant difference in the egg DHA/EPA ratio between groups fed an ARA supplement. However, these ratios were consistent with those found in other fish species at around 2:1 (Sargent 1995). This ratio is considered important because a diet high or deficient in ARA, DHA and EPA can influence levels of eicosanoids in eggs. This in turn can have a negative impact on egg production, larval growth and development through increased stress (Sargent 1995). In the present study, the ratio between EPA/ARA ratio was highest in eggs from the control group and lowest in eggs from the group fed ARA for 2 months. It has been suggested that low EPA/ARA ratio may give better egg quality (Blanco 2005). This would suggest that egg quality in the groups fed ARA should be better though this was not consistently found to be the case. From the present study the ratio of (n-3) / (n-6) showed a significant difference between Group A 7.4 and Group D, 7.0 (ANOVA $F= 8.868$, $p < 0.05$) (Table 3.5). Ratios of 5:1 to 10:1 for (n-3) / (n-6) PUFA have been proposed as optimal (Bromage 1995).

There was a significant difference found between PC percentages and timing of ARA supplementation with the highest in the group fed for 3 months before peak

spawning. In cod, PC is the major egg lipid class (Fraser et al. 1988). Cod and other species such as Atlantic herring (*Clupea harengus*) and halibut have no oil globule in the eggs and yolk sac larvae. These fish have low levels of neutral lipids and consequently high levels of phospholipids, predominantly PC (Salze et al. 2005; Tocher et al. 2008). PC is utilised during both embroyogenesis and early larval development (Tocher et al. 1985; Fraser et al. 1988; Rainuzzo et al. 1992; Finn et al. 1995). In the present study, the group fed ARA for 3 months had significantly higher levels of polar lipid. It has been proposed that polar lipids may promote growth in juvenile cod (Olsen et al. 1991). Therefore, the group fed ARA for 3 months may have been expected to have had better performance compared to the other groups. However, there was no significant correlation between lipid class percentage, ARA or other fatty acids and the duration of supplementation with ARA. There were no other correlations between lipid class percentages and timing of ARA supplement even with pooled groups.

ARA is preferentially concentrated in PI in fish eggs (Bell & Dick 1991). Once ARA has reached its optimum level any further addition will be deposited in other phospholipid classes and TAG rather than more in PI. This could also be a means for eggs to conserve ARA for future larval development. In other species such as the Japanese flounder, larval growth improved with PC percentage, but not with PI or PE increase (Kanazawa 1993).

Therefore, there are no clear patterns between species. The supplementation of diet may result in changes to the lipid class ratio but result in no correlation with ARA level.

3.5 Conclusion

In conclusion, it was found that short term supplementation of broodstock diets with ARA for 1, 2 or 3 months before peak spawning resulted in increased concentrations of ARA in eggs. Supplementation for more than 2 months did not provide any significant increase in egg ARA. This suggests egg ARA may have reached a plateau level then fallen back but was not increased further by a longer period of ARA feeding.

There was no correlation between egg production or egg quality parameters and the length of time supplementation was provided. However, there were higher numbers per batch of floating eggs per kg per female and fertilized eggs per kg female in the three tank groups fed the ARA supplement than in the unsupplemented control group.

These results also suggest that the performance of the control group was affected by the lack of ARA supplementation in the diet as they had the lowest performance when compared to the pooled ARA groups. However, while the supplementation of diets with ARA to 3 % of total fatty acids provided some limited benefit it was not a major factor for improved reproductive performance.

Chapter 4. Evaluation of the effect of astaxanthin (Ax) supplementation on egg quality in farm-reared cod

4.1 Introduction

Carotenoids, particularly Ax, are essential pigments with a wide range of functions (as discussed in Chapter 1,). Ax is the major carotenoid pigment in the marine environment and cannot be synthesised by fish (Schiedt 1998). Therefore it must be obtained entirely through the diet, which must contain sufficient levels (Davies 1985; Matsuno & Hirao 1989; Schiedt 1998). It is also one of the most important carotenoids for cod eggs and larvae and has been shown to be essential for growth of fish and as a pigment source (Torrissen & Christiansen 1995). For example, a lack of carotenoids in the diet of broodstock has been shown to result in low fertility and deformities in larvae (Pavlov et al. 2004). Nutrition is especially important for cod broodstock particularly since farm-reared fish may be conditioned for spawning in tanks and fed formulated feed over a period of several years. More than 600 naturally occurring carotenoids have been identified in vegetables, fruits and seafoods although they mostly originate in plants, photosynthetic bacteria and algae where they are accessory pigments in photosynthesis and photoprotection (Isler 1981).

The influence of nutrient availability on reproductive physiology and broodstock performance in fish has been reviewed previously (Hardy 1985; Bromage 1995; Pavlov et al. 2004) These studies have investigated the effects of a number of nutrient supplements including PUFA, vitamins C and E, and the carotenoid pigment Ax. In cod, differences in carotenoid pigment concentration have previously been identified between wild and farmed cod broodstock (Salze et al. 2005) These nutritional differences were correlated with differences in egg quality, suggesting that sub-optimal levels of carotenoid pigment may cause some egg quality problems in farmed cod

(Salze et al. 2005). For example, Salze et al. (2005) found that carotenoid concentrations were lower in eggs from farmed cod than eggs from wild cod. Similarly, Grung et al. (1993) also found lower concentrations of carotenoid pigment in eggs from farmed cod than wild cod and demonstrated that dietary carotenoid supplementation resulted in an increased carotenoid concentration in the eggs. Numerous functions have been proposed for carotenoids in fish eggs including UV protection, provitamin A activity, improved respiratory function (Craik 1985; Mikulin 2000) and antioxidant protection against free-radical damage (Edge et al. 1997). These findings suggest that carotenoids are important in ensuring normal embryonic development and could also affect hatching rates and larval survival (Torrissen 1984; Craik 1985; George et al. 2001). Carotenoids are also a source of pigmentation in the embryo (Pan et al. 2001) and may be involved in photoreception processes (Rønnestad et al. 1998). Supplementation of broodstock diets with Ax has also been shown to improve egg quality in red sea bream and yellowtail (Watanabe & Miki 1993; Verakunpiriya et al. 1997). Dietary carotenoid supplements have also shown a positive relationship between egg pigmentation and fertilization as well as survival of rainbow trout eggs (Harris 1984; Craik 1985) while (Svensson et al. 2006) found the colouration of female *Gobiusculus. flavescens* (two-spotted goby) was strongly related to the carotenoid content of the eggs.

The aim of the experiment reported here was to evaluate the effect of short-term supplementation of Ax in broodstock diets on a number of egg quality parameters in farmed cod. Duplicate groups of farmed cod broodstock were fed either a control diet with no Ax supplement, or an Ax supplemented diet, for two months prior to peak spawning. Egg numbers were expressed in terms of female biomass to permit

comparisons between stocks. The Ax content of eggs was carried out to examine the effects of dietary treatment on Ax content.

4.2 Materials and methods

4.2.1 Fish husbandry and diets

The experimental design used two treatment groups of Atlantic cod broodstock each housed in duplicate tanks. The control group was fed an unsupplemented diet with no added Ax throughout the spawning period while the treatment group was fed an Ax supplemented feed, at a measured inclusion level in the finished feed of 73.7 mg/kg dry weight, for two months prior to the peak-spawning date. The broodstock were farm-reared fish and were allocated to four fibreglass $7m^3$ tanks in November 2005. Tanks were supplied with seawater at 40 L/min in a flow-though system. The average water temperature during the experimental period was 8 ^oC and the average salinity was 33 ppt (Figure 4.1). The sharp reduction in salinity during March 2006 was most likely due to heavy rain at that time.

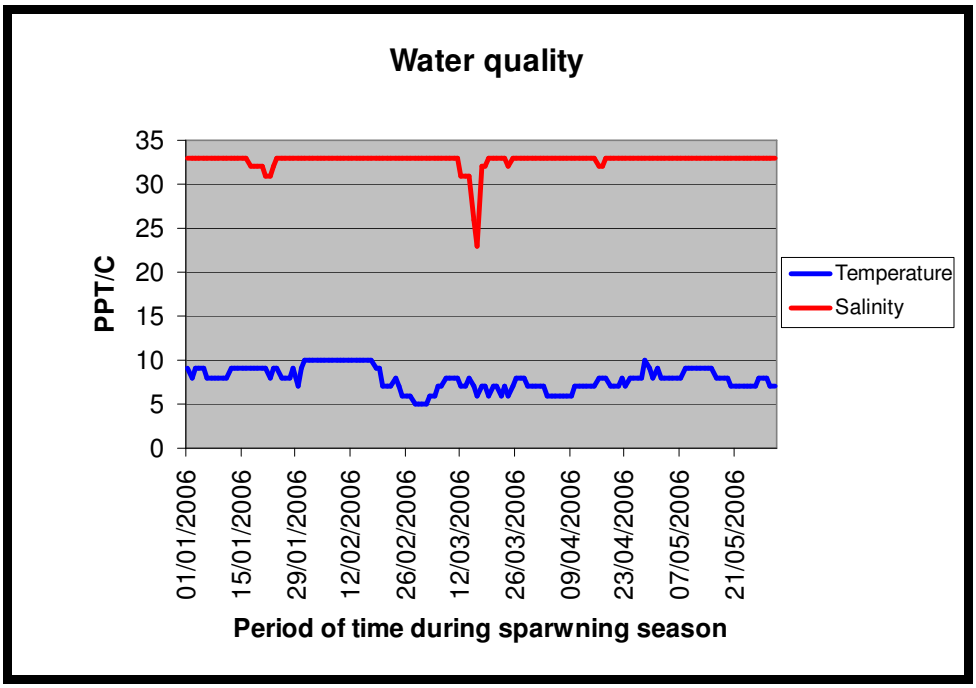

Figure 4.1 Water quality during cod spawning. Temperature (^{o}C) in blue and salinity (ppt) in red.

Experimental design

In January 2006, fish were weighed individually, screened by ultrasound to determine gender and state of maturation and reallocated so that each tank contained a similar number and biomass of males and females. After allocation each tank contained 34 or 35 males and 35 or 36 females (Table 4.1). The biomass in each tank was; Tank 1 unsupplemented 1, 89.4 kg, Tank 2 unsupplemented 2, 89.0 kg, Tank 3 Ax treatment 1, 91.5 kg and Tank 4 Ax treatment 2, 90.0 kg. The average individual fish weight in each tank was 1.29 kg.

Table 4.1 Experimental design and distribution of broodstock fish for experiment 2

Factor	Tank 3.1 Control	Tank 3.3 Control	Tank 3.2 Ax	Tank 3.4 Ax
Ratio of males to females	34 M: 35F	34 M: 36 F	34 M:36 F	35 M: 36F
Total biomass of male fish (Kg)	40.4	39.6	39.6	41.7
Total biomass of female fish (Kg)	49.0	50.4	51.9	48.2
Mean weight female (Kg)	1.40	1.39	1.44	1.34
Total biomass of fish (Kg)	89.4	88.9	91.5	90.0
Female mortality during the spawning period	3	1	4	4

Experimental design: distribution and biomass of broodstock fish by treatment group (M = males, F = females & Kg = kilogram). Control tanks no feed supplementation and Ax tanks feed supplemented with Ax. Mortality of fish during experiment indicated.

4.2.2 Feed

Basal feed was prepared as described in section 2.2. For the Ax supplemented feed, Carophyll Pink® (DSM, Basel, Switzerland), with a nominal Ax content of 10% w/w, was added at a rate of 1g Carophyll Pink per kg dry mix. The concentration of Ax in the finished feed, as measured by HPLC, was 73.7 mg/kg dry weight. Fish were fed to satiation twice daily.

Feed formula for Control group

Vevodar oil was transferred from the storage container to a spray bottle. Five kg dry meal was weighed and transferred to the mixer and 3.5 kg water, added and mixed.

The spray bottle was placed on the balance and tared to zero. When the feed mix had the appearance of gravel, the mixer was stopped and the compacted mixture loosened and crumbled by hand. The mixer was re-started and 60 g Vevodar oil was sprayed onto the mix. The diet was mixed until homogenous. The mixer was stopped when necessary to loosen the compacted feed. When the feed was well-mixed, the mixer was stopped and the feed removed by hand.

Feed formulation for treatment group (supplemented with 100 ppm Ax)

The Ax supplemented diet was prepared as for the control except that 5g Carophyll Pink was added to the diet. Before feeding to the fish the feed was chopped into small pieces (approx 15x15x15 mm) as shown in Figure. 2.2 from General Materials & Methods (Chapter 2).

4.2.3 Egg quality assessment

The spawning period was regarded as the period from 1st March to 31st May 2006, and the peak spawning date was 15th April 2006. Each day during the 92 day spawning period, egg batches were collected and egg quality was assessed using standard techniques to measure total egg production, floating egg production and fertilization rate. Dropout (number of sinking unfertilised eggs) within each tank was measured, over a 24 h period, on five different dates. Samples of floating eggs (good quality and mainly fertilised eggs) were collected on 14 different dates for hatch rate determination and fertilization rate. Ax analysis was carried out on floating eggs collected from each tank on 11 different dates during the course of the spawning period.

4.2.4 Biochemical analysis of astaxanthin concentration in feed and eggs

Carotenoid pigments, including Ax, were extracted from cod eggs largely using the method of (Barua et al. 1993) Barua et al. (1993). Eleven samples of 20 eggs were collected from each of the four broodstock tanks over the spawning period and stored in chloroform/methanol (2:1 v/v) with 0.01% (w/v) BHT. The values presented for Ax are average values for each tank (n =11). Total lipid was extracted from the egg samples by the method of Folch et al. (1957) as explained in Chapter 2.

Carotenoid in diets was extracted after enzymatic digestion with Maxatase enzyme (International Biosynthetics, Rijswijk, Netherlands). Portions of ground diet (1g) were mixed with 10 mL water and 110 mg Maxatase in a 50 mL stoppered glass tube followed by incubation in a water bath at 50 ^{o}C for 30 min. Samples were then extracted with 5 mL of absolute ethanol and 5 mL of ethyl acetate on a vortex mixer for 5 min. The homogenate was centrifuged (1000 x *g*, 5 min) and the supernatant removed to a stoppered glass tube. The pellet was re-extracted in 5 mL ethyl acetate, centrifuged, and the supernatant combined with the first supernatant. Finally, the pellet was re-extracted in 10 mL isohexane, centrifuged, and the supernatant combined with the pooled supernatant. The pooled supernatant was dried under N_2 and vacuum desiccated for 2 h before dissolving the residue in 2 mL of isohexane prior to analysis. The Ax was separated and quantified using the HPLC method described above.

4.2.5 Data analysis

Egg quality indices used for group comparisons included: batch weights of eggs collected, batch weights of floating eggs, fertilization rate and hatch rate, and estimates of mean numbers per batch of eggs spawned, eggs collected, floating eggs, viable (i.e.

floating, fertilised eggs) and hatched eggs. Numbers were calculated in terms of the biomass of female fish to compensate for small differences in broodstock biomass and allow comparison with other stocks. ANOVA, or Kruskal-Wallis non-parametric tests, were used to identify differences in egg quality, or biochemical parameters, between individual groups. Group comparisons were made using ANOVA with tank as a factor nested within each treatment. Where differences were identified, appropriate multiple comparison tests were used to identify differences between the group averages. Spearman's rank test was used to detect any correlation between fatty acid composition and egg quality.

4.3 Results

Total carotenoid pigment concentration in the unsupplemented control diet was 14.8 mg/kg and in the Ax-supplemented diet was 73.7 mg/kg. The concentrations measured in the eggs were 0.98 ± 0.48 and 2.79 ± 0.10 ng/egg for the unsupplemented and Ax supplemented groups, respectively (Fig. 4.2 and 4.3). A significant correlation was detected.between egg Ax content and fertilization rate (Spearman's $r = 0.3061$, $P < 0.01$) in individual egg batches.

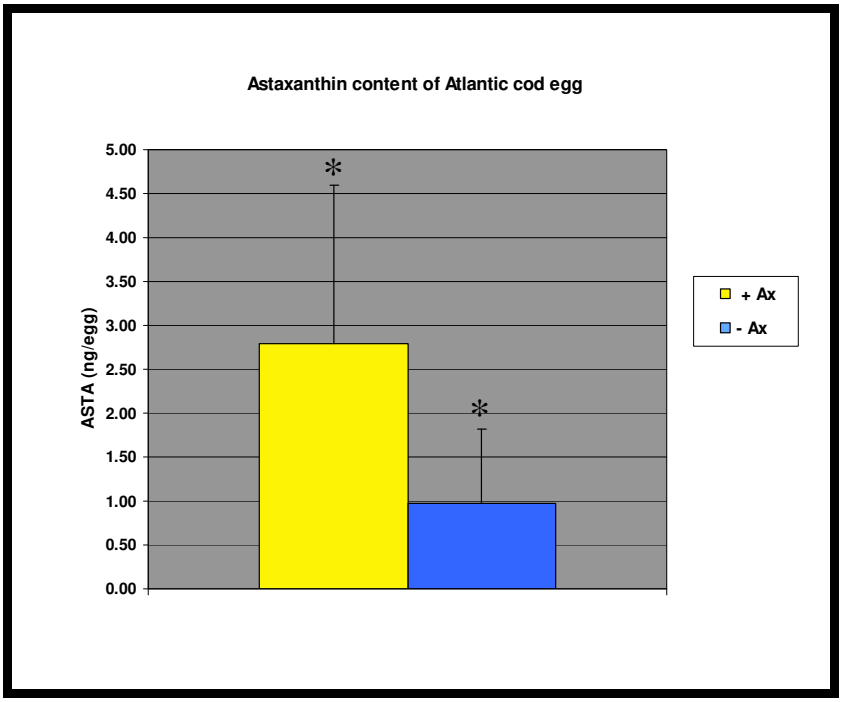

Figure 4.2 Ax content of eggs from control cod broodstock and broodstock fed an Ax supplemented diet for two months prior to peak spawning.

Values are ng Ax/egg (mean ± SD, n =22). Values with * are significantly different p <0.005. Data compare using ANOVA.

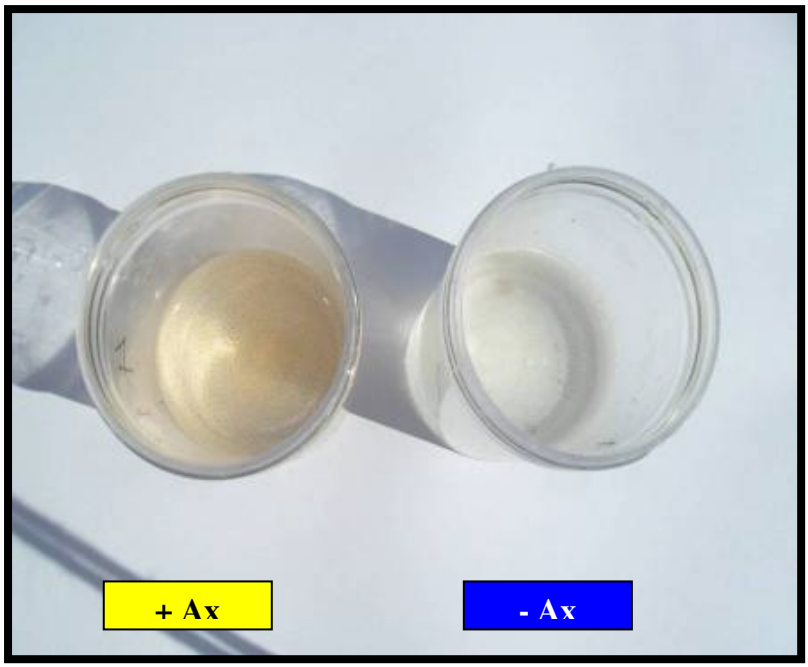

Figure 4.3 Visual comparison of egg colour between broodstock fed diets with (left) and without (right) the Ax supplement.

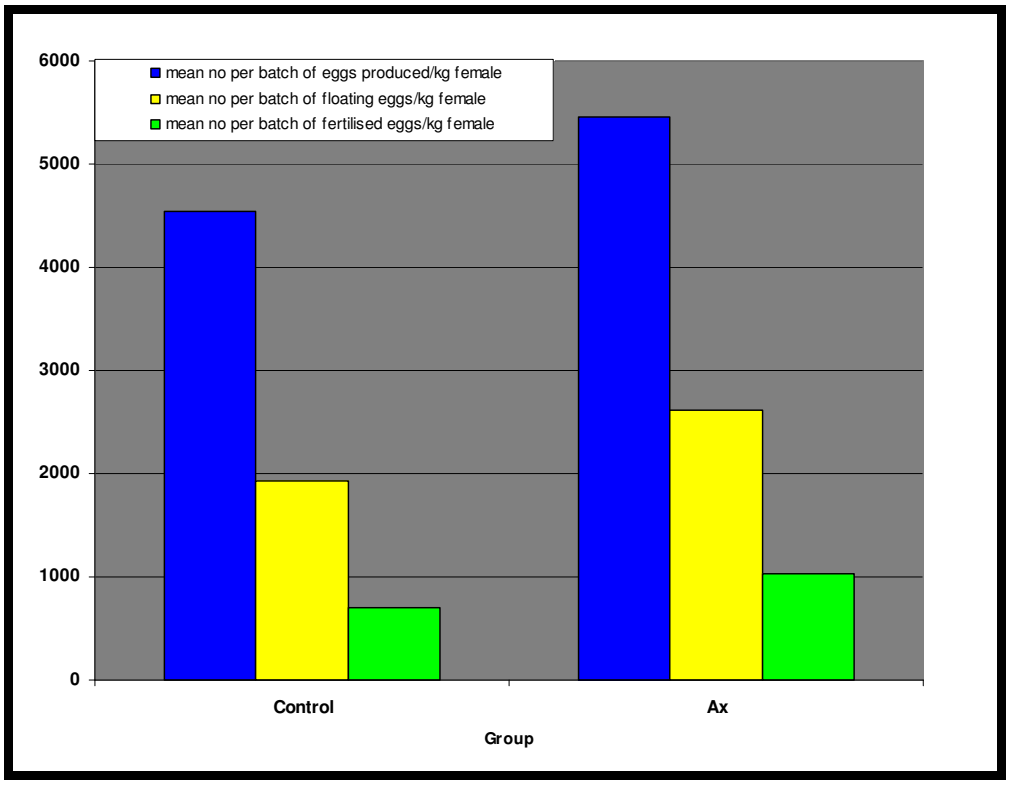

Figure 4.4 Egg production and egg quality parameters in cod broodstock fed a diet with (Ax) and without (Control) added Ax.
Differences in the mean number of eggs spawned, mean number of floating eggs and mean number of fertilised eggs were statistically significant ($P < 0.05$). Comparison made using Kruskal Wallace test.

Table 4.2 and Fig.4.4 show data on egg production and egg viability in the two treatment groups. In the unsupplemented control group, total production was estimated to be 301,032 eggs per kg female. Dropout within the tank was approximately 7% and the number of eggs collected over the season was 280,884 eggs per kg female. A mean of 123,022 eggs per kg female (44 % of those collected) were floating eggs. The mean fertilization percentage of floating eggs was 31% and the total number of viable eggs was 42,573 eggs per kg female (15 % of eggs collected). The mean hatch percentage was 11 % of floating eggs incubated, and the total number of hatched eggs was 13,492 per kg female (5 % of collected eggs). The Ax supplemented group produced

numerically fewer batches of eggs, but the mean number per batch of eggs spawned per kg female was significantly larger ($p< 0.05$). Fertilization percentages were similar but the weight per batch of floating eggs ($p < 0.01$), number per batch of floating eggs per kg female ($p < 0.01$), and number per batch of fertilised eggs/kg female ($p< 0.01$) were all significantly higher in the Ax supplemented group than in the control group. Cumulative egg production for control broodstock and broodstock fed Ax are shown in Fig 4.5. These results show that after 15 days of egg production the broodstock fed an Ax supplement had produced more eggs than control fish.

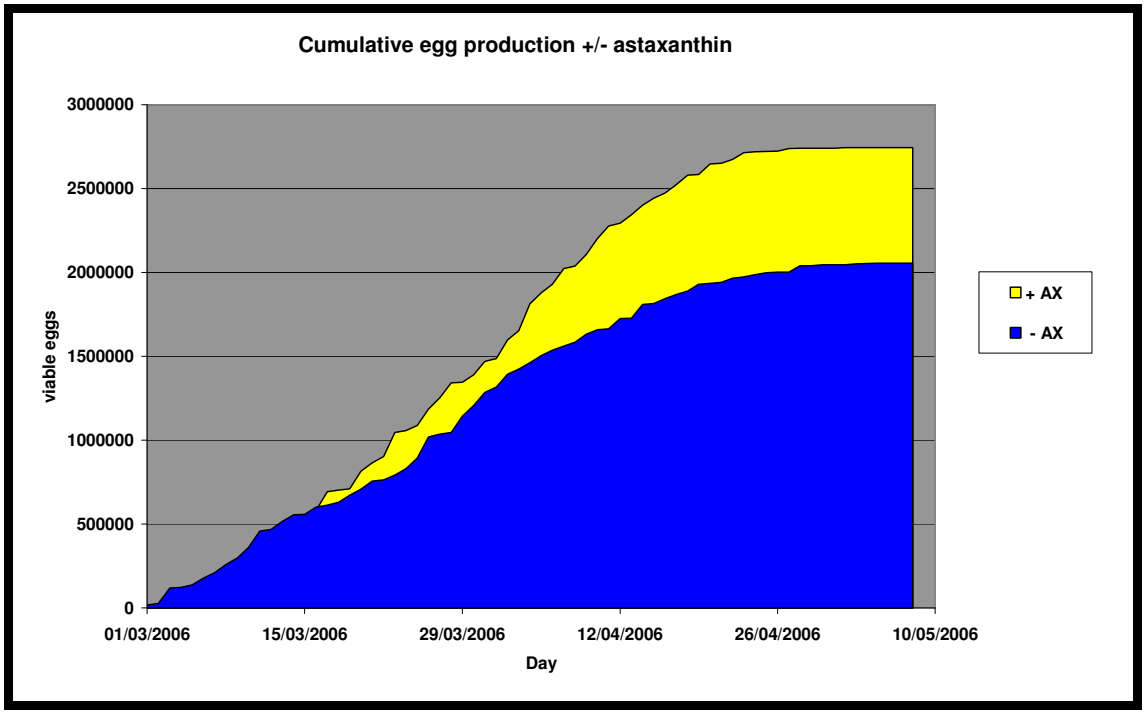

Figure 4.5 Cumulative egg production, over the 90 day spawning period, from control broodstock and broodstock fed an Ax supplemented diet.
Yellow shading indicates egg production from the Group fed Ax supplement. Blue shading indicates egg production from the Control Group (without Ax supplement).

Table 4.2 Egg production and egg quality indicators.

Parameter	Control				Astaxanthin				Pooled			
	Tank 1		Tank 2		Tank 3		Tank 4		Control		Astaxanthin	
	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N
1.Total no. of eggs produced/kg female	333725	69	268339	63	349900	58	321691	66	301,032 ± 46,235	132	335,795 ± 19,947	124
2.Mean no. per batch of eggs produced/kg female	4837 ± 3284	69	4259 ± 3589	63	6033 ± 4120	58	4874 ± 3442	66	4548 ± 409	132	5454 ± 820*	124
3.Total weight of eggs collected (g)	29487	69	24621	63	32338	58	27792	66	27,054 ± 3441	132	30,065 ± 3215	124
4.No. of batches collected	69	69	63	63	58	58	66	66	66 ± 4.2	132	62 ± 5.7	124
5.Mean wt. of collected egg batches	427 ± 296	69	391 ± 329	63	558 ± 388	58	421 ± 304	66	409 ± 26	132	490 ± 97	124
6.Total no. of collected eggs/kg female	312248	69	249520	63	320792	58	301766	66	280,884 ± 44,355	132	311,279 ± 13,453	124
7.Mean no. per batch of eggs collected/kg female	4525 ± 3073	69	3960 ± 3337	63	5531 ± 3778	58	4572 ± 3229	66	4244 ± 400	132	5052 ± 678	124
8.Total weight of floating eggs (g)	10677	67	13169	60	16421	53	13107	63	11,923 ± 1762	127	14,764 ± 2343	116
9.Mean wt. per batch of floating eggs (g)	159 ± 137	67	219 ± 198	60	310 ± 221	53	208 ± 158	63	189 ± 42.4	127	259 ± 72.1**	116
10.Total no. of floating eggs/kg female	112678	69	133366	60	163046	55	142671	63	123,022 ± 14,629	127	152,859 ± 14,407	118
11.Mean no. per batch of floating eggs/kg female	1633 ± 1427	69	2223 ± 2003	60	2964 ± 2208	55	2265 ± 1710	63	1928 ± 417	127	2615 ± 494**	118
12.Mean fertilization rate (% floating eggs)	35	69	28	60	32	55	34	63	31.5 ± 5.0	127	33.0 ± 1.4	118
13.Total no. of fertilised eggs/kg female	40923	66	44223	57	60479	52	54488	61	42,573 ± 2334	127	57,484 ± 4236	113
14.No. of batches with fertilised eggs	66	66	57	57	52	52	61	61	61.5 ± 6.4	123	56.5 ± 6.4	113
15Mean no. per batch of fertilised eggs/kg female	620 ± 684	66	776 ± 824	57	1163 ± 1287	52	893 ± 913	61	698 ± 110	123	1028 ± 191**	113
16.Mean percent hatch (% floating eggs)	10	66	12	57	14	52	13	61	11.0 ± 1.4	123	13.5 ± 0.7	113
17.Total no. of hatched larvae/kg female	11437	69	15547	60	22978	55	18313	63	13,492 ± 2906	129	20,645 ± 3299	118
18.Mean no. per batch of hatched larvae/kg female	166 ± 144	69	259 ± 232	60	418 ± 308	55	291 ± 218	63	212 ± 66	129	354 ± 90	118
Biochemical parameters												
Ax (ng/egg)	1.31 ± 1.06	11	0. 64 ± 0.37	11	2.72 ± 1.24	11	2.86 ± 2.30	11	0.98 ± 0.48	22	2.79 ± 0.10	22

Egg numbers are expressed as numbers per kg female. Values are mean ± SD, n = 2. Significant differences in mean weights or numbers per batch between the control and Ax supplemented groups are shown as * (P<0.05), ** (P<0.01) or *** (P<0.001). Comparison made using Kruskal Wallace test. Control Group fed no supplement, Ax Group fed supplemented diet two months before peak spawning. Pooled data combine tanks for their respective Groups.

4.4 Discussion

A previous study that measured cod egg pigment concentrations identified higher levels of Ax in eggs from wild cod broodstock compared to farmed broodstock held in the same hatchery (Salze et al. 2005). This study showed that eggs from wild caught broodstock wild eggs contained around 3 times more Ax than the eggs from farmed broodstock and that the fertilization percentage in the latter was about half of that seen in the wild eggs. In the present study, short term supplementation of cod broodstock diets with Ax, for a period of two months prior to peak spawning, increased concentrations of carotenoids in the eggs by around 3-fold, indicating efficient and rapid uptake. Whilst fish fed the diet supplemented with Ax produced fewer batches of eggs, the mean number per batch of eggs spawned/kg female was significantly higher (by 20%) and the numbers of floating eggs and numbers of fertilised eggs per kg female in each batch were also significantly improved (by 37 and 47%. respectively). In addition, a correlation between the Ax content of the eggs and fertilization success of individual batches was identified.

These findings confirm that addition of Ax to the cod broodstock diets results in uptake and deposition into eggs and provides significant improvements in egg quality, similar to those found in other fish species. The efficient transfer of Ax from broodstock to egg has been shown previously, in both cod and salmonids, (Torrissen 1984; Grung et al. 1993) although improved egg quality has not been consistently observed in salmonids (Christiansen & Torrissen 1997; Choubert et al. 1998). However, in marine species, including red sea bream and yellowtail, the addition of synthetic Ax

or krill lipid that contains Ax, to broodstock diets was found to clearly improve a number of egg quality parameters (Watanabe et al. 1991; Watanabe & Miki 1993). In red sea bream, the percentage of buoyant and hatched eggs as well as the percentage of normal larvae was significantly increased in eggs from broodstock fed an Ax supplemented diet (Watanabe & Kiron 1995).

Supplementation of broodstock feeds with specific nutrients, particularly contain fatty acids and fat-soluble micronutrients, including carotenoids, can lead to an increase in levels of these nutrients in the developing eggs and, in the case of sea bass, sea bream, yellowtail and halibut, these have been shown to have a measurable impact on egg quality (Ashton H.J et al. 1993; Verakunpiriya et al. 1997; Czesny & Dabrowski 1998; Gallagher et al. 1998; Sargent et al. 2002).

In addition to the benefits reported in fin fish, there is also evidence from studies on crustacean and echinoderm culture that suggest similar benefits of carotenoid supplementation of broodstock diets. Inclusion of dietary carotenoids was shown to improve egg and larval production in the edible sea urchin *Lytechinus variegates,*(George et al. 2001). Supplementation with HUFA and 50 mg/kg Ax resulted in increased total egg production and egg production/female in cultured *Penaeus monodon* broodstock (Huang et al. 2008) Similarly, survival of *Penaeus vannamei* nauplii was increased following a carotenoid supplement while broodstock diets lacking carotenoid resulted in reduced larval feed intake, increased deformities and reduced survival (Wyban et al. 1997).

One explanation for the beneficial effects of Ax on cod egg quality could be that Ax acts as a compound to improve fertilization by stimulating and attracting

spermatozoa (Hartmann et al. 1947). However, the ability of carotenoid pigments to absorb light and, thereby, quench or inactivate singlet oxygen and free radicals, is a more likely reason for their nutritional efficacy (Mayne 1996). The mechanism by which the damaging effects of light (UV and visible), and the subsequent generation of reactive oxygen species, is attenuated, is a consequence of the conjugated polyene structure of carotenoids that allows sequestration and inactivation of these harmful molecules (Nishigaki et al. 1994). This action of carotenoids on control of damaging free radicals has lead to intervention studies in human conditions that have a pro-oxidant aetiology including heart disease, cancer, stroke, cataract, macular degeneration and immune modulation (Mayne 1996). In natural spawning of cod, the eggs are released into the upper layers of the oceans that are both highly illuminated and oxygen-rich, presenting an ideal environment for free radical generation. Thus, the improvements observed in egg and larval quality in farmed cod, when diets are supplemented with Ax, could be explained by better antioxidant protection both in the diet and in the eggs and larvae themselves (Cowey et al. 1985; Pangantihon-Kuhlmann et al. 1998).

A further explanation for the efficacy of Ax supplementation might be related to stress reduction and enhancement of immune function. Larval fish, both in the wild and in hatcheries, can be subjected to both osmotic and thermal fluctuations as well as to pathogenic challenge. In tiger prawn (*Penaeus monodon*), studies have shown that dietary Ax supplementation can improve resistance to both osmotic stress, in the form of salinity fluctuation, and thermal stress as a reduction in temperature from 27 to 5^{o}C (Merchie et al. 1998; Chien et al. 2003). The postulated mechanism for improved stress resistance was related to the increased energy production required to respond to stress

that would generate more oxygen radicals that could be attenuated by the presence of Ax. Ax supplementation has been shown to improve health and immune function in salmon and rainbow trout although the exact mechanism is not known (Thompson et al. 1995; Christiansen et al. 1995). However, a study in mice, using spleen cell suspensions isolated from animals fed control or Ax supplemented diets, showed enhanced T-dependent antigen specific humoral immune responses in the supplemented mouse cells (Jyonouchi et al. 1995). Similar immune enhancement, via modulation of T-dependent antibody responses, has also been observed in humans supplemented with Ax (Jyonouchi et al. 1995). The benefits of Ax supplementation, seen in the present study, suggests that hatcheries should check the status of their cod broodstock with regard to dietary Ax concentrations in the pre-spawning period. If necessary, short term supplementation should be used to boost these nutrients prior to spawning. If the status of the broodstock is unknown, the hatchery should consider sending samples of eggs for analysis at the start of each spawning period. Such tests would assess the nutritional status of eggs prior to spawning, thus allowing corrective action to be taken before spawning commences. More information on the Ax status of eggs from commercial broodstock is required, and should be assessed in relation to egg quality. Records of egg quality in standard form (e.g. no of fertilised eggs per kg female) are necessary to allow effective comparisons between eggs from different broodstock populations.

Future studies should aim to determine the most efficient forms, concentration of Ax and other carotenoids and duration of supplementation required for optimal response. More information is also required on the role of environmental conditions, husbandry and behavioural interactions in relation to spawning of cod broodstock.

4.5 Conclusion

The results indicate that Ax uptake into eggs from the broodstock diet was highly efficient. Fish fed the diet supplemented with Ax produced fewer batches of eggs, but the mean number per batch of eggs spawned/kg female was higher, and numbers of floating eggs and numbers of fertilised eggs per kg female in each batch were also significantly improved. A correlation between the egg Ax content and fertilization success of individual batches was identified. This improvement in egg quality demonstrated the potential value of Ax supplementation of broodstock diets for cod. Ax supplementation produced a 20% increase in the number of eggs per batch spawned, a 37% increase in the number per batch of floating eggs per kg female and a 47% increase in the number per batch of fertilised eggs per kg female. These results clearly demonstrate significant benefits of Ax supplementation of cod broodstock feeds in terms of improved egg quality and larval production.

Chapter 5. Comparison of the relative effect of diets supplemented with arachidonic acid (ARA) and astaxanthin (Ax) on egg quality in wild and farmed cod

5.1 Introduction

As described in the General introduction, broodstock dietary factors are important in determining the quality of spawning success and the lack of certain essential nutrients and fatty acids can have a negative impact on egg quality and larval growth and development (Watanabe 1985; Mourente & Odriozola 1990; Harel et al. 1994; Izquierdo et al. 2001). Food intake of cod during gonadal growth has been shown to affect fecundity (Kjesbu 1991, Karlsen 1995). However, manipulation of PUFA levels in cod broodstock diets have also been shown to increase fecundity, rate and egg quality (Palov 2004; Salze et al. 2005). The correct ratio of DHA: EPA: ARA in the diet is also important because elevating the level of DHA in the diet simultaneously reduces the proportion of EPA, which alters the ratio of EPA:ARA and affects interactions in eicosanoid production between EPA and ARA. ARA is an important precursor of eicosanoids in fish (2-series PG_s) including PGE_2 that is involved in their stress response (Bell et al. 1995; Sargent et al. 1997; Sargent et al. 1999). PGE_2 also helps modulate adaptation to salinity changes (Mustafa & Srivastava 1989). Elevated levels of PGE_2 have been linked to an increase in tissue content of ARA and malpigmentation in common sole (*Solea solea)* larvae (Lund et al. 2008).

Supplementation of broodstock diets with ARA has been shown to improve egg and larval quality in a number of species including Atlantic halibut (Mazorra 2000; Bromage et al. 2001), haddock (*Melanogrammus aeglefinus*) (Castell et al. 2001), and summer flounder (*Paralichthys dentatus*) (Willey et al. 2001). In captive black sea bass (*Cebtropristis striata L.*) broodstock, for example, DHA and ARA

levels in egg lipid were lower than those of wild black sea bass. Fertilization success was directly correlated with levels of DHA and ARA and was higher in wild black sea bass than in the captive fish. This study also showed in the two sources of broodstock (South and North captive black sea bass) fed different feed formulae, that the diet lipids were broadly reflected in the egg lipid composition (Gloria et al. 2009).

The egg phospholipid profile and reproductive success of Atlantic cod broodstock from two different stocks were compared by Pickova et al (1997). In this experiment, wild, Skagerrak, broodstock performed better than wild caught, Baltic, broodstock that had been raised on a formulated diet for two years. The results of this study also showed that the egg lipid profile from the wild broodstock contained twice as much ARA as the captive broodstock. From this study ARA was found to be positively correlated with the hatching success rate (Pickova et al. 1997).

Carotenoids, and Ax in particular, are essential for marine fish. The functions of carotenoids include enhanced reproduction success, pigmentation, antioxidant activity, egg respiration, cell growth and proliferation, as a precursor of vitamin A and in visual acuity (Tacon 1981; Craik 1985; Torrissen 1990; Pavlov et al. 2004). Fish are unable to synthesize carotenoids so they must be provided in the diet (Christiansen & Torrissen 1997). Supplementation of broodstock diet of rainbow trout with Ax increase the concentration in the eggs and improve egg quality and survival whilst lowering the mortality rate (Ahmadi et al. 2006). Mikulin and Soin (1975) showed that Ax may improve egg quality through its role in metabolic processes during embryonic development. It has been found that high concentrations of carotenoids provide antioxidant protection to lipid-rich tissues, though this protection

may be indirect. In cod, carotenoid pigments are important for egg quality with lower levels in eggs from broodstock of farm origin compared to those of wild origin broodstock (Salze et al. 2005). Supplementation of broodstock diets with Ax has been found to give increased concentrations in the eggs (Grung 1993). Many positive effects of broodstock dietary supplementation with Ax have been found in species such as red sea bream and yellowtail (Verakunpiriya et al. 1997; Watanabe & Miki 1993); for example, diet supplementation with Ax improved fecundity and egg quality in yellowtail and striped jack (Watanabe & Vassallo-Agius 2003).

Vitamin E is is an essential nutrient for fish nutrition and provides antioxidant protection in the lipid phase, for example for HUFA-rich cell membranes (Jobling 2004). It has been found that vitamin C (ascorbic acid), vitamin E and some B-complex vitamins are found in high concentrations in gondal tissue indicating an important role in reproduction (Blom & Dabrowski 1996; Sandnes et al. 1998; Jobling 2004) . Teleost fish eggs are rich in phosholipids containing n-3 HUFAs (Furuita et al. 2000; Jobling 2004), which are vulnerable to peroxidative damage (Benzie 1996). The presence of high levels of vitamins C and E in the ovaries during development of the oocytes and egg (Blom & Dabrowski 1996) may help to reduce peroxidative damage (Jobling 2004). The level of vitamin E in broodstock feed is correlated with vitamin E in their eggs. If broodstock are given diets with high levels of HUFAs they should also be given higher levels of vitamin E to prevent oxidation (Pavlov et al. 2004) and egg hatching rates and larval success have been linked to vitamin E levels (Pavlov et al. 2004). If vitamin E levels are not sufficient it has been found to reduce the number of spawning fish, egg hatching success and juvenile survival (Watanabe 1985). The supplementation and improvement of broodstock diets before spawning

can significantly affect fecundity, egg quality and larval survival (Watanabe et al. 1984). This is an important consideration for hatchery operators who wish to improve economic and production efficiency.

Another factor that has been shown to affect egg quality and survival of larvae is the source of the broodstock. Broodstock of wild origin have been found to have better spawning success than broodstock of farm origin (Pavlov et al. 2004). The reason for this could be due to levels of specific lipids in the wild fish which are retained over many years. However, it could also be due to factors such as the greater size of the wild broodstock, a better diet early in life before entering the hatchery (micro nutrients may be retained), sub-optimal diet for farm reared broodstock, genetic differences between broodstock of different origin, high variability in individual broodstock performance, different ages of broodstock and different stocking densities. Pickova et al (1997) proposed that broodstock source rather than diet could explain differences in phospholipid content and performance of cod eggs. In addition, "over-ripening" of eggs (caused by a delay between ovulation and fertilization of the eggs) has been linked to poor egg quality (McEvoy 1984; Bromage et al. 1994).

This experiment aimed to examine the role of broodstock source on spawning success in Atlantic cod fed ARA and Ax supplemented feed in order to evaluate to what extent the dietary modifications had succeeded in reducing the difference in egg quality between wild and farmed fish. The opportunity was also taken to compare vitamin E and TBARS as a measure of lipid oxidation in eggs from wild and farmed cod broodstock.

5.2 Materials and methods

5.2.1 Fish and husbandry

The experiment was carried out at the Machrihanish Marine Environmental Laboratory (MMERL). The wild and farm broodstock origin were described in chapter 2. Duplicate groups of wild caught and farm-reared broodstock were allocated to four 12m^3 tanks in November 2006. The experimental tanks were 4 metre diameter and contained 12 m^3 of seawater. The total tank depth was 1.5 m but water depth was kept to 1.2 m. Each tank was covered and individually equipped with four (36 W) fluorescent tube lights and two (50 W) halogen lamps at about 70 cm above the water surface. The environmental conditions for all fish were SNP from fluorescent lights. Dawn and dusk were simulated by automatic dimming. A flow of seawater was maintained at all times but during summer, when the water temperature rose, the flow was reduced to allow a water cooler to operate and maintain temperature. The average temperature was 9C^o (n= 150, SD ± 1.2) and ranged between 6C^o -12C^o. The average salinity was 32 ppt (n=150, SD ± 0.8) and ranged between 30 ppt – 33 ppt. Oxygen levels were maintained at greater than 6.5 ppm throughout the spawning season (Figure 5.1).

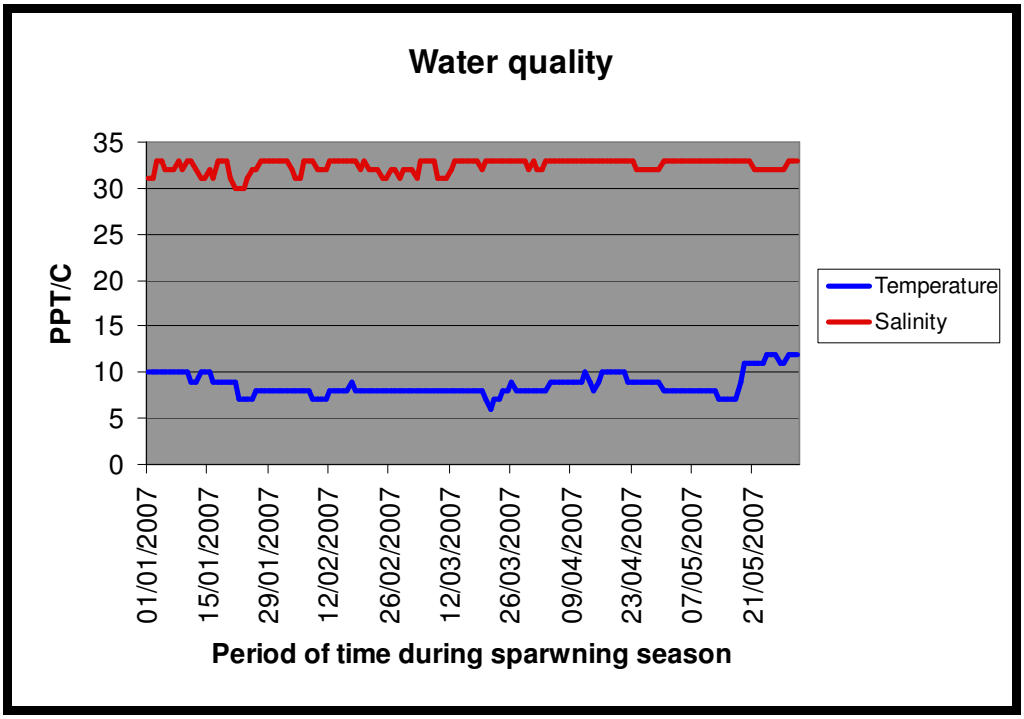

Figure 5.1 Water quality during cod spawning. Temperature (^{o}C) in blue and salinity (ppt) in red.

5.2.2 Experimental design

In January 2007, the fish were individually weighed, screened by ultrasound to determine sex and state of maturation, and reallocated so that each tank contained a similar number and biomass of males and females. The number of fish in each tank is shown in Table 5.1.

The farm fish were allocated to tanks so that eighteen male and seventeen females were contained in each. The mean farm fish biomasses in each tank were 111.4 kg and 106.3 kg. Wild fish were eleven or twelve male and eleven females in each tank. The mean wild fish biomasses in each tank were 116.2 kg and 112.3 kg (Table 5.1). The average individual fish weight in each tank was 3.88 kg. The

experiment used one diet treatment group with two sources of broodstock (wild and farmed) with duplicate tanks. During the experiment there were ten mortalities, mainly female fish unable to release eggs and results were adjusted to account for this (Table 5.1).

Table 5.1 Design for experimental tanks showing distribution and biomass of broodstock fish by treatment group.

(M = males, F = females & Kg = kilogram). Farm tanks contain broodstock from farm origin and wild tanks broodstock from wild origin. Mortality of fish during experiment indicated.

Factor	Farm (tank 4.1)	Farm (tank 4.2)	Wild (tank 4.3)	Wild (tank 4.4)
Ratio of males to females	18 M: 17F	18 M: 17F	11 M: 11 F	12 M:11 F
Total biomass of male fish (kg)	50.3	46.9	56.9	52.2
Total biomass of female fish (kg)	61.1	59.4	59.3	60.1
Mean weight of females (kg)	3.59	3.49	5.39	5.46
Total biomass of fish (kg)	111.4	106.3	116.2	112.3
Female mortality during the spawning period	1 Female	4 Females	2 Females	3 Females

5.2.3 Experimental diet

All fish received the same diet which was based on a commercial moist feed formula (Vitalis® Marine Broodstock Mix, Skretting, Wincham, UK) specially prepared to contain no supplementary ARA or Ax. Feed was prepared by the addition

of water (0.6 L/kg dry mix), ARA (Vevodar® oil, 8.7 g/kg) and Ax (Carophyll Pink, 1.2 g/kg).

Feed formulation

The broodstock feed was prepared in a standardised way. Vevodar oil was transferred from the storage container to a spray bottle. Ten kg dry meal was weighed and transferred to the mixer. Then 12 g of Carophyll Pink was weighed out and dissolved in 3 litres of tapwater. The mixer was switched on then the Carophyll pink solution was added. The container for Carophyll Pink was topped up with 3 litres of tap water and the contents added to the mixer. The spray bottle was tared to "0" on the balance. When the feed mix had the appearance of gravel the mixer was stopped and the compacted mixture loosened and crumbled by hand. The mixer was re-started and 87 g Vevodar oil was sprayed onto the mix. The weight of the spray bottle was checked frequently to avoid adding too much. The diet was mixed until homogenous. The mixer was stopped as necessary to loosen the compacted feed. When well mixed, the mixer was stopped and the feed removed by hand. Before feeding to the fish the feed was chopped into small pieces (approx 15 x 15 x 15 mm) as shown in Figure 2.2 in Chapter 2.

The experimental diet was fed from approximately 2 months prior to the peak spawning date starting on 31st January 2007. Fish were fed to satiation twice daily. The mean concentration of ARA in feed, as measured by GC as 3.19 % of total fatty acids (equivalent to 0.41 g/kg finished feed) and Ax concentration as 80.2 mg/kg finished feed. The EPA concentration in the feed was 6.31 % total fatty acids (0.82 g/kg), and the EPA/ARA ratio was 2.0.

5.2.4 Egg quality assessment

The spawning period was taken as the period from 1st February to 11th May 2007, and the peak spawning date as 22nd March 2007. Eggs were collected each day during the 104 day period. The egg quality was measured for all batches collected and egg quality was assessed using the standard methods described in Chapter 2. The measurements used included total egg production, floating egg production and fertilization rate. The numbers of dead eggs remaining in each tank (dropout) were also measured over a 24 h period on 8 different days. Batches of eggs from all 4 tanks collected on 22 different dates were incubated to determine hatching rates. Samples of floating eggs were collected on 12 different dates for biochemical analysis.

5.2.5 Biochemical analysis

Total lipid content, fatty acid composition, and Ax content were measured in feed. Total lipid content, fatty acid composition, Ax, Vitamin E, TBARS, lipid class and PGE_2 were determined in egg samples using standard laboratory procedures (Chapter 2).

5.2.6 Data analysis

A number of egg quality indices were calculated to allow comparisons between groups. These included batch weights of eggs collected, batch weights of floating eggs, fertilization rate and hatch rate, and estimates of mean numbers per batch of eggs spawned, eggs collected, floating eggs, viable (ie floating, fertilised eggs) and hatched eggs. In order to compensate for small differences in broodstock biomass and allow comparison with other stocks, these indices were calculated in

terms of the biomass of female fish. A full description of the indices used is given in Chapter 2.

Statistical tests used to identify differences (related to fish origin (Wild and Farmed)) included ANOVAor Kruskal-Wallis non-parametric tests, with tank as a factor nested within each treatment. Multiple comparison tests were used to identify differences between the group averages. The Mann- Whitney test was used to compare differences between two groups and ANOVA for comparing eggs produced / batch. Correlation between fatty acid composition and egg quality was tested using Spearman's rank test.

Data from lipid analysis were analysed for homogeneity using Levene's test. Those sets that did not have normal distribution were transformed using arcsine, square root or $\log_{10}$ functions before further analysis. Statistical analyses were carried out using SPSS 16.0 (SPSS Inc, 2007).

5.3 Results

5.3.1 Egg quality and fecundity

Egg production and egg viability were different between the farmed and wild stocks. For example, the number of batches collected was 72 and 77 out of 100 days of spawning in farm fish and 64 and 74 batches in wild fish.

Farmed fish produced eggs more frequently than wild fish but there was no significant difference between the two groups in the mean number of eggs produced

per batch. Spawning started on 10th February for farm fish and 18th Feb for wild fish. Spawning stopped on the 3rd May for farm fish and the 11th May for wild fish. The cumulative production or viable eggs in farm fish was less than for the wild fish (Fig 5.2).

The principal egg quality and fecundity indices for the two groups in this experiment are summarized in Table 5.2 and Figures 5.2 and 5.3.

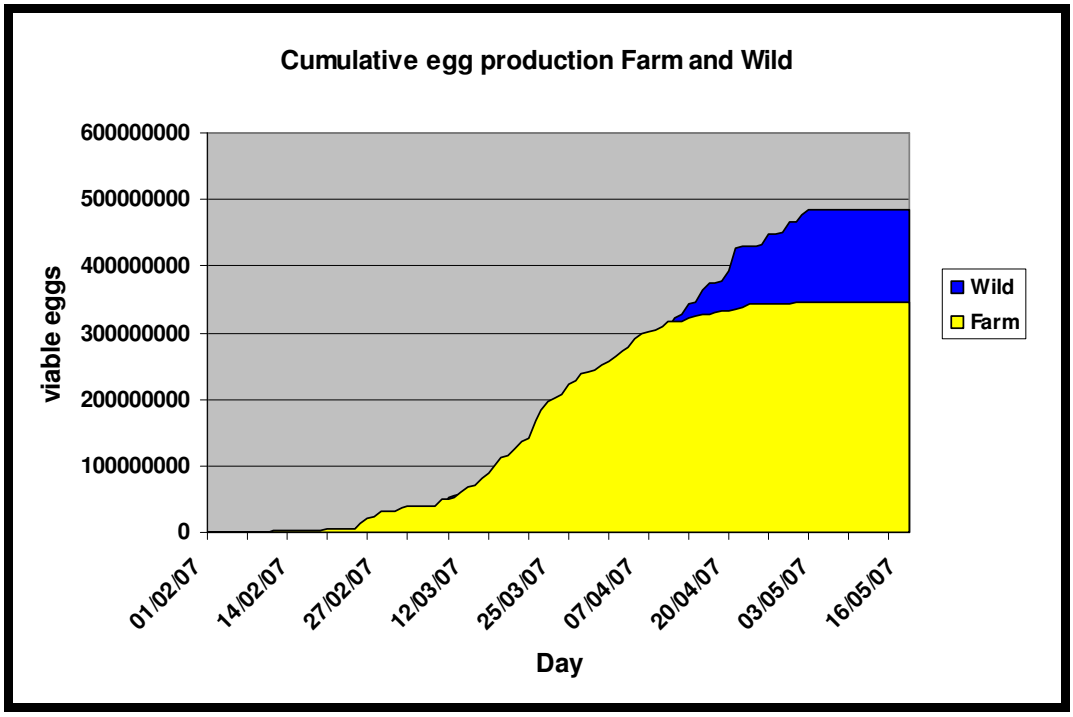

Figure 5.2 Cumulative egg production over the spawning period, from wild and farm origin broodstock fed supplemented diet.
Yellow shading indicates egg production from the Farm origin Group. Blue shading indicates egg production from the Wild origin Group.

The total number of eggs produced during the spawning season was 505,584 eggs per kg female in farmed fish and 594,017 eggs per kg female in wild fish. No

significant difference was detected in total egg production between wild and farmed fish.

More eggs per kg female were collected from wild fish (341,114 egg/kg) than farmed fish (243,433 egg/kg) though this difference was not statistically significant. However, when the mean number per batch of eggs collected per kg of female were compared significant differences were found ($F = 13.51$, $p < 0.001$).

Wild fish produced more floating eggs per kg of female (131,727 egg/kg) than farmed fish (88,678 eggs/kg). The mean weight per batch of floating eggs was significantly different between wild and farm fish ($F = 8.04$, $p < 0.01$). In addition, the mean number per batch of floating eggs per kg female was also significantly different between wild and farm fish ($F= 210.65$, $p < 0.001$).

Wild fish produced a greater number of fertilized eggs per kg female (94,205 egg/kg) compared to farm fish (62,991 egg/kg). There were significant differences in the mean number per batch fertilized eggs per kg female when wild and farm groups were compared (F- 136.55, $p < 0.001$). However, percentage hatch was not significantly different between farmed and wild groups (15 % of floating eggs for farm fish and 14 % for wild fish).

There were no statistically significant differences between wild and farmed fish for the remaining indices of egg quality and fecundity. For example, the total weight of eggs collected was 26,452 g in farmed fish and 35,285 g in wild fish. The total weight of floating eggs/kg female were 9,694 g and 13,646 g for farmed and wild fish, respectively, and the mean percentage fertilization rate was similar between

both groups at 63 % in farmed fish and 65 % in wild fish. The number of batches with fertilised eggs in farmed fish was 64 % and wild 61 % and the mean percent hatch or percentage floating eggs in farmed fish was 15 % and in wild 14 %. The total number of hatched larvae/kg female was 13,302 and 17,124 for farmed and wild fish, respectively, and the mean number per batch of hatched larvae/kg female in farmed fish was 195 and 270 for wild fish (Table 5.2).

Table 5.2 Egg numbers are expressed as numbers per kg female.

Parameter	FARM				WILD				POOLED			
	Tank 4.1		Tank 4.2		Tank 4.3		Tank 4.4		Farm		Wild	
	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N	Value ± SD	N
1.Total no. of eggs produced/kg female	330447	77	680721	72	329592	64	838441	74	505584 ± 247681	149	584017 ± 359811	138
2.Mean no. per batch of eggs produced/kg female	4291 ± 3269	77	9454 ± 8144	72	5150 ± 3693	64	11330 ± 10717	74	6873 ± 3651	149	8240 ± 4370	138
3.Total weight of eggs collected (g)	32269	77	20635	72	24099	64	46470	74	26452 ± 8226	149	35285 ± 15819	138
4.No. of batches collected	77	77	72	72	64	64	74	74	75 ± 3.5	149	69 ± 7.1	138
5.Mean wt. of collected egg batches	419 ± 316	77	287 ± 251	72	376 ± 264	64	628 ± 562	74	353* ± 93	149	502* ± 178	138
6.Total no. of collected eggs/kg female	285932	77	200935	72	233737	64	448492	74	243433 ± 60102	149	341115 ± 151855	138
7.Mean no. per batch of eggs collected/kg female	3713 ± 2829	77	2791 ± 2404	72	3652 ± 2619	64	6061 ± 5733	74	3252*** ± 652	149	4856*** ± 1703	138
8.Total weight of floating eggs (g)	13898	73	5489	63	8454	58	18837	69	9694 ± 5946	136	13646 ± 7342	127
9.Mean wt. per batch of floating eggs (g)	190 ± 212	73	87±84	63	146 ± 128	58	273 ± 297	69	139** ± 73	136	209** ± 90	127
10.Total no. of floating eggs/kg female	123539	73	53816	63	81462	58	181991	69	88678 ± 49302	136	131727 ± 71085	127
11.Mean no. per batch of floating eggs/kg female	1692 ± 1891	73	854 ± 804	63	1404 ± 1241	58	2638 ± 3023	69	1273*** ± 593	136	2021*** ± 873	127
12.Mean fertilization rate (% floating eggs)	70	73	56	63	68	58	61	69	63 ± 9.9	136	65 ± 4.9	127
13.Total no. of fertilised eggs/kg female	96557	69	29425	59	59020	55	129390	66	62991 ± 47469	128	94205 ± 49759	121
14.No. of batches with fertilised eggs	69	69	59	59	55	55	66	66	64 ± 7.1	128	61 ± 7.8	121
15.Mean no. per batch of fertilised eggs/kg female	1399 ± 1592	69	499 ± 566	59	1073 ± 1121	55	1960 ± 2651	66	949*** ± 636	128	1517*** ± 627	121
16.Mean percent hatch (% floating eggs)	16	73	14	63	14	58	13	69	15 ± 1.4	136	14 ± 0.7	127
17.Total no. of hatched larvae/kg female	19766	73	7534	63	11405	58	23659	69	13302 ± 8649	136	17124 ± 8665	127
18.Mean no. per batch of hatched larvae/kg female	271 ± 293	73	120 ± 115	63	197 ± 166	58	343 ± 383	69	195 ± 107	136	270 ± 103	127

Egg numbers are expressed as numbers per kg female. Values within a row and within a broodstock source are significantly different at * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). Comparison made using Kruskal Wallace test. Wild Group are fish of wild origin, Farmed group are fish of farm origin. Pooled data combine tanks for their respective Groups.

5.3.2 Fatty acid composition of eggs

The results of fatty acid analysis of eggs from wild and farmed groups are summarized in Tables 5.3 and Fig 5.2. The concentrations of ARA and DHA were not significantly different between the farmed and wild groups. EPA concentrations were higher in eggs from farmed fish than the wild ($F= 2.003$, $p < 0.05$) and the ratios of DHA/EPA and EPA/ARA were significantly different between farm and wild fish. DHA/EPA ratio was 2.7 for farmed and 2.5 for wild ($F= 18.50$, $p<0.001$) and the EPA/ARA ratio was 4.0 for farmed and 4.4 for wild fish ($F= 9.47$, $p<0.05$).

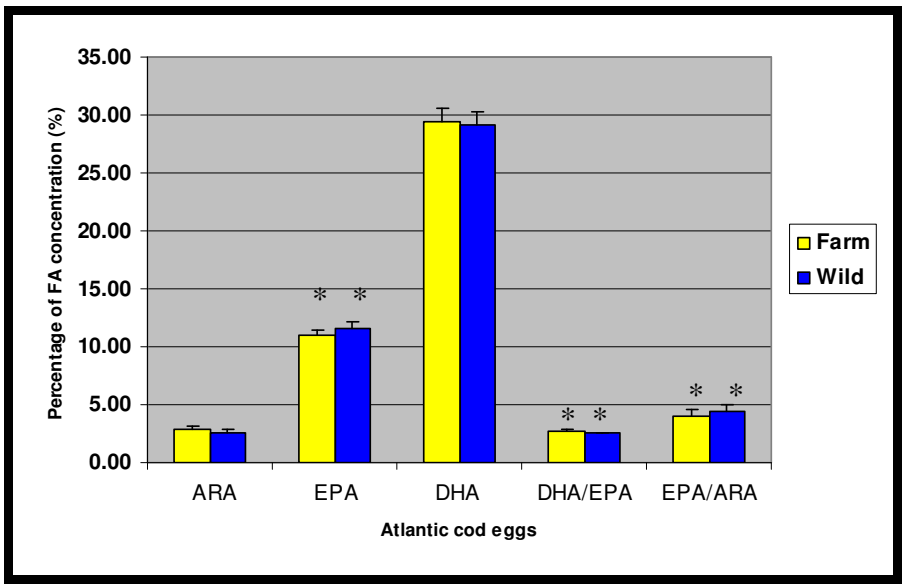

Figure 5.3 Concentrations of the fatty acids ARA, EPA and DHA and the ratios DHA/EPA and EPA/ARA in eggs from broodstock of farm origin compared to wild origin fish fed a diet supplemented with ARA and Ax.

Value with * are significantly different ($p<0.05$). Comparison made using ANOVA test.

Table 5.3 Fatty acid analysis of farmed origin and wild origin broodstock eggs. Values are % weight of total fatty acids.

Fatty acid	Farm	Farm	Wild	Wild	Farm Pooled	Wild pooled
14:0	1.7 ± 0.2 [a]	1.5 ± 0.2 [b]	1.5 ± 0.1 [bc]	1.5 ± 0.1 [bd]	1.6 ± 0.2	1.5 ± 0.1
16:0	22.4 ± 0.9 [a]	20.8 ± 0.3 [b]	20.8 ± 0.4 [bc]	22.2 ± 0.8 [ad]	21.6 ± 1.0	21.5 ± 0.9
18:0	2.5 ± 0.1	2.3 ± 0.2	2.3 ± 0.3	2.5 ± 0.3	2.4 ± 0.2	2.4 ± 0.3
Total saturated	27.1 ± 1.1 [a]	25.1 ± 0.5 [b]	25.1 ± 0.6 [bc]	26.6 ± 0.9 [ad]	26.1 ± 1.3	25.9 ± 1.1
16:1n-9	1.3 ± 0.3	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.2 *	1.2± 0.1*
16:1n-7	1.7 ± 0.7	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	1.9 ± 0.5 *	2.0 ± 0.1 *
18:1n-9	12.5 ± 0.4	12.4 ± 0.2	12.4 ± 0.6	12.7 ± 0.6	12.5 ± 0.3	12.6 ± 0.6
18:1n-7	3.1 ± 0.1	3.2 ± 0.2	3.3 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.2
20:1n-9	3.0 ± 0.2	2.9 ± 0.2	2.8 ± 0.1	2.8 ± 0.2	2.9 ± 0.2	2.8 ± 0.2
22:1n-11	0.9 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.8 ± 0.2
24:1n-9	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Total monounsaturated	23.0 ± 0.8	23.0 ± 0.5	23.1 ± 0.9	23.3 ± 0.8	23.0 ± 0.7	23.2 ± 0.8
18:2n-6	3.3 ± 0.1 [a]	3.1 ± 0.1 [b]	3.2 ± 0.2 [bc]	3.0 ± 0.1 [bd]	3.2 ± 0.1	3.1 ± 0.2
20:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:4n-6	2.7 ± 0.3	2.9 ± 0.4	2.6 ± 0.3	2.7 ± 0.2	2.8 ± 0.4	2.7 ± 0.3
22:5n-6	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
Total n-6 PUFA	6.1 ± 0.4	6.7 ± 0.3	6.5 ± 0.3	6.5 ± 0.5	6.7 ± 0.4	6.5 ± 0.4
18:3n-3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
18:4n-3	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0
20:4n-3	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
20:5n-3	10.8 ± 0.5 [b]	11.1 ± 0.4 [ab]	11.9 ± 0.4 [ab]	11.4 ± 0.5 [a]	11.0 ± 0.5*	11.7 ± 0.5*
22:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1
22:5n-3	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.2
22:6n-3	28.6 ± 1.0 [b]	30.3 ± 0.7 [a]	29.6 ± 1.0 [a]	28.5 ± 1.0 [bc]	29.4 ± 1.2	29.0 ± 1.2
Total n-3 PUFA	42.5 ± 1.0 [b]	44.5 ± 0.6 [a]	44.6 ± 1.4 [a]	42.9 ± 1.0 [bc]	43.5 ± 1.3	43.7 ± 1.5
Total 16 PUFA	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Total PUFA	49.9 ± 1.2 [b]	51.9 ± 0.6 [a]	51.8 ±1.4 [a]	50.1 ± 0.9 [bc]	50.9 ± 1.4	50.9 ± 1.4
(n-3) / (n-6)	6.3 ± 0.4	6.7 ± 0.4	6.9 ± 0.4	6.6 ± 0.5	6.5 ± 0.2	6.8 ± 0.5
DHA/EPA	2.6 ± 0.2 [ab]	2.7 ± 0.1 [a]	2.5 ± 0.1 [b]	2.5 ± 0.1 [bc]	2.7 ± 0.2 *	2.5 ± 0.1 *
EPA/ARA	4.0 ± 0.6 [ab]	3.9 ± 0.5 [b]	4.5 ± 0.5 [a]	4.3 ± 0.6 [ab]	4.0 ± 0.5 *	4.4 ± 0.5 *

Values are mean ± S.D. Farm (n=12), wild (n= 12) and Pooled (n= 24). Values within a row with the different superscript letter are significantly different $p < 0.05$ Includes 15:0, 20:0 and 22:0.

Values within a row with * are significantly different $p<0.05$, comparison only between farm pooled(n=24) and wild pooled (n=24).

Comparison made using ANOVA. Wild Group are fish of wild origin, Farmed group are fish of farm origin. Pooled data combine tanks for their respective Groups.

Table 5.4 Biochemical analysis of eggs from broodstock of farmed origin and wild origin.

	FARM		WILD		POOLED	
	Farm	Farm	Wild	Wild	Farm	Wild
Biochemical analysis	**Value ± SD**	**Value ± SD**	**Value ± SD**	**Value ± SD**	**Value ± SD**	**Value ± SD**
Astaxanthin (ng/egg)	4.6 ± 2.2	2.7 ± 2.1	1.6 ± 1.2	3.6 ± 1.8	3.7 ± 1.34	2.6 ± 1.41
Vitamin E (α-tocopherol) (mg)	3.8 ± 0.5	3.7 ± 0.6	3.2 ± 0.9	3.5 ± 0.7	3.8 ± 0.5*	3.4± 0.7*
TBARS (µmol MDA/egg)	0.2 ± 0.14	0.2 ± 0.24	0.4 ± 0.55	0.3 ± 0.30	0.2 ± 0.03	0.3 ± 0.06
PGE_2 (pg/egg)	8.8 ± 4.6	8.7 ± 4.4	10.2 ± 8.2	9.1 ± 4.0	8.8 ± 4.4	9.6 ± 6.3
Lipid class analysis						
Phosphatidylcholine	29.0 ± 5.1	31.3 ± 4.3	33.1 ± 4.6	33.6 ± 3.8	30.2 ± 4.8	33.4 ± 4.1
Phosphatidyethanolamine	14.4 ± 2.2 [a]	13.2 ± 1.8 [a]	13.0 ± 2.0 [b]	12.6 ± 2.3 [ab]	13.8 ± 2.0	12.8 ± 2.1
Phosphatidylinositol	3.6 ± 1.1	3.2 ± 1.7	3.4 ± 1.0	3.0 ± 1.1	3.4 ± 0.3	3.2 ± 0.2
Cholesterol	18.4 ± 2.2	18.0 ± 2.5	16.4 ± 2.1	16.5 ± 1.9	18.2 ± 2.3	16.4 ± 1.9
Triacylglycerol	18.5 ± 3.6	18.4 ± 3.3	18.0 ± 3.2	19.7 ± 2.6	18.4 ± 3.4	18.8 ± 3.0
Free fatty acids	2.6 ± 2.5	1.3 ± 0.6	1.3 ± 0.6	1.8 ± 2.0	2.0 ± 1.9	1.5 ± 1.4
Total polar	49.9 ± 8.6	50.2 ± 6.7	52.1 ± 6.9	51.0 ± 6.6	50.0 ± 0.2	51.5 ± 0.8
Total neutral	50.1 ± 8.6	49.8 ± 6.7	47.9 ± 6.9	49.0 ± 6.6	50.0 ± 0.3	48.5 ± 0.8

Values are mean ± S.D. Farm (n=12), Wild (n= 12), and Pooled (n=24). Values within a row with the same superscript letter are significantly different. Values within a row with * are significantly different $p<0.05$, comparison only between farm pooled(n=24) and wild pooled (n=24).
Comparison made using ANOVA. Wild Group are fish of wild origin, Farmed group are fish of farm origin. Pooled data combine tanks for their respective Groups.

The results of Ax concentrations in cod eggs are summarised in Table 5.4. The concentration of Ax was slightly higher in farm fish (3.7 ng/egg) than the wild fish (2.6 ng/egg) but this difference was not statistically significant

Correlation analysis showed a significant positive correlation between Ax and the number per batch of floating eggs/ kg female (Spearman's $r = 0.2953$, $p<0.05$) and number per batch of fertilized eggs/kg female ($r = 0.2983$, $p<0.05$). However, there was no correlation between Ax content and any fatty acid concentrations or ratios.

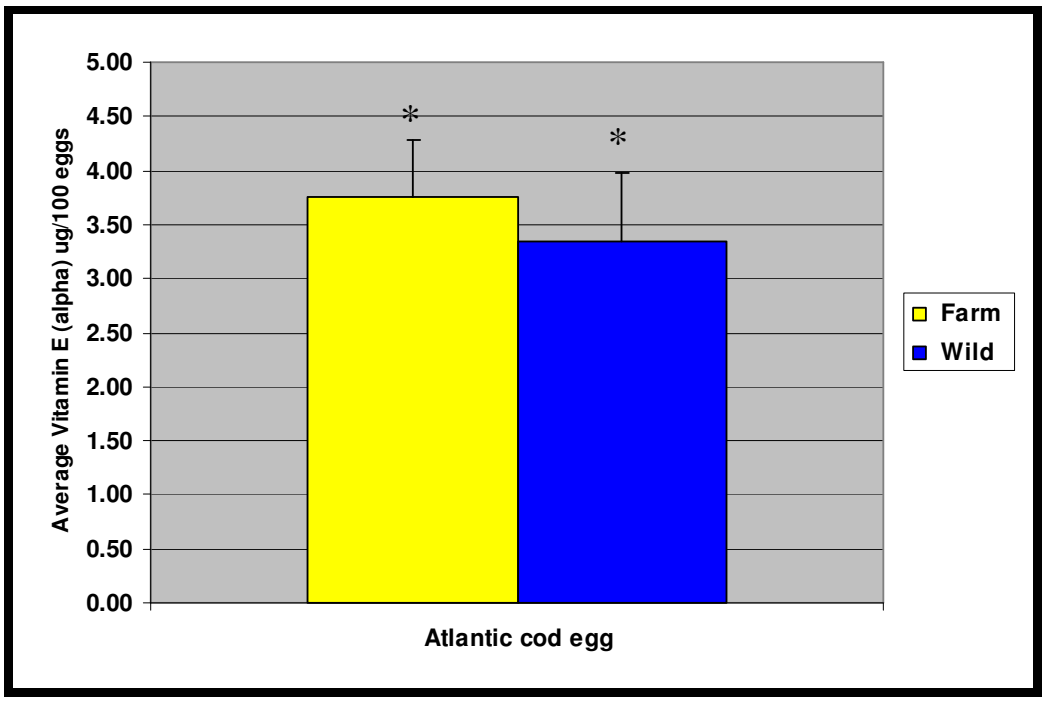

Figure 5.4 Mean vitamin E content in farmed and wild eggs (µg/100 eggs).

Farm Group refers to broodstock of farm origin compared to wild origin fish fed a diet supplemented with ARA and Ax. Value with * are significantly different ($p<0.05$). Comparison made using ANOVA test. Bars indicates standard deviation of the mean.

The results of egg vitamin E content (α-tocopherol) are summarised in Table 5.4 and Fig 5.3. There was a significant difference between farmed fish and wild fish with respect to egg vitamin E content. Farm fish had higher egg vitamin E content (3.8 µg/100 eggs of α-tocopherol) ($F= 0.14$, $p< 0.05$) than the wild fish (3.4 µg/100 eggs of α-tocopherol).

There was no correlation between vitamin E with any fatty acids, egg quality parameter or fecundity.

The results of concentrations of MDA were similar in farm (0.22 µmol MDA/egg) and wild fish (0.33 µmol MDA/egg) (Table 5.4). This difference was not statistically significant. There was no correlation between MDA and any fatty acids, egg quality parameters or fecundity.

The results of PGE_2 concentration in cod eggs are summarised in Table 5.4 and Wild fish had higher mean prostaglandin concentrations (9.6 mg/egg) than the farm fish (8.8 mg/egg) although again there were no significant differences between the groups.

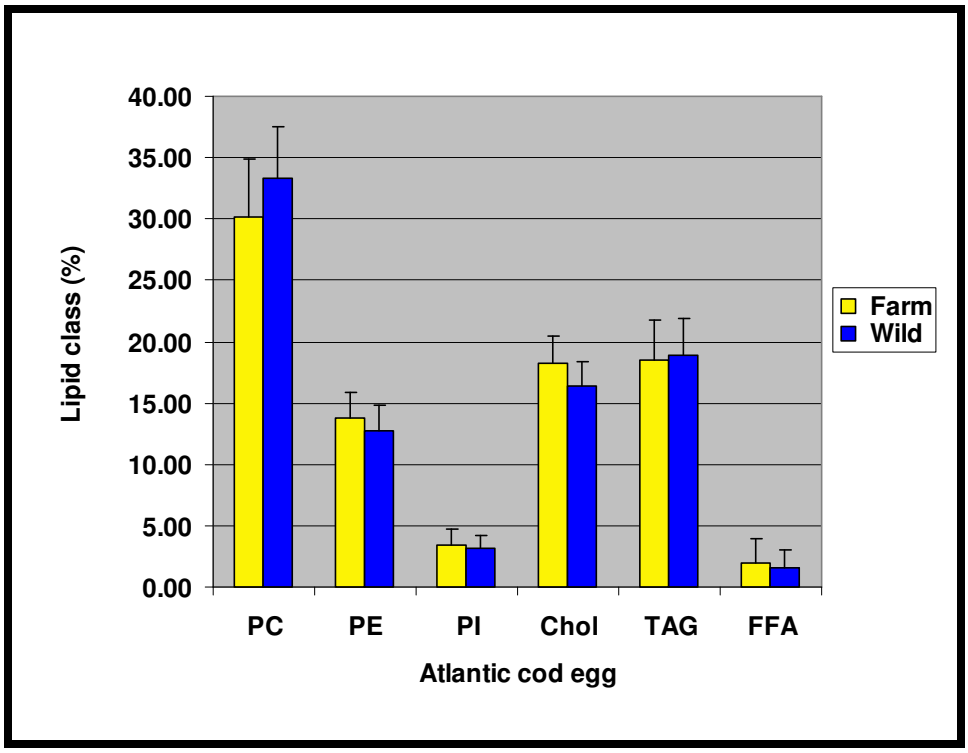

Figure 5.5 Mean lipid class composition of wild and farmed cod eggs. Farm Group refers to broodstock of farm origin compared to wild origin fish fed a diet supplemented with ARA and Ax.

Comparison made using ANOVA test (no significant differences found). Bars indicates standard deviation of the mean.

The results of lipid class analysis of eggs are summarised in Table 5.4 and Fig 5.4. Significant differences in PE were found between each tank $p<0.05$ [highest in farm fish (14.4 %) and lowest in wild fish (12.6 %)]. However, when the results were pooled there were no significant differences found between farm and wild fish (Table 5.4 and Fig 5.5).

Correlation analysis was carried out between egg quality parameters and the levels of specific fatty acids including ARA, EPA and DHA and their ratios (DHA/EPA and

EPA/ARA). The analysis showed no statistically significant correlation between levels in wild and farmed fish.

5.4 Discussion

5.4.1 Egg quality and fecundity

The results of this experiment show that broodstock of wild origin perform better with respect to a number of egg quality and fecundity indices when compared to broodstock of farm origin, despite the supplementation with ARA and Ax. This general finding confirms the observations previously reported by hatchery operators and of a previous study (Salze et al. 2005) and suggests that factors other than status of these nutrients are having a practical impact on egg quality in farm reared broodstock.

The main results of this study show that wild fish produced a similar number of batches of eggs (75 and 69 in 100 days) but the weight of eggs collected was 33 % more than those produced by farm fish. Individual cod have been recorded to spawn 15- 20 batches in 50-60 days (Kjesbu 1989). The higher spawning rate in the present experiment is because the fish were kept in a group and the number of females spawning at any one time would be more than one. Wild fish also produced more eggs per batch with the mean number per batch of eggs collected per kg female being 49 % more than farm fish. The eggs produced by wild fish were of better quality, as indicated by the higher weight of floating eggs per batch, which was 51 % higher than farm fish. In addition, the number of

floating eggs was 59 % greater per kg female. Older fish are known to produce more viable eggs than younger fish (Kjørsvik 1994). Lastly, the mean number per batch of fertilized eggs per kg female was 60 % higher in wild than farm fish. These results were similar to black sea bass where the percentage of fertilized eggs from wild fish were higher than in captive fish (Gloria et al. 2009). However, it should be noted that the mean percentage hatch was not significantly different between farm and wild cod broodstock.

The reasons for these differences are not clear from this result. However, it could be due to the greater size of the wild broodstock, a better diet early in life before entering the farm (micro and other nutrients may be retained), a poor diet for farm reared broodstock, genetic differences between broodstock of different origin, high variability in individual fish performance and age differences and stocking density (Kjørsvik 1994; Pavlov et al. 2004).

5.4.2 Fatty acid composition of eggs

The results of this experiment show that there were some minor significant differences in egg fatty acid and vitamin E content between farmed and wild fish. However, there were no differences in Ax, PGE_2, TBARS levels and lipid classes between the farm and the wild origin broodstock fed a diet supplemented with ARA and Ax. It should be noted that there were small significant differences in levels of some fatty acids (16-1n-9 and 16-1n-7), EPA and in the ratio of DHA/EPA and EPA/ARA. Previous comparisons of the lipid contents in eggs for wild and captive Atlantic cod also found no significant differences between fish origin (Salze et al. 2005). This is a similar result to that

found in turbot when wild fish were compared with captive fish fed formulated diets (Silversand et al. 1996) and in Striped trumpeter (*Latris lineate*) wild and captive broodstock compared with fish fed either commercial diet or a chopped fish diet (Morehead et al. 2001).

EPA concentrations were higher in eggs from farmed fish than the wild ($F= 2.003$, $p <0.05$). This has been found in other species, for example the Striped trumpeter, whose broodstock egg lipid profile was significantly different in EPA and DHA between wild and captive, and between captive fish fed different diets (Morehead et al. 2001; Bransden et al. 2007). The beneficial effect of EPA and ARA on rate has been proposed by several investigators. From the present study wild fish had a 60 % better rate than farm fish. This suggests that measuring only EPA levels in the egg alone cannot be used as an indicator of good egg quality in cod. However, the relative proportions of EFA (such as ARA, EPA and DHA) has been suggested as a good measure of quality in cod eggs (Pavlov et al., 2004). In a previous study, Salze et al. (2005) found the ratio of EPA/ARA was linked to egg quality, with lower ratios (approximately 5:1) in eggs from wild origin fish displaying better performance than eggs from farm origin fish with a high EPA/ARA ratio of approximately 16:1. In the present study, EPA/ARA ratio for farm fish was found to be lower (4.0:1) than for wild fish (4.4:1), with wild fish performing better than farm fish although the differences in the EPA/ARA ratio were fairly minor. This was due to higher EPA and lower ARA levels in wild fish compared to the farmed. If the egg ARA and Ax concentrations in the present study are compared to those of Salze et al. (2005) it might be expected that the eggs from farmed fish would have performed equally well as the wild

fish, but this was not found to be the case although the differences seen between groups were small.

It has been proposed that the optimum ratio of DHA/EPA in broodstock diets of cod should be a 2:1 as this is normally found in most fish eggs (Bromage 1995; Cowey et al. 1985; Tocher & Sargent 1984). In a previous study Salze et al. (2005) found no signifcant differences in DHA/EPA ratios in eggs from wild origin broodstock (approximetaly 1.8:1) and farm reared broodstock (2.0:1). In the present study, the ratio of DHA/EPA for farm fish was significantly higher (2.7:1) than wild fish 2.5:1), so a low DHA/EPA ratio was not responsible for the differences observed. These results suggest that further study of the importance of DHA/EPA ratios in broodstock diets and eggs is needed. Both EPA and ARA are involved in cell-mediated functions and are precursors of eicosanoids including PGE_3 and PGE_2, respectively (Izquierdo et al. 2001; Tocher 2003). The results of this experiment show the wild fish had a slightly higher PG concentration in eggs (9.6 pg/egg) than farm fish (8.8 pg/egg) alhough this difference was not significant.

In the present study, the concentrations of Ax in eggs was slightly higher in farm fish (3.7 ng/egg) than in wild fish (2.6 ng/egg) (though this difference was not statistically significant). Differences in the levels of egg Ax have been observed in previous studies and in the present study (Chapter 4). The main reasons for these differences could relate firstly to the respiratory function of carotenoids. The second reason could be the need of pelagic eggs to avoid predation in an environment where colouration may be a disadvantage. Pavlov (2004), suggested that this explained why in general, eggs from pelagic spawning

species are colourless compared to demersal spawning species. The results of the present study, though not statistically significant, suggests that the farm fish had higher levels of Ax. However, this might simply be due to the previous dietary history of the two broodstocks which suggests that diet over the long term may be an important factor to consider.

The correlation between Ax and egg quality parameters for both wild and farmed cod confirm that Ax supplementation can be used to improve reproductive performance in cod broodstock. This finding confirms the observation in previous studies and Chapter 4. Ax has a role in reproduction perhaps acting as a hormone which increases rates (Christiansen & Torrissen 1997). Dietary carotenoid supplements have also shown a positive relationship between egg pigmentation and fertilization, as well as survival, of rainbow trout eggs (Harris 1984; Craik 1985; Ahmadi et al. 2006).

There were significant differences between farmed fish and wild fish in vitamin E levels. Farm fish has higher vitamin E contents in their eggs compared to wild fish. However, there were no correlations between vitamin E levels and egg quality parameters. Furthermore TBARS were similar suggesting that differences in vitamin E status and lipid oxidation do not explain difference in egg quality between wild and farmed fish. Higher concentrations of vitamin E are thought to reduce the percentage of abnormal eggs and increase overall fecundity (Izquierdo et al. 2001). The amount of vitamin E needed by adult fish depends on levels of PUFA in the diet and their degree of unsaturation (Baker & Davies 1996). If there are high levels of PUFA, for example, and not enough vitamin E

then broodstock egg quality and survival can be reduced (Fernandez-Palacios et al. 1998). Vitamin E in fish eggs enters the oocytes from muscle and other tissue nutrient redistribution during oogenesis (Palace & Werner 2006). High vitamin E diets can lead to increased accumulation in all tissues (Palace & Werner 2006). Before spawning, a large portion of Vitamin E is redistributed to the ovaries in fish (King 1985).

Lipid classes from the present study were similar in both farmed and wild fish. There were no correlations with any other fatty acids or egg quality parameters. Salze et al (2005) found a correlation between ARA content that was positively related to egg quality performance parameters such as and hatching success rate. This was similar to the pattern in black sea bass (Gloria et al. 2009). However, it has been proposed that once ARA has reached its optimum level any further increases will be deposited in other phospholipid classes and TAG, rather than in PI, and without further improvement in egg quality. In this case it would be expected that ARA would reach a maximum level in eggs as dietary ARA is increased. This would explain the differences between the results of the present study and those of other authors in that both groups in the present study may have exceeded the optimal level of dietary ARA.

This finding shows that when both farm and wild broodstock are fed identical diets supplemented with ARA and Ax, the eggs produced had very similar levels of all fatty acids as might be expected. Even though differences in egg fatty acid profiles between wild caught and captive broodstock were reported for species such as the European sea bass, turbot and striped bass (Harrell & Woods 1995; Silversand et al. 1996; Bell et al. 1997)

this would normally be the result of fatty acid concentrations in the diet provided (Bell et al. 1997; Gloria et al. 2009).

It is not clear why wild and farm fish broodstock fed the same diet had significantly different fecundity and egg quality. It is known that fish egg quality is affected by many factors other than diet and these include environmental factors, genetics, stress levels, husbandry, condition of broodstock, age, body size and previous reproductive experience, with the exception of genetics most of these can also be influenced by annual cycles (Kjørsvik 1994; Marshall et al. 1998; Yaragina & Marshall 2000; Lloret & Ratz 2000; Thorsen et al. 2003; Pavlov et al. 2004). The findings of the present study are consistent with the suggestion of Pickova et al. (1997) that the source of the stock may be a more important factor than diet in determining egg fatty acids in phospholipids of eggs.

5.5 Conclusion

In conclusion, this experiment has shown that the performance of wild origin and farm origin broodstock does vary significantly even when fed identical diets supplemented with ARA and Ax. Wild origin broodstock performed better on a number of egg quality and fecundity indices as has been observed in hatchery situations. However, although composition was made for the large size of the wild fish it was not possible to take the greater age and spawning experience of the wild broodstock in to account, and this may have influenced the results.

There were few significant differences in levels of fatty acids between eggs of farm and wild broodstock except for some minor fatty acids like 16-1n-9 and 16-1n-7, and the EPA content, ratios of DHA/EPA, EPA/ARA and vitamin E levels. Ax showed a positive relation to fertilization rate in both wild and farmed fish. This suggests that a diet supplemented with ARA and Ax was being used in a similar way by the wild and farmed fish.

These results also confirm that for hatcheries, broodstock of wild origin will give better reproductive performance over farm fish even although they they have both received a supplemented diet. Although this too may be due to they greater age, size and spawning experience. In addition, percentage of floating eggs may not be ideal as an indicator of egg quality as differences did not translate into improved percentage of hatching.

Chapter 6. Fatty acid and carotenoid content of Atlantic cod eggs from Scottish hatcheries and their relationship with egg and larval quality

6.1 Introduction

The role of broodstock nutrition and its impact on fecundity, gonadal growth, egg quality and larval growth has been discussed in the previous chapters. One of the most important elements of the diet of cod broodstock has been shown to be EFA in particular the HUFA, ARA, EPA, DHA and pigments such as Ax. The experiments described in the previous chapters have shown that supplementation of diets with ARA leads to improved fecundity and some parameters of egg quality. However, despite the use of supplementation in these experiments there remains a large variation in the performance of broodstock (both from wild and farmed stocks).

A number of authors have reported significant effects of abiotic factors on the performance of fish broodstock including season, temperature and spawning cycle effects (Howell et al. 2004; Yanes-Roca 2006; Yanes-Roca et al. 2009). These may affect performance of broodstock and should be considered by hatchery operators. For example, low temperatures during vitellogenisis can improve egg quality in some species (Bransden et al. 2007; Yanes-Roca et al. 2009). Temperature also affects the rate of biological processes and metabolic rate. It can also affect the timing of spawning by impacting on the development of gonads, particularly in species in which changes in temperature (such as increasing spring temperature) are important triggers of final maturation. Growth rates and reproduction timing are linked to optimal temperatures (Howell et al. 2004) and the optimal temperature for spawning of cod is 4 - 6 ^{o}C (Jobling & Pedersen 1995). Egg and larval quality problems can therefore be related to factors including environmental conditions,

disease, pollutants and genetic variations (in broodstock) and not just broodstock diet (Sargent et al. 1995). In nature, season can also affect broodstock performance indirectly through the quality of the food consumed (Sargent 1995). Fish lipid content can change with the type of the food available in different seasons (Sargent et al. 1999). For example, in many species, fish are known to accumulate lipids during spring to late summer when food is abundant and their gonads start to develop. These fish typically spawn in spring with the fertilized eggs hatching to coincide with a plentiful supply of summer plankton. Many marine fish have the lowest levels of lipids when food availability is low, for example, in winter when it is used for metabolic energy as well as for reproduction (Lovern & Wood 1937; Bromage 1995). Generally, the energy used by fish for growth is greater than that used for metabolic activities such as osmoregulation, although long term migrations to spawning grounds can also use considerable energy. The formation of gonads and gametes in fish can use a lot of energy and at a time when food supplies are low. Fish like cod are usually lipid rich when they enter winter but by the next spring after spawning will have low lipid levels, with the liver being the major lipid store (Sargent 1995). Such seasonal changes in lipid content of the diet and its impact on broodstock breeding performance are of less importance to fish hatchery operators because the broodstock diet is controlled. Prior to spawning, diets of captive fish can be supplemented with fatty acids to ensure that diet levels are optimal.

Previous authors have shown the importance of the source of a stock with regard to breeding success and fatty acid content of eggs. Fish of the same species but from different geographical areas can have very different lipid profiles (Morehead et al. 2001; Gloria et al.

2009). This can be due to the different diets of these fish or possibly to genetic variation. For example, wild black seabass (*Cebtropristis striata* L.) broodstock from different areas (North and South USA) consuming different feed components had egg lipid levels which reflected those of their diets (Gloria et al. 2009). Similar results have been found in Atlantic cod with egg phospholipid profiles and reproductive success of broodstock varying according to their geographical source (Pickova et al. 1997). Pickova (1997) found differences in performance between wild (Skagerrak stock) broodstock and wild caught (Baltic stock) broodstock that had been raised on a farm diet for two years. Egg lipid profiles of captive fish were found to contain half the levels of ARA found in the wild broodstock.

When egg batches are examined from early spawning cod eggs, they were found to be smaller and of lower quality than those from later batches (Kjesbu et al., 1990 Merteindottuir and Syeinarsson 1998). Towards the end of spawning batches of eggs in cod were found to have poor quality (Kjesbu 1989). This reduction in egg quality towards the end of the spawning season is due to “exhaustion” of energetic reserves of fish. The effect of lipid content on egg quality and the spawning season has been used to establish the most productive months in terms of egg viability (Yanes-Roca et al. 2009).

The aim and objective of this experiment was to determine whether egg lipid content and fatty acid composition, especially ARA, EPA, DHA, DHA/EPA, EPA/ARA ratio and carotenoid (Ax) level varied according to hatchery location in the UK and spawning time through the collection and analysis of egg samples throughout a whole spawning season.

6.2 Materials and Methods

6.2.1 Fish and husbandry

Egg batches were collected and analysed from two locations. The first was from Machrihanish Marine Farms Ltd (MMF) (collected between 4/10/07 and 24/10/07). The second was from Viking Fish Farms (VFF) (collected between 4/10/07 and 29/5/08).

The aim of the experiment was to collect 50 egg batches from each of the two sites. However, operational problems at MMF prevented egg collection after October 2007. Ninety two egg batches were received from VFF and twelve from MMF.

The location of both farms,VFF and MMF, is shown in Fig 6.1. Both are on the West coast of Scotland and 155 miles apart.

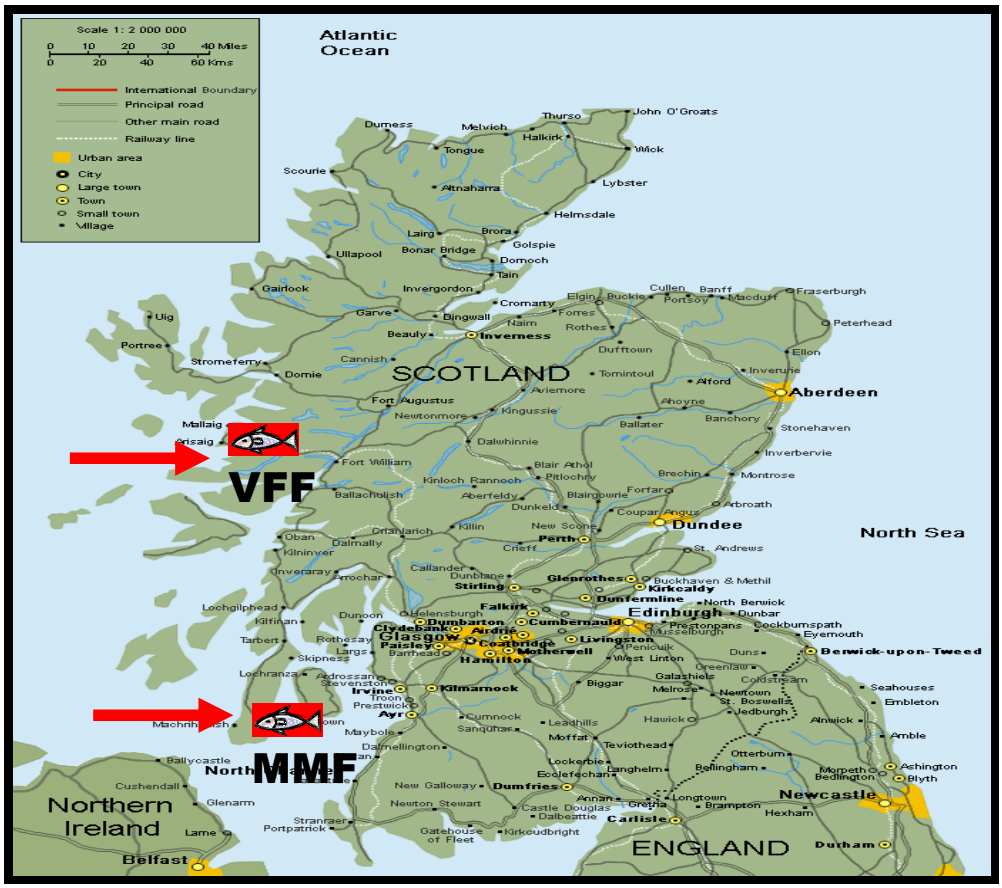

Figure 6.1 Location of the two cod farms where egg samples were collected. Arrows indicates VFF and MMF farms.

The fish were maintained under the normal management conditions of each farm. There was no attempt to standardize husbandry or environmental conditions between the farms. The environmental conditions for broodstock on the farms were SNP from fluorescent lights, a flow of seawater at all times with a water cooler in operation to maintain lower temperature in summer. The average water temperature for VFF during the experimental period was 8 ^{o}C. The environmental data for MMF was not available due to operational problems. The broodstock from VFF came from a variety of sources described

in Table 6.1. This table shows the wide variety of sources, environmental conditions and diets for fish from VFF.

Limited environmental data were available from VFF farm including ambient temperature data and daylength (Fig 6.2). Fish in tanks RU1, RU2 and RU3 were raised on ambient + 3 months daylength.

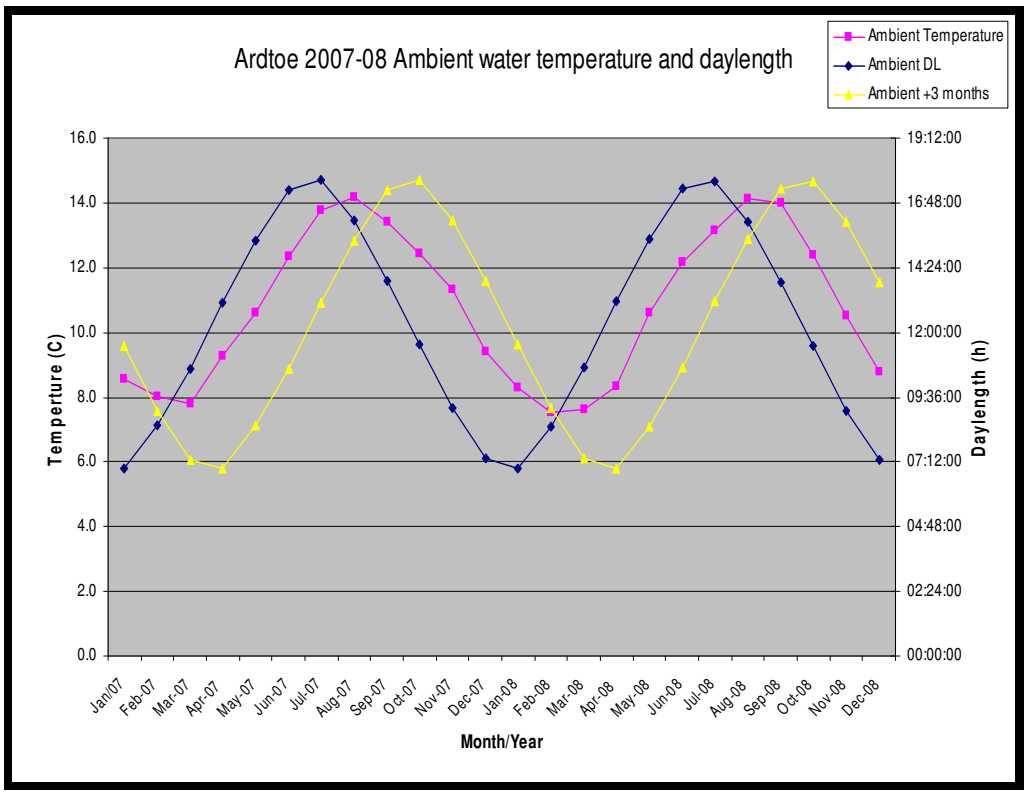

Figure 6.2 Ambient sea water temperature for VFF farm, Ardtoe during 2007-2008 sampling season. Ambient daylength and ambient + 3 months indicated.

Table 6.1 Summary of broodstock source, feed regime and environmental bacgroud from VFF.

Spawning Season	Tank code no.	Stock name	Source of broodstock	Year class	Number of fish	Temperature	Broodstock diet	The average Weight and lengths for fish
Autumn	R1	MMF/ Ardtoe	Hatchery	2004	30	8 °C Chill early August	Pellet mix Skretting Vitalis repro 17 mm and Dana 15 mm broodstock diet	
Autumn	F3	Ardtoe	Hatchery	2003	13	Chill early August	15 mm and 17 mm (pellet as above)	
Autumn	F1	Ireland/Aultbe	Wild	not aged 1st x spawn	Ireland 10 fish Aultbea 7	Chill on 11 September	Marine sausage	
Autumn	F2	Ardtoe/ MMF	Hatchery	2004	43	Chill by 1.5 ^{0}C	Pellet 17/15 and 17/13 mm	
						Ambient	Skretting and Dana	
Spring	New stock into F3	Firth of Forth	Wild	2004/05	13 Firth of Forth	Ambient	Sausage diet	1284 g, 483 mm on 12 Sept 2007
		Loch Nevis	Wild	2004/05	15 Loch Nevis			978 g, 417 mm on 12 Sept 2007
Spring	R2	Ardtoe	Orkney	2002	57	Ambient	Biomar 12 mm, Skretting co Europa 13 mm, Skretting Vitalis 17 mm	4013 g, 648 mm on 11 May 2006
Spring	R3	Millport, Loch Linnhe	Wild	2002	7 Millport,6 Loch Linnhe	Ambient	Sausage diet	5408 g, 721 mm on 22 Aug 2007
Spring	Big Tank	Shetland	Hatchery	2005	110	Ambient	Biomar 12mm/Skretting 13 mm Co Europa Skretting 17 mm Vitalis	3083 g, 628 mm on 30 Aug 07
Summer (3 month delayed)	RU1	Ardtoe	Hatchery	2003		Chill flow to waste (no recycle)	Pellet 17/15 mm, Skretting and Dana	
Summer (first spring spawning season)	RU2	Ardtoe	Hatchery	2005		Chill for short time	Pellet 13 and 17/15 Skretting	
Summer	RU3	Ardtoe	Hatchery	2001		No chill 2007	Marine sausage diet	

6.2.2 Feed

Broodstock had been fed a variety of feed from commercial suppliers (Pelleted feeds from Skretting (Vitalis), Danafeed, Biomar (Europa)) and farm made (Marine sausage diet) sources according to normal production protocols for each farm. It was not possible to standardise feed or feeding regime. Analysis of fatty acid contents was carried out on farm produced marine sausage feed from VFF (Table 6.2). No feed samples were available from MMF due to operational problems.

Table 6.2 Mean fatty acid composition of marine sausage diet from VFF (Values are % weight of total fatty acids).

Fatty acid	**VFF Feed**
14:0	6.3 ± 0.1
16:0	15.8 ± 0.2
18:0	2.9 ± 0.1
Total saturated	25.7 ± 0.4
16:1n-9	0.0 ± 0.0
16:1n-7	4.7 ± 0.1
18:1n-9	11.5 ± 0.1
18:1n-7	2.4 ± 0.1
20:1n-9	8.7 ± 0.1
22:1n-11	13.5 ± 0.5
24:1n-9	1.4 ± 0.0
Total monounsaturated	42.7 ± 0.4
18:2n-6	1.9 ± 0.0
20:3n-6	0.1 ± 0.0
20:4n-6	0.8 ± 0.0
22:5n-6	0.3 ± 0.0
Total n-6 PUFA	3.6 ± 0.0
18:3n-3	1.3 ± 0.0
18:4n-3	2.8 ± 0.1
20:4n-3	0.9 ± 0.0
20:5n-3	7.4 ± 0.0
22:4n-3	0.0 ± 0.0
22:5n-3	1.4 ± 0.0
22:6n-3	13.4 ± 0.0
Total n-3 PUFA	27.3 ± 0.1
Total 16 PUFA	0.8 ± 0.1
Total PUFA	31.6 ± 0.1
(n-3) / (n-6)	7.5 ± 0.1
DHA/EPA	1.8 ± 0.0
EPA/ARA	9.7 ± 0.2

Values are mean ± S.D. n=4. Total saturated includes 15:0, 20:0 and 22:0

6.2.3 Egg quality assessment

The egg quality was measured for all batches collected at both VFF and MMF. Egg quality was assessed by farm staff using the standard methods described in chapter 2. The quantities measured included total egg production, floating egg production and fertilization rate. Analysis of fatty acids was carried out on a sample of floating eggs from ninety two samples from VFF and twelve batches collected from MMF. Fertilization rates were measured in all samples collected according to the protocol described in Chapter 2.

6.2.4 Biochemical analysis

The fatty acid and Ax composition in eggs and total lipid content of feed were measured using standard laboratory procedures as explained in Chapter 2.

6.2.5 Data analysis

A number of egg quality indices were calculated to allow comparisons between the two sources. These included batch weights of eggs collected, batch weights of floating eggs, fertilization rate and estimates of mean numbers per batch of eggs spawned, eggs collected, floating eggs, viable (ie floating, fertilised eggs) and hatched eggs. A full description of the indices used is given in Chapter 2.

In order to compensate for the low number of samples collected at MMF the data from twelve matching days (samples) from VFF were sub sampled for comparison (VFF 12).

Statistical tests used to identify differences between groups included ANOVA or Kruskal-Wallis tests. Multiple comparison tests were used to identify differences between the group averages. The Mann -Whitney test was used to compare differences between control and pooled groups and ANOVA for comparing eggs produced / batch. Correlation between fatty acid composition and egg quality was measured using Spearman's rank test. Statistical analyses were carried out using Minitab 14.0 (Minitab Inc, 2004).

Data from lipid analyses were analysed for homogeneity using Levene's test. Those sets that did not have normal distribution were transformed using arcsine, square root or log 10 functions before further analysis. Statistical analyses were carried out using SPSS 16.0 (SPSS Inc, 2007).

6.3 Results

6.3.1 Fatty acid composition of eggs

The fatty acid concentrations in eggs from tanks at MMF and VFF (all data and sub samples) are shown in Table 6.3. Analysis of fatty acid percentages allows biochemical comparisons of egg quality between farms.

Comparison of the twelve samples from MMF (12) with twelve samples collected over the same period (4 – 24 October 2007 for MMF and 4 October – 6 November 2007 for VFF) from VFF (12) show that the average level of ARA was significantly higher, in MMF eggs at 2.2 %, ($F= 176.483$, $p<0.001$) than VFF eggs at

1.4%. While the average for EPA from VFF was 14.5 % ($F=205.719$, $p<0.001$) which was significantly higher than MMF at 11.9 %.

DHA in eggs from both locations was similar (MMF 28.1 % and VFF 27.5 %). The ratio of DHA/EPA at MMF was higher at 2.4 % ($F=165.767$, $p<0.001$) than at VFF at 1.9 %. Consequently, the ratio of EPA/ARA from VFF was 10.5 ($F=570.972$, $p<0.001$) being higher than MMF at 5.4 (Table 6.3).

Comparison of the twelve samples from MMF (12) with the full set of ninety two samples from VFF (92) showed that eggs from MMF had a higher percentage of ARA and the DHA/EPA ratio than VFF eggs. The average content of ARA in eggs from MMF was 2.2 % ($F= 155.46$, $p<0.001$), which was significantly higher than eggs from VFF at 1.5 %. The average content of EPA in eggs from VFF was 13.5 % which was significantly higher than those from MMF at 11.9%. DHA from both places was similar (MMF 28.1 % and VFF 28.8 %).

The ratio of DHA/EPA in eggs from MMF was higher at 2.4 ($F= 5.282$, $p<0.05$) than those from VFF at 2.2. Consequently the ratio of EPA/ARA in eggs from VFF was 8.9 ($F= 101.421$, $p<0.001$) and in those from MMF 5.4 (Table 6.3) (Figure 6.3).

Table 6.3 Mean fatty acid composition (% by weight of total fatty acids) of Atlantic cod eggs from MFF 12 and VFF 92 and sub samples from VFF 12.

Fatty Acids	**MMF(12)**	**VFF(12)**	**VFF(92)**
14:0	1.6 ± 0.2	1.6 ± 0.2	1.7 ± 0.3
16:0	22.3 ± 1.0	21.9 ± 0.9	21.7 ± 1.4
18:0	2.4 ± 0.3[b]*	3.1 ± 0.4[a]	3.1 ± 0.4*
Total saturated	27.0 ± 1.2	27.2 ± 1.1	27.1 ± 1.7
16:1n-9	1.1 ± 0.1[b] *	1.2 ± 0.1[a]	1.2 ± 0.2*
16:1n-7	1.8 ± 0.1[b]*	2.3 ± 0.1[a]	2.1 ± 0.3*
18:1n-9	13.3 ± 0.5[a]*	12.2 ± 0.6[b]	12.4 ± 0.7*
18:1n-7	3.1 ± 0.2	3.0 ± 0.3	3.2 ± 0.4
20:1n-9	2.8 ± 0.2[a]*	1.9 ± 0.2[b]	1.9 ± 0.4*
22:1n-11	1.2 ± 0.2[a]*	0.4 ± 0.3[b]	0.5 ± 0.3*
24:1n-9	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
Total monounsaturated	23.7 ± 0.6[a]*	21.7 ± 0.8[b]	21.9 ± 1.2*
18:2n-6	2.9 ± 0.3[b]	3.6 ± 0.4[a]	2.8 ± 1.5
20:3n-6	0.1 ± 0.0 [b]	0.2 ± 0.1 [a]	0.2 ± 0.1
20:4n-6	2.2 ± 0.2 [a]*	1.4 ± 0.1 [b]	1.5 ± 0.2*
22:5n-6	0.2 ± 0.0 [b]*	0.3 ± 0.0 [a]	0.3 ± 0.0*
Total n-6 PUFA	5.7 ± 0.5	5.9 ± 0.6	5.2 ± 1.5
18:3n-3	0.4 ± 0.1 [b]*	0.5 ± 0.0 [a]	0.5 ± 0.1*
18:4n-3	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1
20:4n-3	0.5 ± 0.0 [a]	0.4 ± 0.1 [b]	0.5 ± 0.2
20:5n-3	11.9 ± 0.3 [b]*	14.5 ± 0.5 [a]	13.5 ± 1.2*
22:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	1.5 ± 0.1 [a]	1.4 ± 0.1 [b]	1.5 ± 0.2
22:6n-3	28.1 ± 1.1	27.5 ± 1.1	28.8 ± 2.4
Total n-3 PUFA	42.9 ± 1.4 [b]*	44.7 ± 1.4 [a]	45.2 ± 2.4*
Total C_{16} PUFA	0.7 ± 0.1 [a]	0.5 ± 0.1 [b]	0.6 ± 0.2
Total PUFA	49.3 ± 1.5 [b]*	51.1 ± 1.7 [a]	51.0 ± 2.2*
(n-3) / (n-6)	7.5 ± 0.7	7.7 ± 0.8	9.5 ± 3.4
DHA/EPA	2.4 ± 0.1 [a]*	1.9 ± 0.1 [b]	2.2 ± 0.3*
EPA/ARA	5.4 ± 0.5 [b]*	10.5 ± 0.5 [a]	8.9 ± 1.3*

Values are mean ± S.D. MMF12 (n=12), VFF12 (n= 12), VFF92 (n=92). For comparison between MMF12 and VFF12, values within a row the different superscript letter shows significant difference at $p<0.05$. Total saturated ncludes 15:0, 20:0 and 22:0.
For comparison between MMF12 and VFF92, values within a row with * are significantly different at $p<0.05$.
Comparison made using ANOVA.

The main fatty acids are show in Figure 6.2 .

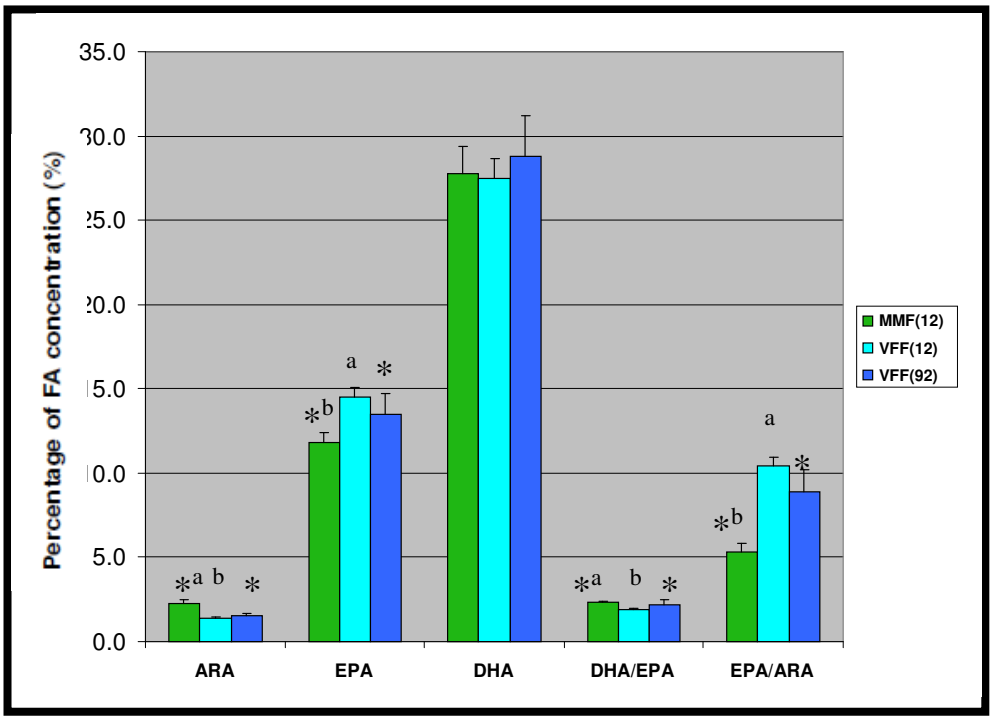

Figure 6.3 Mean percentage of the essential fatty acids ARA, EPA, DHA and their ratios in eggs collected from broodstock fish at MMF and VFF farms.

MMF12 refers to samples from MMF farm, VFF92 refers to all samples from VFF farm and VFF12 refers to the sub sample from VFF92 data. Comparison within group were made using ANOVA. * indicates data significantly different at $p< 0.05$ the different superscript letter shows significant difference at $p<0.05$. Bars indicate standard deviation of the mean.

6.3.2 Season and fatty acid composition of eggs

The fatty acid levels of eggs from VFF by season are shown in Table 6.4. One-way ANOVA was used to compare the levels of fatty acids and pigments.

Comparison of means was carried out between seasons. Eggs collected in autumn had a significantly higher percentage of 18:1n-7 than summer eggs, and significantly higer levels of total monounsaturated and 22:1n-11 than eggs collected in spring. Total n-3 PUFA was different when compared between spring and summer. Summer-collected eggs had significantly higher percentages of 14:0, 18:0, 18:2n-6 and total n-6 PUFA, (Table 6.4).

Table 6.4 Mean fatty acid composition (% by weight of total fatty acids) of Atlantic cod eggs collected at VFF according to season.

Fatty acid	Autumn	Spring	Summer
14:0	1.7 ± 0.3[b]	1.5 ± 0.1[c]	2.0 ± 0.4[a]
16:0	21.6 ± 1.5	21.9 ± 0.8	21.7 ± 0.9
18:0	3.0 ± 0.3[c]	3.2 ± 0.3[b]	3.7 ± 0.3[a]
Total saturated	26.9 ± 1.8	27.2 ± 1.0	28.1 ± 1.5
16:1n-9	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.1
16:1n-7	2.2 ± 0.2[a]	1.8 ± 0.4[b]	2.3 ± 0.3[a]
18:1n-9	12.5 ± 0.6	12.5 ± 0.9	12.1 ± 0.3
18:1n-7	3.3 ± 0.4[a]	3.3 ± 0.3[a]	2.9 ± 0.3[b]
20:1n-9	1.9 ± 0.5	1.7 ± 0.3	1.8 ± 0.2
22:1n-11	0.6 ± 0.2[a]	0.4 ± 0.2[b]	0.7 ± 0.4[a]
24:1n-9	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
Total monounsaturated	22.1 ± 1.1[a]	21.4 ± 1.2[b]	21.5 ± 1.3[ab]
18:2n-6	2.7 ± 1.4[b]	2.7 ± 1.7[b]	4.3 ± 0.3[a]
20:3n-6	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	1.5 ± 0.2	1.6 ± 0.1	1.6 ± 0.1
22:5n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
Total n-6 PUFA	5.1 ± 1.4[ab]	4.9 ± 1.7[b]	6.6 ± 0.5[a]
18:3n-3	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
18:4n-3	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:4n-3	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.0
20:5n-3	13.4 ± 1.1	13.8 ± 1.3	13.1 ± 0.7
22:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	1.5 ± 0.2	1.6 ± 0.1	1.5 ± 0.1
22:6n-3	28.8 ± 2.6	29.4 ± 1.8	27.3 ± 1.8
Total n-3 PUFA	45.2 ± 2.6[ab]	46.0 ± 1.0[a]	43.2 ± 2.3[b]
Total 16 PUFA	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.1
Total PUFA	50.9 ± 2.4	51.5 ± 1.5	50.4 ± 2.7
(n-3) / (n-6)	9.3 ± 3.2	10.8 ± 4.1	6.6 ± 0.3
DHA/EPA	2.2 ± 0.3	2.2 ± 0.3	2.1 ± 0.1
EPA/ARA	9.1 ± 1.4	8.5 ± 1.0	8.3 ± 0.4

Values are mean ± S.D. Autumn (n=65), Spring (n= 21), and Summer (n=6). Values within a row with the different superscript letter are significantly different $p < 0.05$. Total saturated includes 15:0, 20:0 and 22:0. Comparison made using ANOVA.

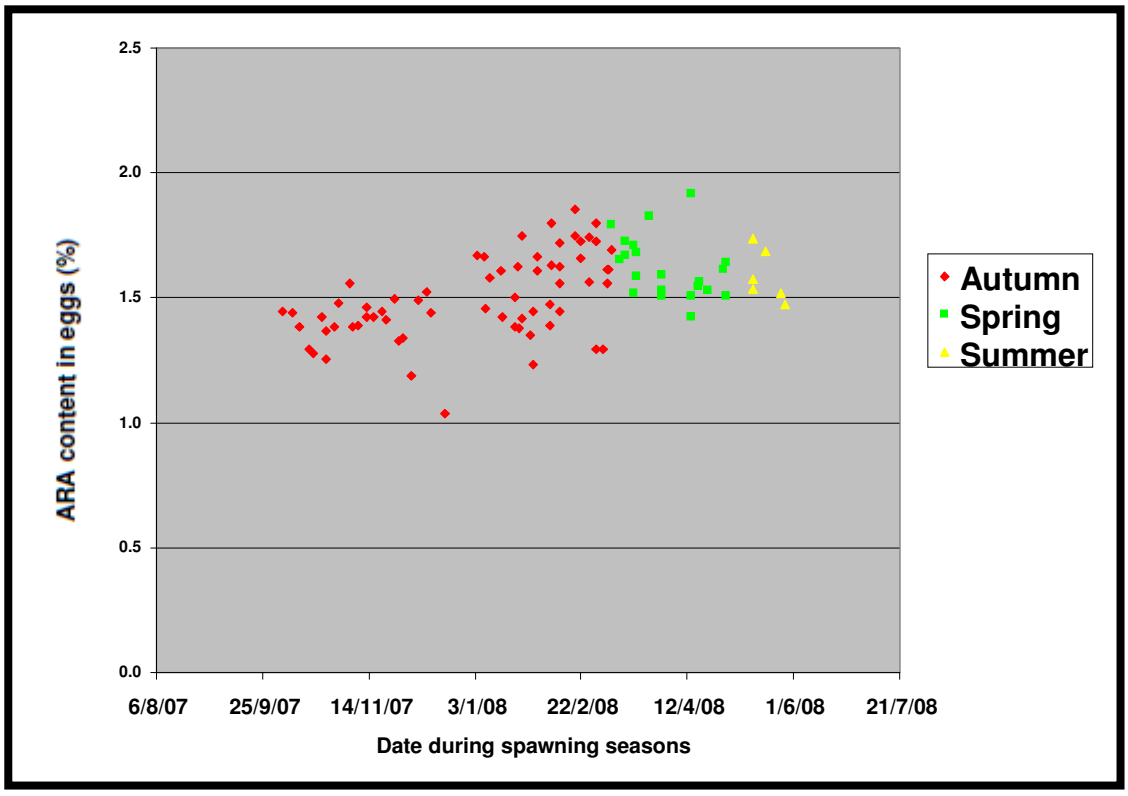

Figure 6.4 ARA content in egg collected over the spawning season in VFF eggs. Autumn (red), Spring (green) and Summer (yellow) spawned eggs are indicated.

ARA was measured from broodstock and eggs collected at VFF on different dates in autumn, spring and summer. Figure 6.3 shows the ARA content (as percentage of total lipids) in 92 samples across spawning seasons from VFF.

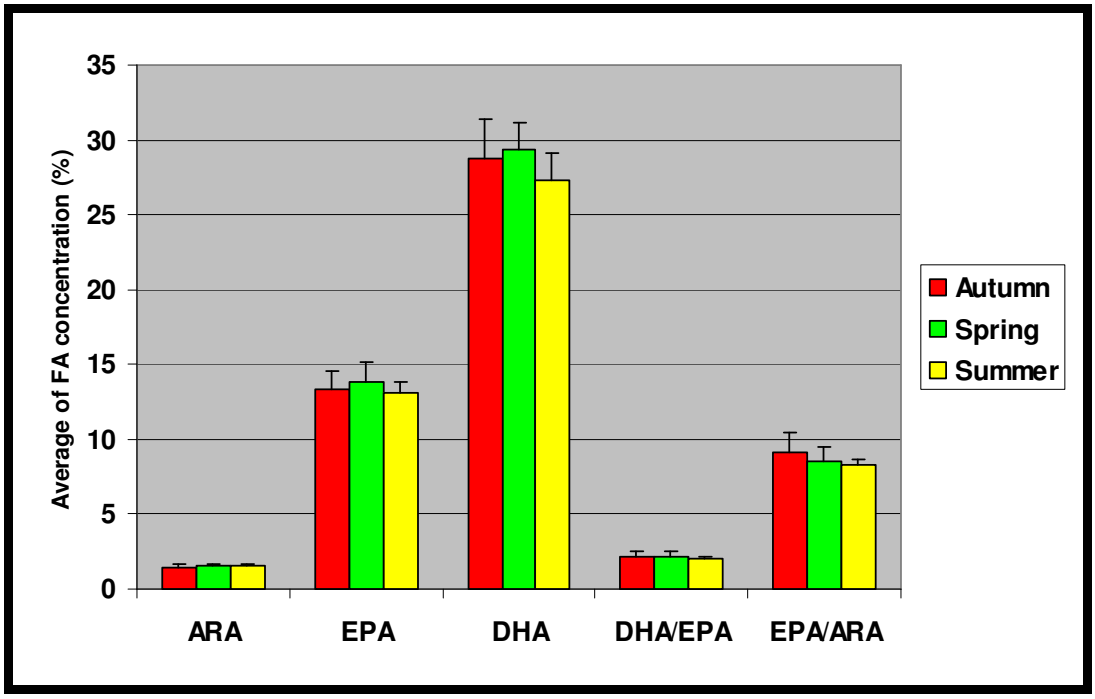

Figure 6.5 The mean of the main essential fatty acids present over the spawning season in VFF eggs.

Autumn (red), Spring (green) and Summer (yellow) spawned eggs are indicated. Bars are standard deviation of the mean. Comparison made by using ANOVA (no significant differences were found).

The average of ARA, EPA, DHA and DHA/EPA and EPA/ARA ratios in VFF eggs were not significantly different between the three seasons (Figure 6.5).

6.3.3 Ax levels in eggs collected at MMF and VFF farms

The Ax levels were significantly higher in eggs from the MMF farm when compared to those from VFF, with the former being 3-fold higher than the latter (using the comparison of the 12 samples from MMF and 92 samples from VFF). The average of Ax from MMF was 1.32 ng/egg ($F=54.021$, $p<0.001$) and VFF 0.44 ng/egg. However, there was no significant difference between the two hacheries when the 12 MMF and 12 VFF sub samples were compared (Table 6.5 and Figure 6.5).

Table 6.5 Mean Ax levels in egg samples from MFF and VFF farms.

MMF12 refers to samples from MMF farm, VFF92 refers to all samples from VFF farm and VFF12 refers to the sub sample from VFF92 data. Standard deviation of the mean indicated.

Carotenoid						
	MMF (12)		**VFF (12)**		**VFF (92)**	
	Ave ± SD	N	Ave ± SD	N	Ave ± SD	N
Ax(ng/egg)	1.32 ± 0.46[a]	12	1.17 ± 0.55	12	0.44 ± 0.47[b]	92

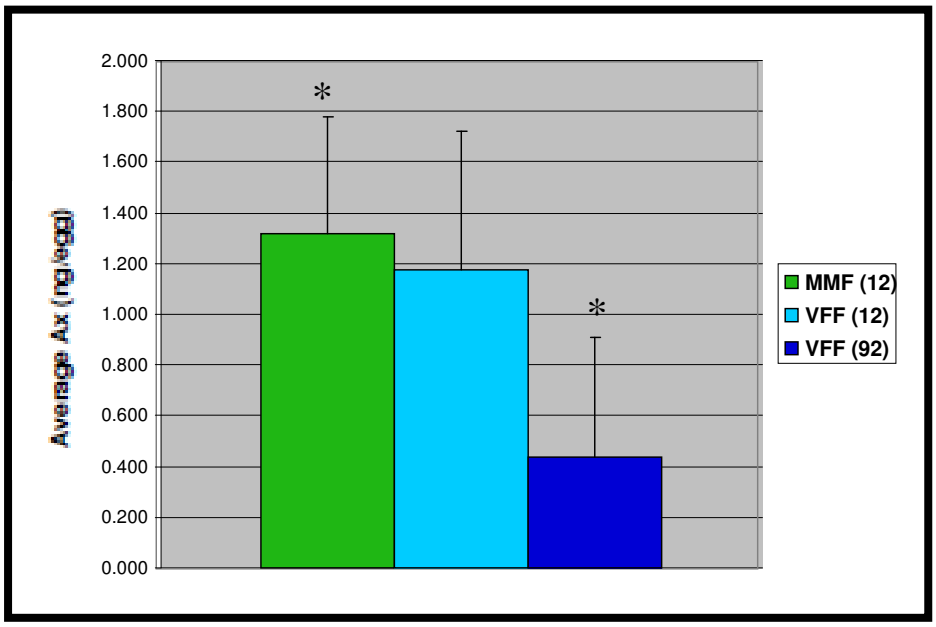

Figure 6.6 Mean Ax concentrations between MMF and VFF farms.

MMF12 refers to samples from MMF farm, VFF92 refers to all samples from VFF farm and VFF12 refers to the sub sample from VFF92 data. Bars are standard deviation of the mean. Comparison made by using ANOVA.* indicate statistically significant differences between MMF12 and VFF92 at $p < 0.05$.

There was no significant difference in Ax levels in eggs collected between the three seasons of autumn, spring and summer (Table 6.6). Also there was no correlation between Ax levels and other egg quality parameters such as fertilization rate, floating eggs, sinking eggs or total weight for all the data collected across the seasons. The average values for egg Ax concentration were highest in the autumn spawning fish, although there was considerable variation between batches across all seasons. The Kruskal-Wallis test analysis was carried out to compare the levels of Ax and volume of egg quality parameters.

Table 6.6 Effect of seasons on mean Ax concentrations in egg samples from VFF. Standard deviation of the mean indicated.

Carotenoid						
	Autumn		**Spring**		**Summer**	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
Ax(ng/egg)	0.49 ± 0.51	62	0.29 ± 0.33	21	0.28 ± 0.13	6

Distribution of egg Ax concentrations over the spawning season. Ax was measured from broodstock and eggs at VFF according to season (Table 6.6). The Kruskal-Wallis test analysis was carried out to compare the levels of Ax and volume of egg quality parameters.

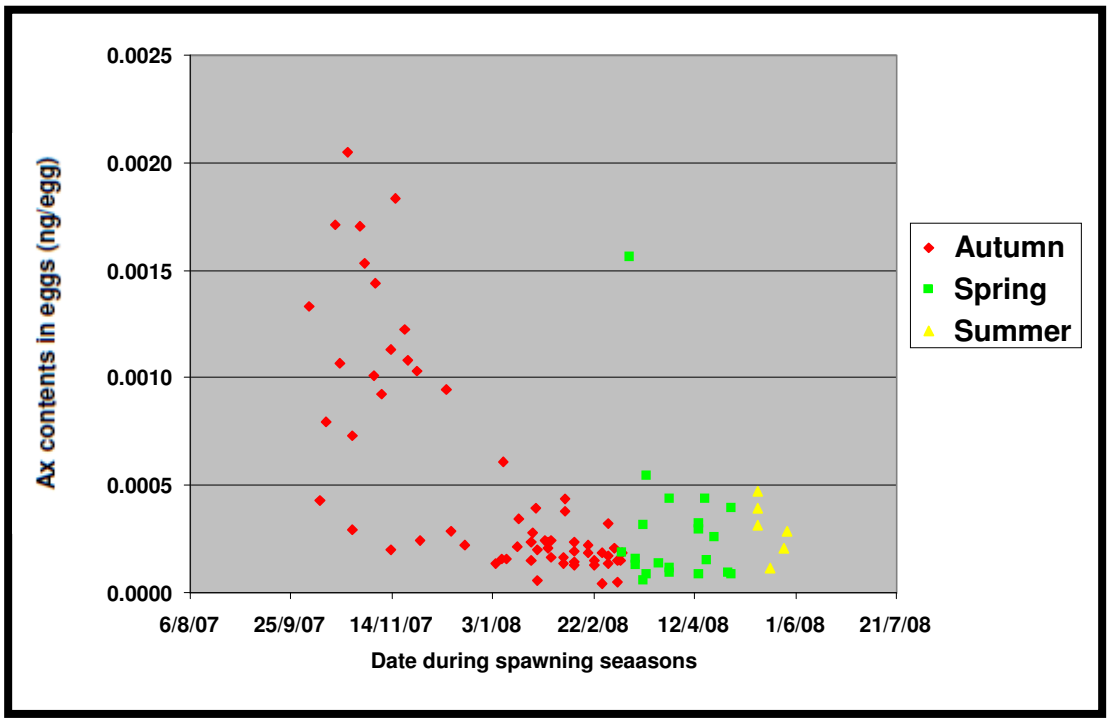

Figure 6.7 The Ax concentration over the spawning season for VFF eggs. Autumn (red), Spring (green) and Summer (yellow) spawned eggs are indicated.

The variation in the egg Ax concentrations between the three seasons was shown in Figure 6.6.

6.3.4 Fecundity and egg quality comparing seasons

Data on egg quality and fecundity from VFF by seasons are shown in Table 6.7. The results show differences in all parameters between seasons.

Table 6.7 Seasonal variation in egg quality parameters from eggs collected from VFF between October 2007 and May 2008. Standard deviation of the mean indicated.

	Autumn		Spring		Summer	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
Total weight of eggs (g)	1308 ± 1135	58	1095 ± 851	11	285 ± 58	3
Average weight of sinking eggs (g)	485.5 ± 535.0 [a]	55	138.1 ± 131.5[b]	10	113.7 ± 60.5[c]	3
Average weight of floating eggs (g)	907.9 ± 781.2	54	1025.8 ± 865.6	10	171.3 ± 56.5	3
Average fertilization rate of eggs (%)	81.4 ± 28.3	59	89.6 ± 19.3	18	66.6 ± 33.2	5

The results show a significantly increased number of sinking eggs recorded in the autumn spawners compared to the spring and summer groups although the latter contained only a small number of egg batches. However, the percentage of floating eggs was highest in the spring spawners at 94 % with autumn spawners having 69 % and summer spawners 60 % of floating eggs. Fertilization rate of eggs was not significantly different between the seasons.

6.4 Discussion

6.4.1 Fatty acid concentrations and farm location

Significant differences in the fatty acid profile of the two hatcheries were observed from this study.

The lipid analysis of the eggs showed that the MMF eggs were significantly higher in ARA, lower in EPA and the EPA/ARA ratio compared to the VFF eggs. Both ARA and EPA had the same trend when compared using the MFF/VFF12 or VFF92

samples. DHA/EPA and EPA/ARA ratios were significantly different between the two hatcheries. There may be a number of reasons for this observation. Firstly, the relationship between the concentration of ARA in egg lipids and that of broodstock has been described in a number of species (Tocher and Sargent, 1984). The positive impact on egg and larval quality of higher ARA and lower EPA/ARA ratio has also been described (Bruce et al. 1999; Mazorra et al. 2003). In addition, it was not possible to compare egg quality perameters between MMF and VFF because data on broodstock sex, size and number per tank were not available. In gilthead seabream a positive relationship between fatty acid levels in broodstock diets and egg quality was reported (Mourente & Odriozola 1990). In this study, analysis of the feed from VFF showed lower levels of ARA (0.8 %), compared to the values seen in earlier chapters of this thesis. However, it was not possible to collect feed samples from the MMF farm, and its ARA content, at the time of egg collection, was unknown. As shown in Chapter 3, supplementation of broodstock diets with ARA can alter the levels in eggs. This factor should be considered by hatchery operators, especially when broodstock are being kept in captivity and fed artificial diets (Morehead et al. 2001).

6.4.2 Fatty acid concentrations and seasons

Levels of ARA, EPA, DHA and DHA/EPA and EPA/ARA ratios in eggs were not significantly different across the seasons. The ratio between n-3 / n-6 PUFA in spring showed the highest values. According to Sargent, (1995) the ratio of these should be between 5:1 and 10:1, whilst in spring spawned eggs from VFF the ratio was 10.8. However, there was no significant difference in the ratio between seasons. The ratio of DHA/EPA should be ~2:1 and was similar in all seasons i.e. 2.2:1, 2.2:1 and 2.1: 1

respectively in autumn, spring and summer. However, there were no significant differences between seasons.

The captive fish from the farms were kept with controlled temperature and photoperiods maintained at all times. Thus, this could probably explain why season had no effect on the farms. There was no correlation between biochemical measures of egg quality and morphological parameters such as fertilization rate, floating egg or sinking egg numbers.

6.4.3 Fatty acids and fecundity

While the results show differences in all parameter factors between MMF and VFF it was not possible to compare this due to lack of data on broodstock sex, size and number per tank.

6.4.4 Ax

There was no significant difference in the levels of the carotenoid pigment Ax between the two hatcheries when the MMF 12 and VFF 12 samples were analysed. However, when the MMF 12 samples were compared to the VFF 92 samples there were significant differences with MMF (1.32 ng/egg) being 3-fold higher than VFF (0.44 ng/egg). Levels of Ax varied across the seasons with higher levels in autumn spawned eggs. These data were from VFF farm only. According to the record from the farm these data were collected from various sources (11 tanks) and tanks were fed different diets e.g. commercial pellets from Skretting Vitalis, Danafeed, Biomar and Europa and farm produced marine sausage diet (Table 6.1). This observation could explain the variation at different dates.

Evidence from the literature suggests that increased egg carotenoid concentrations are linked to improved egg and larval quality in Atlantic cod (Salze et al., 2005). The mechanism by which Ax exerts beneficial activity for egg and larval quality is not fully clarified but some possible explanations exist. There were no significant differences across the seasons due to Ax and no correlation between Ax and other egg quality parameters such as fertilization rate, floating eggs and sinking eggs.

6.5 Conclusion

In this study, levels of lipid and the fatty acid profile of eggs from two hatcheries were found to vary significantly. This variation was found between batches obtained from the different farms and from the same farm at different times of the spawning cycle. The farms used broodstock sources from different locations that were fed with a variety of supplemented or unsupplemented diets. Both these factors could explain the differences in fatty acid levels between farms.

However, others factors besides the broodstock diet may be important in determining the lipid content and fatty acid profile of the eggs. For example, the fatty acid composition of fish egg lipids is not only determined by the broodstock diet but also varies between different stocks (Pickova et al. 1997).

In the present study, season appeared not to affect fatty acids in the captive fish. This could be because fish were kept in a controlled environment for temperature, oxygen, light and diet. Whilst wild fish depend a lot on the food they eat which varies with season, ARA levels in eggs during spring were slightly higher than other seasons.

Although improvements in broodstock performance may be influenced by individual nutrients it is also likely that broodstock source and genetics, season of sampling and photoperiod as well as diet broodstock water temperature and other environmental conditions also influence overall performance and these are all worthy of further study to elucidate their individual and collective roles in egg quality.

The results highlight the difficulty in collecting morphological data on egg quality and husbandry data (fish age, size and sex) and feed samples and to measure these factors systematically on different farms. The analysis of egg fatty acid profile though is possible and combined with morphological measurements could strengthen egg quality assessment carried out by hatcheries.

Chapter 7. General Discussion, Conclusions and Future Perspectives

7.1 General

One of the main problems in cod hatcheries is low larval quality resulting in poor survival and growth (Brown et al. 2003). Hatcheries currently address this issue by using wild caught cod broodstock because they are believed to have significantly better performance. This use of wild origin broodstock can introduce disease and means that artificial selection to improve stock cannot be carried out. Whilst these problems may be due to environmental or other factors, poor egg and larval quality can be due to poor broodstock nutrition (Vassallo-Agius et al. 2001; Izquierdo et al. 2001). Broodstock nutrition has a major impact on spawning success with nutrients such as EFAs and antioxidants of particular importance. HUFA contents in particular have been found to be important in reproduction and development of eggs and larvae, with low levels in broodstock diets having a negative affect on fecundity, egg quality, hatching success, numbers of normal larvae and incidence of deformity (Watanabe 1985). ARA, EPA and DHA are the most important HUFA involved in successful reproduction (Sargent et al., 2002). Levels of ARA and carotenoid pigments (Ax) have been shown to be linked to hatching success and egg quality in different stocks of wild cod (Pickova et al. 1997). Levels of ARA and Ax have been found to be lower in eggs from broodstock of farm origin compared to those of wild origin broodstock (Salze et al. 2005).

This study investigated the impact of dietary supplementation with ARA and Ax on egg quality in cod. Four experiments were conducted. In the first experiment (Chapter 3) the impact of dietary supplementation with ARA was investigated in wild cod broodstock. An ARA supplemented diet was fed to broodstock for 1, 2 or 3 months prior to peak spawning. The experiment looked at effects on egg quality parameters in

order to determine the optimum period of supplementation for best reproductive performance. The second experiment (Chapter 4) investigated the impact of dietary supplementation with carotenoid (Ax) on egg quality parameters in farm origin cod broodstock. The third experiment (Chapter 5) investigated the impact of dietary supplementation with both ARA and Ax on farmed and wild origin broodstock egg quality parameters. The fourth experiment (Chapter 6) investigated whether egg lipid content and fatty acid composition, especially ARA, EPA, DHA and DHA/EPA and EPA/ARA ratio and carotenoid (Ax) levels varied according to hatchery location in the UK and with spawning time. In this experiment egg samples were collected from two cod hatcheries throughout a spawning season.

7.2 The impact of ARA and Ax supplementation on egg quality and fecundity

The results of the present study show that, in general, supplementation of cod broodstock diet with ARA and Ax had a positive impact on fecundity and some eggs quality parameters. However, the effect of ARA supplementation was not consistent across all egg quality parameters.

In the first experiment, ARA supplementation of broodstock diet for 1, 2 or 3 months before peak spawning had no significant impact on the number of eggs produced when compared to the control group. When data from groups receiving the ARA- supplemented diet were pooled, they produced 1.29 times more eggs per batch when compared with the control group. Batches of eggs from fish that received the ARA supplemented diet has significantly more floating fertilized eggs, 29 % more mean number of floating eggs/kg female and 41 % more mean number per batch of

fertilised eggs than the control group. However, these results were not consistent between the groups supplemented with ARA for different periods of time.

The reasons for these inconsistencies could be natural variability between broodstock populations, though other factors such as genetics, broodstock age (Evans et al., 1996), differences between spawning seasons, stage in spawning cycle and other environmental factors which can influence egg quality. Similar results have been reported before. For example, Rosenlund, (2006) found that when cod broodstock were fed an ARA supplemented diet (0.5, 1, 2 or 4% ARA) it had no effect on gonadosomatic index in male or female fish or egg quality parameters but did increase fecundity in the group fed 1% ARA.

In the second experiment, supplementation of cod broodstock diet with Ax for a period of two months before peak spawning resulted in a 20 % greater mean number per batch of eggs spawned/kg female, a 37 % greater number of floating eggs and 47 % more fertilised eggs per kg female. A significant correlation was also found between egg Ax content and fertilization success in individual batches. These results confirm that Ax has an important role in cod reproduction. This has been demonstrated in other species, such as rainbow trout, where dietary carotenoid supplements resulted in a positive relationship between egg pigmentation and fertilization and improved survival (Harris 1984; Craik 1985; Ahmadi et al. 2006). Previous research has suggested Ax may act as a substance which increases fertilization rates (Christiansen & Torrissen 1997).

In the third experiment, the diets of wild and farm origin cod broodstock were supplemented with ARA and Ax. The results showed that broodstock of wild origin performed better over a range of egg quality and fecundity indices when compared to

broodstock of farm origin. For example, wild origin fish had a 49 % higher mean number per batch of eggs collected per kg female than farm origin fish. In addition, wild origin fish produced a 51 % greater weight of floating eggs per batch and a 60 % higher mean number per batch of fertilized eggs per kg female, than in farm origin fish. However, mean hatching rates were not significantly different between farm and wild origin fish. The reasons for these differences are not clear from these results. A similar result has been reported in previous studies (Salze et al. 2005) and has been observed in black sea bass where the percentage of fertilized eggs from wild fish were higher than in captive fish (Gloria et al. 2009). Reasons for these findings could be due to factors such as the greater size and age of the wild origin broodstock, a better diet early in life before entering the farm (micro and other nutrients may be retained), a poor diet for farm reared broodstock, genetic differences between broodstock of different origin, high variability in individual fish performance and different age ranges and stocking density (Kjørsvik 1994; Pavlov et al. 2004). For example, it has been reported that cod have a very high potential fecundity and a positive relationship is found between relative fecundity and fish size (Kjesbu et al. 1991). In addition (Vadstein et al. 1993) suggested that problems with survival, growth and quality of cod larvae could be due to bacterial infections during the egg and yolk-sac stage. In many species, high mortality of larvae has been linked to the presence of bacterial pathogens (Muroga et al. 1990), and these effects may be unrelated to the quality of eggs and larvae.
In the last experiment, levels of ARA and Ax in eggs from two cod hatcheries were measured along with data on egg production over a complete spawning season. The results of this study found differences in the egg production parameters measured with more eggs produced by MMF broodstock. However, MMF had operational problems which meant they could not provide data on the number and weight of female fish

contributing to the data, whilst at VFF the lack of specialised ultrasound equipment also meant that it was not possible for them to determine fish gender. Therefore, at neither farm was it possible to adjust data for these factors and report true fecundity. These findings highlight the difficulty encountered measuring fecundity on cod hatcheries that employ different production techniques.

7.3 Fatty acid and Ax composition in eggs

The results of the present study show that dietary supplementation of cod broodstock diet with ARA and Ax successfully increased levels in the eggs produced by the broodstock.

In the first experiment, supplementation of broodstock diet for 1, 2 or 3 months before peak spawning with ARA successfully resulted in transfer of ARA to eggs, resulting in increased concentrations in eggs when compared to the control group. Those fed a supplemented diet for 2 months prior to peak spawning had the highest levels of ARA in their eggs. These findings show that farms can successfully supplement the diet of broodstock with ARA in the three months prior to peak spawning and that this will be rapidly transferred into the eggs. Although supplementation for only two months is enough to increase egg ARA during spawning. Increasing ARA levels in the diet did not impact significantly on the level of other fatty acids in eggs. This is largely because ARA is a relatively minor component of the overall egg fatty acid composition. The results also show that there were differences in levels of egg lipid classes from the broodstock fed ARA supplement for 3 months (for example, with significantly higher levels of polar lipid). The percentages of PC compared to time of ARA supplementation showed highest PC in the group fed ARA

for 3 months before peak spawning. PC is the main egg lipid class in cod (Fraser et al. 1988) which has low levels of neutral lipids and consequently high levels of phospholipids (Salze et al. 2005; Tocher et al. 2008). PC is utilised during egg and larval development (Finn et al. 1995; Fraser et al. 1988; Rainuzzo et al. 1992; Tocher et al. 1985) and it has been proposed that polar lipids may promote growth in juvenile cod (Olsen et al. 1991). However, there were no other correlations between lipid class percentages and timing of ARA supplement even with pooled groups. Correlations were also found between lipid class percentages including PE and EPA/ARA ratio.

The results of the second experiment showed that supplementation of broodstock diet with Ax results in uptake and deposition of Ax into eggs and provides significant improvements in egg quality, similar to those found in other fish species. The concentrations measured in the eggs from fish fed the Ax supplemented diet were almost three times greater than those from the control group (2.79 ng/egg compared to 0.98 ng/egg). A significant correlation was also detected between egg Ax content and fertilization rate in individual egg batches. The improved egg quality in farmed cod fed diets supplemented with Ax may be the result of improved antioxidant protection for these fish and their eggs (Cowey et al. 1985; Pangantihon-Kuhlmann et al. 1998).

In the third experiment, egg fatty acid, vitamin E, Ax, PGE_2, TBARS levels and lipid classes were compared between farm origin and wild origin broodstock fed a diet supplemented with both ARA and Ax. The results showed that there were significant differences in egg fatty acid and vitamin E levels between the wild and farmed origin fish but there were no differences in levels of Ax, PGE_2, TBARS level and lipid classes.

For example, the level of EPA was greater in eggs from wild origin broodstock than those of farm origin broodstock. This result has been found in other species such as

the striped trumpeter (*Latris lineate*) with EPA and DHA levels in eggs from broodstock of wild and captive origin, as well as captive origin fish fed different diets were significantly different (Morehead et al. 2001; Bransden et al. 2007). The EPA/ARA ratio for farm fish was lower (4.0) than wild fish (4.4) because of higher EPA and low ARA levels in eggs from wild origin fish compared to those of farm origin. Pavlov (2004), found the relative proportions of EFAs (such as ARA, EPA and DHA) differed and could be a good measure of egg quality in cod. In addition, lower ratios of EPA/ARA have been linked to better egg quality in cod (Salze et al., 2005) as was shown in the first trial described in this thesis.

Vitamin E concentrations were higher in the eggs of farmed fish than in those from wild fish, and there was no difference in the content of TBARS. This suggests that vitamin E is not a limiting nutrient in relation to cod egg quality, and that there was no difference in the production of MDA (as assessed by TBARS) due to lipid oxidation within the eggs.

In the fourth experiment levels of ARA and Ax in eggs from two cod hatcheries were measured along with data on egg production. The results showed significant differences in egg fatty acid profile between the two hatcheries. For example, MMF eggs were found to be higher in ARA, lower in EPA and the EPA/ARA ratio compared to the VFF eggs. The ratios of DHA/EPA and EPA/ARA were also found to differ significantly between the two hatcheries. This relationship has been described in a number of species (Tocher and Sargent, 1984). Higher ARA and lower EPA/ARA ratios had a positive impact on egg and larval quality in Atlantic halibut (Bruce et al., 1999; Mazorra et al., 2003). One possible reason for the differences in egg ARA levels could be the level of ARA in the broodstock diet. It was found that feed from the VFF

hatchery had low levels of ARA (0.8 %) which could explain the differences between this farm and MFF. Feed samples from MMF farm were unfortunately not available for analysis so its ARA content was unknown. However, supplementation of broodstock diet at MFF with ARA was carried out during that season (W.Roy, personal communication). There was no correlation between egg quality and morphological parameters such as fertilization rate, floating egg or sinking egg numbers from the two farms. Seasonal data were collected only from the VFF hatchery. The levels of fatty acids such as ARA, EPA, DHA and DHA/EPA and EPA/ARA ratios in eggs were not significantly different across the seasons.

Levels of Ax in eggs from the two hatcheries were not significantly different when the samples collected over the same period were compared. However, if all the samples from MFF were compared with all the samples from VFF, MMF eggs had three times the concentration of Ax. At VFF farm, there was no significant difference in Ax in eggs between seasons. There was no correlation between egg quality parameters such as fertilization rate, floating eggs and sinking eggs and egg Ax levels.

These results could be explained by the fact that fish were kept under controlled farm conditions (temperature, photoperiod and diet). In the wild, the diet of fish will vary according to season.

7.4 General conclusions

The first experiment investigated the impact of dietary supplementation with ARA for 1, 2 or 3 months prior to peak spawning, on egg quality in wild cod in order to

determine the optimum period of supplementation for best reproductive performance. The results showed that:

- Short term supplementation of broodstock diets with ARA for 1, 2 or 3 months before peak spawning resulted in increased concentrations of ARA in eggs

- Supplementation for more than 2 months did not provide any significant increase in egg ARA

- There was no correlation between egg production or egg quality parameters and the length of time supplementation was provided. However, there were higher numbers per batch of floating eggs per kg per female and fertilized eggs per kg female in the three tank groups fed the ARA supplement than in the single unsupplemented control tank group

- The performance of the control group was affected by the lack of ARA supplementation in the diet as they had the lowest egg quality paremeters when compared to the pooled ARA groups

The supplementation of diets with ARA to 3 % of total fatty acids provided some limited benefit but it was not a major factor for improved reproductive performance

The second experiment investigated the effect of short-term supplementation of Ax in broodstock diets on a number of egg quality parameters in farmed cod. The results showed that:

- Ax uptake into eggs from the broodstock diet was highly efficient

- A correlation between the egg Ax content and fertilization success of individual batches was identified. This improvement in egg quality demonstrated the potential value of Ax supplementation of broodstock diets for cod

- Higher numbers per batch of eggs spawned per kg female, numbers per batch of floating eggs per kg female, and numbers per batch of fertilised eggs per kg female were measured in groups of farm origin fish fed the Ax supplement

The third experiment compared the relative effect of diets supplemented with optimum levels of ARA and Ax on egg quality in wild and farmed cod. The results show that:

- The performance of wild origin and farm origin broodstock does vary significantly even when fed identical diets supplemented with ARA and Ax

- Wild origin broodstock performed better on a number of egg quality and fecundity indices as has been observed in some commercial hatchery situations

- There were few significant differences in levels of fatty acids between eggs of farm and wild broodstock except for some minor fatty acids like 16-1n-9 and 16-1n-7, and the EPA content, ratios of DHA/EPA, EPA/ARA and vitamin E levels

- The level of Ax in eggs (pooled data) were positively correlated with fertilization rates. Vitamin E concentrations in the eggs from farmed fish were not lower than in eggs from wild fish, and TBARS were no higher in eggs from farmed fish, suggesting that a lack of vitamin E or other antioxidant niutrients in the diet was not responsible for differences in egg quality.

- For hatcheries, broodstock of wild origin may appear to give better reproductive performance over farm fish even though they have both received a supplemented diet. However, if the wild origin broodstock are larger this would also result in greater number of eggs and improve reproductive performance.

The last experiment compared whether egg lipid content and fatty acid composition, especially ARA, EPA, DHA, DHA/EPA, EPA/ARA ratios and carotenoid (Ax) level varied according to hatchery location in the UK and spawning season through the collection and analysis of samples of eggs. The results show that:

- Levels of lipid and the fatty acid profile of eggs from the two hatcheries were found to vary significantly

- Variation was found between batches obtained from different farms and from the same farm at different times of the spawning cycle

- The farms used broodstock from different geographical locations and fed the fish a variety of supplemented or unsupplemented diets. Both these factors could explain the differences in fatty acid level between farms

- Other factors besides the broodstock diet may be important in determining the lipid content and fatty acid profile of the eggs.

The present study encountered a number of problems which meant that comparison of results between farm and wild broodstock was difficult. These problems can be addressed in future experiment by ensuring that the following are considered; -

- The age and size of broodstock should be matched
- More replicate tank can be used
- Environmental condition should be standardised (such as temperature, salinity and photoperiod regimes)
- Morphological and biochemical egg quality parameter should be measured for all trail, including analysis of egg ripeness (McEvoy 1992)
- Improvement to the broodstock holding tank such as deeper water and dimmer light along with improve egg collector.

7.5 Future perspectives

Cod farming and hatchery operation in particular still requires further research to address key problems which constrain production. Broodstock management and nutrition are areas where significant work is needed in cooperation between industry and research. The results of the present study suggest the following areas for future research:

- Dietary supplementation for cod broodstock with ARA and Ax should be considered in order to improve spawning success. If levels of these are unknown prior to spawning then testing could allow assessment of broodstock nutritional status so that early corrective action can be taken

- More research is required on the ARA and Ax status of eggs from commercial broodstock. Efforts should be made to standardise recording of egg quality parameters in order to allow better comparisons between broodstock populations

- The ARA and Ax requirement of cod broodstock should be established experimentally especially with regards to earlier stages of gonad maturation

- The most efficient chemical forms and concentrations of ARA and Ax for use in cod broodstock feeds should be determined

- More research is needed on the effect of environmental conditions, husbandry and behavioural interactions in relation to spawning success of cod broodstock

- Hatcheries should check the status of their cod broodstock with regard to dietary Ax concentrations (pre-spawning) and if needed provide short term supplementation

- ARA and Ax levels should be considered when developing diet formulations for cod production, especially for broodstock nutrition, immune function and egg and larval quality.

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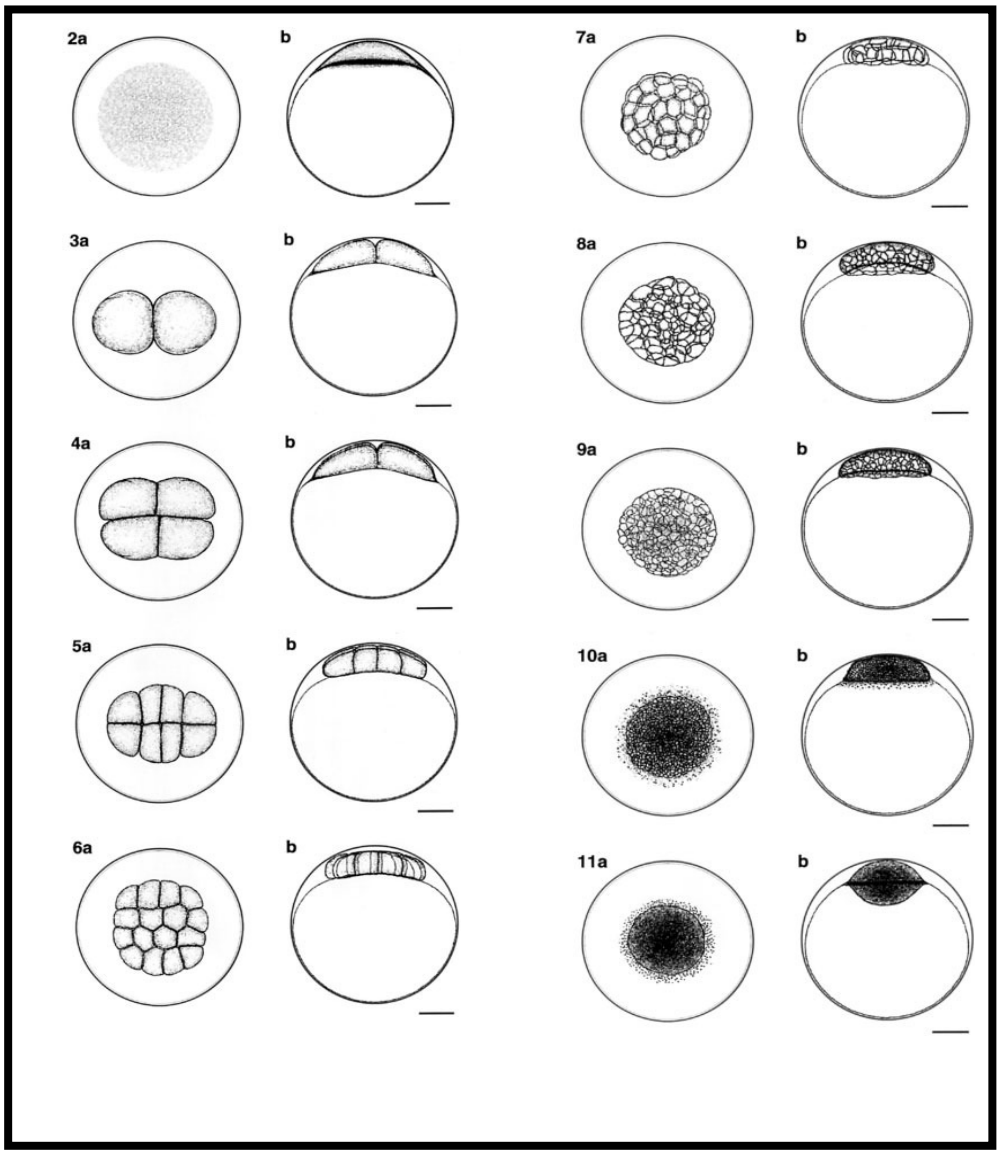
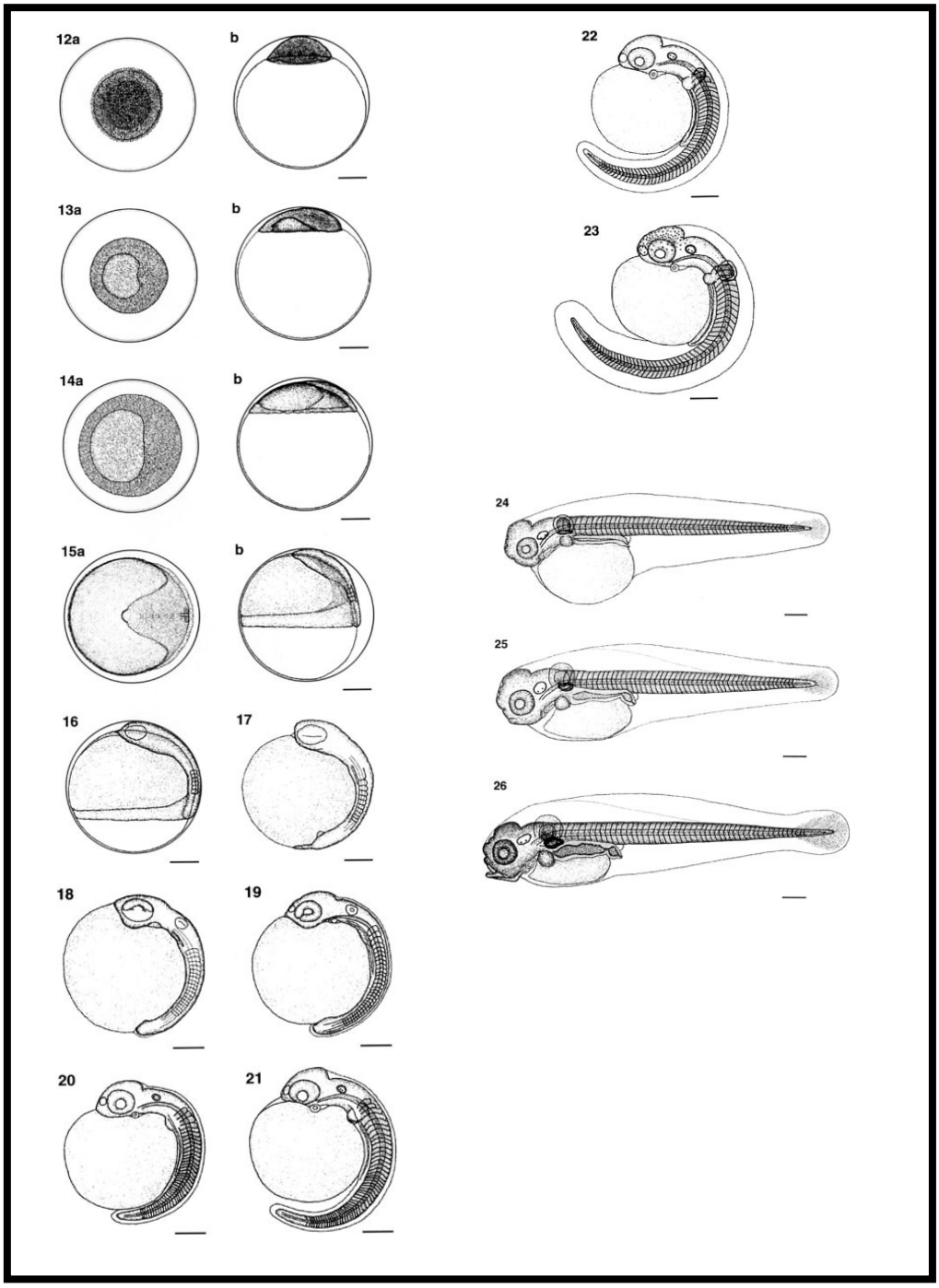
Appendix I. Stages of embryonic development in the Atlantic cod





Figs. 2–26. Stages of embryonic development in the Atlantic cod *Gadus morhua* (bar _ 300 _m). In Figs. 2–15, **a** shows the dorsal view, **b** shows the lateral view. Figs. 16–26 show lateral views only. Fig. 2: 1-cell stage (315 minutes post fertilization mpf). Fig. 3: 2-cell stage(405mpf). Fig. 4: 4-cell stage (540 mpf). Fig. 5: 8-cell stage (675 mpf). Fig. 6: 16-cell stage (810 mpf).

* Modified from Hall et al. 2004, Stages of Embryonic Development in the Atlantic Cod *Gadus morhua*. Journal of morphology 259:255–270.

Appendix II. Feed details

Data on Skretting Feed used for cod experiments

Company address:-

Skretting, Shay Lane, Longridge

Preston UK

Feed hygiene regulation

Approval no AGB193E1318

VMR Approval n0 2015414

Feed type:-

Marine Broodstock Mix

A complete feeding stuff for fish. Feed according to instructions in Skretting technical literature. Contains fishmeals, cannot be fed to ruminants

UFAS-compound feeds

Certificate Reg NO190

Oil 15.00%; Protein 60.00%; Ash 13%; Fibre 1%; Phosphorous 1,90%; Copper 10mg/kg; Vitamin A 12 000 iu/kg; Vitamin D3 2000 iu/kg, Vitamin E (as alpha-tocopherol acetate) 500 iu/kg Vitamin present until best before Jan 22 2007 date of manufacture Mar 16 2006

Contains in descending order:-

Fish meal, Crustacean meal, Fish Proteine Concentrate, Wheat gluten, Fish Oil, Vitamins, Lecithin, Glucan, Minerals, Nucleotide,

Contains permitted antioxidants (Ethoxyquin, BHT, BHA)

Also contains selenium

Store in cool dry place

Net weight indicated on delivery net

Registered Office Town (UK)

Ltd; Wincham, Northwich Cheshire UK

Appendix III. Publications and presentations arising from the project

- Sawanboonchun, J., Roy, W. J., Robertson, D. A., & Bell, J. G. 2009, The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (Gadus morhua, L.), Aquaculture News, vol 35.

- Sawanboonchun, J., Roy, W. J., Robertson, D. A., & Bell, J. G. 2008, The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (Gadus morhua, L.), Aquaculture, vol. 283, no. 1-4, pp. 97-101.

- Roy, W., Bell, G, Sawanboonchun, J. Davie, A, Franco, J, Fernandes, D, Gnassou, J and Robertson, D. (2007). Cod broodstock nutrition, Arachadonic acid and astaxanthin as determinants of egg quality, Final Report. (Scottish Aquaculture Research Forum No.014).

- Oral presentation 1st Annual international Aquaculture students conference 29th October 2008: Atlantic cod broodstock nutrition: The role of arachidonice and astaxanthin as determinants of egg quality.

- Poster show in Aquaculture UK 2008 conference in Aviemore 21st May 2008: The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (*Gadus morhua*, L.).

- Poster show in 1st Annual international Aquaculture students conference 29th October 2008: The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (*Gadus morhua*, L.) (won a prize).

- Poster show in International Symposium- Aquaculture-Asustainable future – Edinburgh 21-22 April 2009: The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (*Gadus morhua*, L.).

In progress

- Poster show in larvi 2009, 5th fish & shellfish larviculture symposium Ghent University, Belgium.7 - 10 September 2009: The impact of dietary supplementation with arachidonic acid on egg quality in Atlantic cod broodstock (*Gadus morhua*, L.)