SARF014: COD BROODSTOCK NUTRITION: ARACHIDONIC ACID AND ASTAXANTHIN AS DETERMINANTS OF EGG QUALITY

FINAL REPORT

Prepared for The Scottish Aquaculture Research Forum

BY

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Executive Summary

Cod hatcheries rely to a large extent on eggs produced by wild-caught broodstock since egg quality from farm-reared broodstock tends to be poor. Reliance on wild fish creates a risk of disease introduction and difficulties with stock improvement by artificial selection. Effective techniques to improve the quality of eggs obtained from farmed broodstock are therefore necessary for the industry to progress. Differences have been identified in concentrations of arachidonic acid (an essential polyunsaturated fatty acid), and astaxanthin (a biologically active carotenoid pigment) between eggs from wild and farmed cod. Reports in the literature make clear the importance of these nutrients in relation to egg quality in fish.

This project investigated the impact of dietary supplementation with arachidonic acid and astaxanthin on egg quality in cod. Three experiments were conducted. The first experiment investigated the effect of feeding a diet supplemented with arachidonic acid, 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 second experiment evaluated the effect of short-term supplementation of astaxanthin in broodstock diets on egg quality in farmed cod. The third experiment compared the relative effect of a diet containing added arachidonic acid and astaxanthin diets on egg quality in wild and farmed cod.

Control groups of wild and farmed fish in the first two experiments provided baseline egg quality indicators against which the effects of dietary manipulations could be assessed. Egg numbers were expressed in terms of female biomass to permit comparisons between stocks. Biochemical analysis of eggs was carried out to examine the effects of treatment on nutritional status, particularly fatty acid composition and astaxanthin content.

In Experiment 1, supplementation of the feed with arachidonic acid (ARA) resulted in a rapid increase in the arachidonic acid content of the eggs. Maximum concentrations were measured in eggs from fish fed the supplement for 2 months prior to peak spawning indicating that short term supplementation was effective in boosting arachidonic acid concentrations in eggs immediately prior to spawning. There was no correlation between the

duration of arachidonic acid supplementation and the number or quality of eggs produced, but groups fed the ARA supplement produced higher numbers per batch of floating eggs per kg female and fertilised eggs per kg female than the untreated control group. In this experiment, the performance of the control group was not seriously compromised by the lack of ARA supplementation of the feed. Egg arachidonic acid concentrations measured in this group suggest that levels were adequate for acceptable reproductive performance.

In Experiment 2, uptake of astaxanthin into the eggs was similarly rapid. Fish fed the diet supplemented with astaxanthin 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 improved.
A correlation between the astaxanthin content and fertilisation success of individual batches was identified. This experiment demonstrated the potential value of high levels of astaxanthin in broodstock diets for cod.

In Experiment 3, the optimised diet containing added arachidonic acid and astaxanthin was fed for two months prior to peak spawning and concentrations of these nutrients in eggs were elevated to the desired levels. Performance of farmed fish fed the optimised diet was improved in relation to the previous benchmark data for farmed fish, and in relation to the wild broodstock. Egg production increased by a factor of 1.50 and numbers of fertilised eggs increased by 1.54 in comparison with the previous year.

These results are discussed in relation to the current needs of the cod farming industry to establish a reliable supply of high quality eggs from farmed broodstock.

1. Introduction

1.1 Commercial production of cod eggs

Cod hatcheries require a supply of good quality eggs as seed. Poor quality eggs give increased rates of mortality and deformity during egg and larval rearing which result in reduced production efficiency and fish welfare problems. Farm observations indicate that wild-caught cod tend to produce better quality eggs and larvae than farmed broodstock and as a result most commercial hatcheries now obtain eggs from wild-caught broodstock. However, reliance on wild broodstock creates a risk of introduction of disease, limits the potential for stock improvement by artificial selection and raises the concerns over the sustainability of an industry heavily reliant on wild caught broodstock. As a result, there is a need to understand and attempt to correct the causes of poor egg quality in farmed cod.

Many parameters have been reported to influence egg quality in farmed fish, including broodstock nutrition, environmental conditions and husbandry practices (Bromage 1995). Where nutritional factors are involved,

manipulation of broodstock diets can provide a practical means of maintaining and improving egg quality. In cod broodstock operations, where individual fish may be fed artificial feed and conditioned for spawning in tanks over a period of several years, nutritional factors are relevant to fish of both farmed and wild origin.

Previous work, funded by the UK Sea Fish Industry Authority and carried out by our group in 2004, demonstrated differences in arachidonic acid and carotenoid pigment concentrations in eggs from wild and farmed cod. These nutrients are known to influence egg quality in other fish species and the current project sought to investigate this function in more detail.

1.2 Previous state of knowledge

Several reviews have considered the influence of nutrient availability on reproductive physiology and broodstock performance in fish (Hardy 1985, Watanabe 1985, Bromage 1995, Pavlov *et al* 2004). Most attention has been paid to the influence of the polyunsaturated fatty acids, vitamins C and E, and the carotenoid pigment astaxanthin.

Manipulation of broodstock diets offers a practical means to improve egg quality in cod culture. Supplementation of broodstock feeds with specific nutrients, particularly specific fatty acids and fat-soluble micronutrients, 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 (Czesny and Dabrowski 1998; Ashton *et al* 1993; Watanabe and Miki 1993; Verakunpiriya *et al* 1997; Gallagher *et al* 1998; Sargent *et al* 2002).

In a Seafish-funded project carried out by our group in 2004, differences in arachidonic acid concentration, eicosapentaenoic acid/arachidonic acid ratio (EPA/ARA) and carotenoid pigment concentration were identified between wild and farmed cod broodstock. These nutritional differences were correlated with differences in egg quality, suggesting that sub-optimal levels of arachidonic acid and carotenoid pigment may be implicated in egg quality problems in farmed cod (Salze *et al* 2005).

The importance of polyunsaturated fatty acids (PUFA) in reproduction and development of eggs and larvae of marine fish is well known, and many studies have shown the effects of sub-optimal levels of PUFA on fecundity, egg quality, hatching success, numbers of normal larvae and incidence of deformity (eg Watanabe 1985; Sargent 1995; Rainuzzo *et al* 1997). Marine fish have a limited capacity for interconversion of fatty acids so the fatty acid composition of the diet is important to ensure adequate provision. An adequate supply and appropriate concentrations of arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are generally regarded as important for successful reproduction and embryonic development (Sargent *et al* 2002).

Salze *et al* (2005) found no differences in DHA concentrations, or DHA/EPA ratio in eggs from wild and farmed fish, but the ARA concentration was lower and the EPA/ARA ratio higher in poor eggs from farmed fish. Similarly, Pickova *et al*. (1997) showed that ARA concentration was correlated with hatching success and other egg quality parameters in different stocks of wild cod. ARA is a precursor of eicosanoids, an important group of compounds with a wide range of biological functions, which includes a role in final maturation of oocytes. Eicosanoid function is inhibited by a range of compounds derived from EPA which act to regulate their activity. The EPA/ARA ratio is therefore significant as an indicator of potential eicosanoid activity and physiological function. Subsequently, analysis of fatty acid profiles and batch quality of eggs obtained from various commercial sources suggests that the correlation between EPA/ARA ratio and egg quality is geographically widespread and not restricted to a single site or particular stock of fish (D. Robertson and G. Bell unpublished observations).

Supplementation of broodstock diets with arachidonic acid has been shown to improve egg quality in halibut, sea bass and Japanese flounder (Bruce *et al.* 1999; Furuita *et al.* 2003; Mazorra *et al.* 2003; Alorend 2004). In a recent study using F1 generation farm-reared cod fed diets containing 0.8 % or 3.2 % ARA for approximately 5 months prior to spawning, no difference was found in numbers of viable eggs and egg quality remained poor in both groups (Blanco 2004; Bell *et al* 2005). However, farm observations suggest that ARA supplementation produces an improvement in egg quality in cod broodstock (D. Robertson unpublished observations).

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) 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. Possible functions of carotenoids in fish eggs include UV protection, provitamin A and antioxidant activity and respiratory function (Craik 1985; Mikulin 2000). The effects of carotenoid supplementation on egg quality in salmonids are well known. Supplementation of broodstock diets with the carotenoid pigment, astaxanthin, has also been shown to improve egg quality in red sea bream and yellowtail (Watanabe and Miki 1993; Verakunpiriya *et al*. 1997). There are no reports of the effects of carotenoid supplementation on egg quality in cod.

1.3 Objectives

The specific objectives of the current study were as follows:

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

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

3. To compare the relative effect of diets supplemented with optimum levels of arachidonic acid and astaxanthin on egg quality in wild and farmed cod.

2. Materials and methods

2.1. Determination of the optimum level of ARA in relation to egg quality in wild-origin cod

Fish and husbandry

Wild-caught fish, acclimatised to farm conditions and held in tanks for at least 12 months, were allocated to four 12m^3 tanks in November 2005. In January 2006, the experimental 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. After allocation each group contained 16 males and 8 or 9 females. Fish were fed to satiation twice daily.

Feed

The basal diet was based on a commercial moist feed formulation (Vitalis ® Marine Broodstock Mix) specially prepared to contain no supplementary astaxanthin or added arachidonic acid and supplied by Skretting UK. The feed was supplied as a dry mix which was constituted for feeding by the addition of water (0.7 L/kg dry mix) and astaxanthin (Carophyll Pink 0.5g/kg dry mix). 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.

Experimental design

The experiment used four treatment groups with one replicate tank per treatment. Group A 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.

Egg quality assessment

The spawning period was taken as the period 1 March to 31 May 2006, and the peak spawning date as 15 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 fertilisation 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. A volume of 2.0 ml of floating eggs (approx 1000 eggs) was incubated in a 500 ml glass beaker in a water bath at approximately 10ºC with continuous low level aeration, and regular partial water changes. After 10 days, numbers of hatched larvae were counted and hatch rate was calculated.

Biochemical analysis

The fatty acid composition and total lipid content of feed and egg samples was measured using standard laboratory procedures.

Data analysis
Egg quality indices used for group comparisons included batch weights of eggs collected, batch weights of floating eggs, fertilisation 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 assuming 500 eggs/g.

Analysis of variance, 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. Spearman's rank test was used to detect any correlation between fatty acid composition and egg quality.

2.2 Evaluation of the effect of astaxanthin supplementation on egg quality in farm-reared cod.

Fish and husbandry
Farm-reared fish were allocated to four 7m3 tanks in November 2005. In January 2006, 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. After allocation each group contained 34 or 35 males and 35 or 36 females.

Feed
The basal feed was, again, Marine Broodstock Mix, specially prepared to contain no added astaxanthin as described above. In this experiment, the feed was constituted for feeding by the addition of water (0.7 L/kg dry mix) and arachidonic acid (Vevodar® oil, 12 g/kg). Carophyll Pink (DSM, Switzerland), with a nominal astaxanthin content of 10%, was used as a source of astaxanthin (ASTA) for the ASTA-supplemented feed, added at a rate of 1g/kg dry mix. Fish were fed to satiation twice daily.

Experimental design
The experiment used two treatment groups each in duplicate tanks. Group A was fed an unsupplemented control diet throughout the spawning period. Group B was fed astaxanthin supplemented feed for approximately two months prior to the peak-spawning date.

Egg quality assessment
The spawning period was taken as the period 1 March to 31 May 2006, and the peak spawning date as 15 April 2006. Each day during spawning, egg batches were collected and egg quality was assessed using standard techniques as described above.
Dropout within each tank was measured on five different 24 hr periods. Samples of floating eggs were collected on 14 different dates for hatch rate determination.

Astaxanthin analysis was carried out on floating eggs collected from each tank on 11 different dates during the course of the spawning period.

Biochemical analysis
Astaxanthin concentrations were measured in samples of feed and eggs using standard laboratory procedures.

Data analysis
Data were summarised as described above. Group comparisons were made using analysis of variance with tank as a factor nested within each treatment. Other analyses were as described above.

2.3 Comparison of the relative effect of diets supplemented with ARA and astaxanthin on egg quality wild and farmed cod

Fish and husbandry
Duplicate groups of wild caught and farm-reared broodstock, were allocated to four 12m^3 tanks in November 2006. 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. After allocation each tank of wild fish contained 11 females (mean weight 5430 g) and 11 or 12 males (mean weight 4740 g) and each tank of farmed fish contained 17 females (mean weight 3543 g) and 18 males (mean weight 2701 g).

Feed
All fish received the same diet which was based on the commercial Marine Broodstock Mix formulation (Skretting, UK), supplemented with additional astaxanthin and arachidonic acid. Feed was prepared by the addition of water (0.6 L/kg dry mix), arachidonic acid (Vevodar® oil, 8.7 g/kg) and astaxanthin (Carophyll Pink, 1.2 g/kg). The experimental diet was fed from approximately 2 months prior to the peak spawning date. Fish were fed to satiation twice daily.

Egg quality assessment
The spawning period was taken as the period 1 February to 11 May 2007, and the peak spawning date as 22 March 2007. Each day during spawning, egg batches were collected and egg quality was assessed as described above. Batches of eggs from all four 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.

Biochemical analysis
Fatty acid composition, total lipid content and astaxanthin content were measured in feed samples. Fatty acid composition, total lipid, astaxanthin, vitamin E and TBARS (thiobarbituric acid reactive substances, a measure of lipid oxidation) were determined in egg samples using standard laboratory procedures.

Data analysis

Data were summarised as described above. Analysis of variance, with tank as a factor nested within each treatment, was used to test for differences related to fish origin (wild and farmed). Spearman's rank test was used to detect any correlation between arachidonic acid content, astaxanthin content and EPA/ARA ratio and egg quality.

3. Results

3.1 Determination of the optimum level of arachidonic acid in relation to egg quality in wild-origin cod

Arachidonic acid concentrations 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 1 and Figures 1 and 2 summarise the principal egg quality indices in the four treatment groups in this experiment.

In Group A (control) total egg production was 590185 eggs per kg female. Approximately 36% were lost within the tank, and 376339 were collected from the surface outlet. Eggs were present in the collector on 61 days during the 92 day spawning period. Approximately 47% of the collected eggs sank and were discarded, and the remaining 200195 egg per kg female were assessed for fertilisation. The average fertilisation rate in the floating fraction was 55 % and a total of 125222 eggs per kg female (33% of those collected) were viable (ie floating, fertilised) eggs. The average hatch rate of the floating eggs was 24 %, equivalent to a total of 48462 hatched larvae per kg female (12.8% of eggs collected).

Differences in total numbers of eggs produced were a result of differences in both the number and size of batches. Groups A and B produced more batches than Groups C and D, and Groups B and D produced the highest number of eggs per batch. Thus, egg production was highest in Group B and lowest in Group C. Significant differences in numbers of eggs produced per batch (ANOVA $F=3.457$; $p<0.05$) were detected between Groups B and C, but, interestingly, there was no difference in number of eggs collected per batch.

The mean number per batch of eggs collected per kg female was significantly higher in Group B than in Group A ($F=3.013$; $p<0.05$), but there was no consistent increase in egg production in the other groups which received the ARA supplement and no evidence of any correlation between the total number of eggs produced by each group and the duration of ARA-supplementation.

Two points indicate that egg quality was significantly better in groups which received the ARA-supplemented diet. Firstly, the average weight per batch of floating eggs was significantly higher in Groups C and D than in Groups A and B ($F=9.140$, $p<0.001$) , and the average number per batch of floating eggs per kg female was significantly higher in the pooled ARA-treated groups than in the control group (Mann Whitney $U=4023$; $p<0.05$). The mean number of floating eggs in Groups B-D was a factor of 1.29 times higher than in Group A. Numbers of floating eggs per batch were higher in all groups which received the ARA supplement (Figure 1). Secondly, the average number per batch of viable eggs per kg female was significantly higher in the ARA-treated groups

than in the control ($t=2.417$, $p<0.05$). The mean number of viable eggs in Groups B-D pooled was a factor of 1.41 times higher than in Group A and, again, numbers of viable eggs per batch were higher than the control in all groups which received the ARA supplement (Figure 2).

Table 1. Egg production and egg quality indicators in Experiment 1. Egg numbers are expressed as numbers per kg female. Differences in mean weights or numbers per batch are shown as * (p<0.05), ** (p<0.01) or *** (p<0.001).

Group	A	B	C	D	B-D
Treatment	No ARA Control	+ARA 1 month	+ARA 2 months	+ARA 3 months	+ ARA pooled
total no of eggs produced/kg female	590185	738132	356707	572813	555884
no of batches produced	61	62	50	55	
mean no per batch of eggs produced/kg female	9675	11905**	7134**	10415	9818
total weight of eggs collected (g)	24660	34220	20729	26537	27162
no of batches collected	61	62	50	55	
mean wt of collected egg batches (g)	404	552	415	482	483
total no of collected eggs/kg female	376339	556097	320337	442727	439720
no of batches collected	61	62	50	55	
mean no per batch of eggs collected/kg female	6169*	8969*	6407	8049	7809
total wt of floating eggs (g)	13118	17352	12161	13877	14463
no of batches with floating eggs	61	61	49	53	
mean wt per batch of floating eggs (g)	215	284	248	262	265
total no of floating eggs/kg female	200195	281981	187931	227011	232308
no of batches with floating eggs	61	61	49	53	
mean no per batch of floating eggs/kg female	3282*	4623	3835	4283	4247***
mean fertilisation rate (% floating eggs)	55%*	56%	70%*	60%	62%
total no of fertilised eggs/kg female	125222	171736	140817	152231	154928
no of batches with fertilised eggs	59	57	48	50	
mean no per batch of fertilised eggs/kg female	2122*	3013	2934	3045	2997**
mean hatch rate (% floating eggs)	24	18	20	16	18
Total no of hatched eggs/kg female	48462	52005	38526	36225	42252
No of batches with floating eggs	61	61	49	53	
Mean no per batch of hatched eggs/kg female	794	853	786	683	774
ARA (ug per egg)	0.11*	0.13	0.19*	0.17*	0.16
EPA (ug per egg)	0.92	0.80	0.78	1.00	0.86
DHA (ug per egg)	2.00	1.77	1.64*	2.18*	1.86
DHA/EPA ratio	2.18	2.22	2.09	2.20	2.17
EPA/ARA ratio	8.37***	6.28***	4.39***	6.06***	5.63

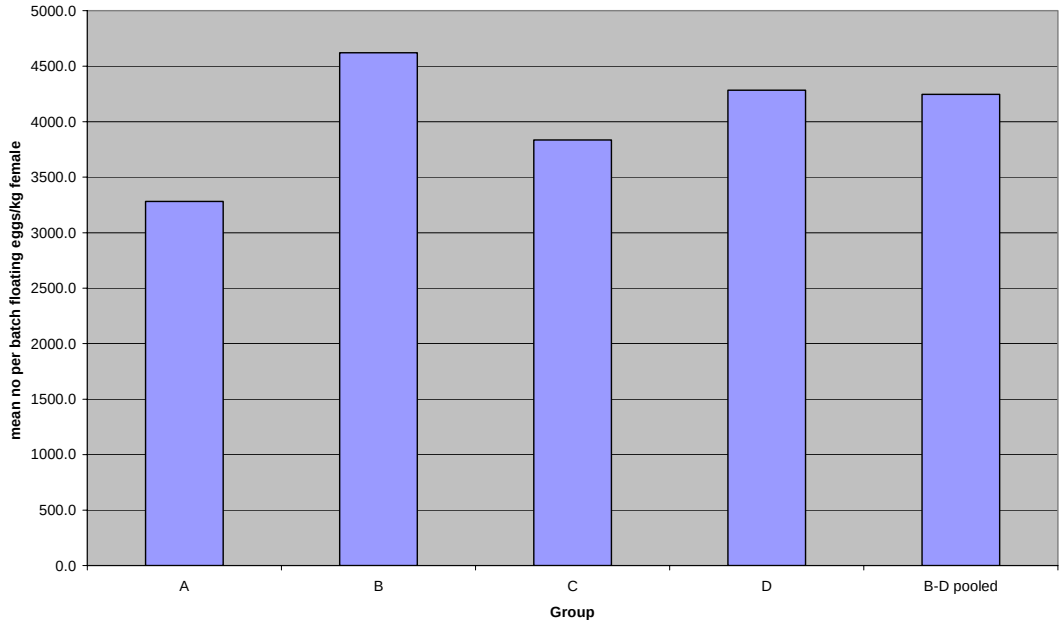


Figure 1. Mean number per batch of floating eggs/kg female in Experiment 1. Groups B-D were fed a diet supplemented with arachidonic acid. Differences between Group A and Groups B-D pooled are statistically significant

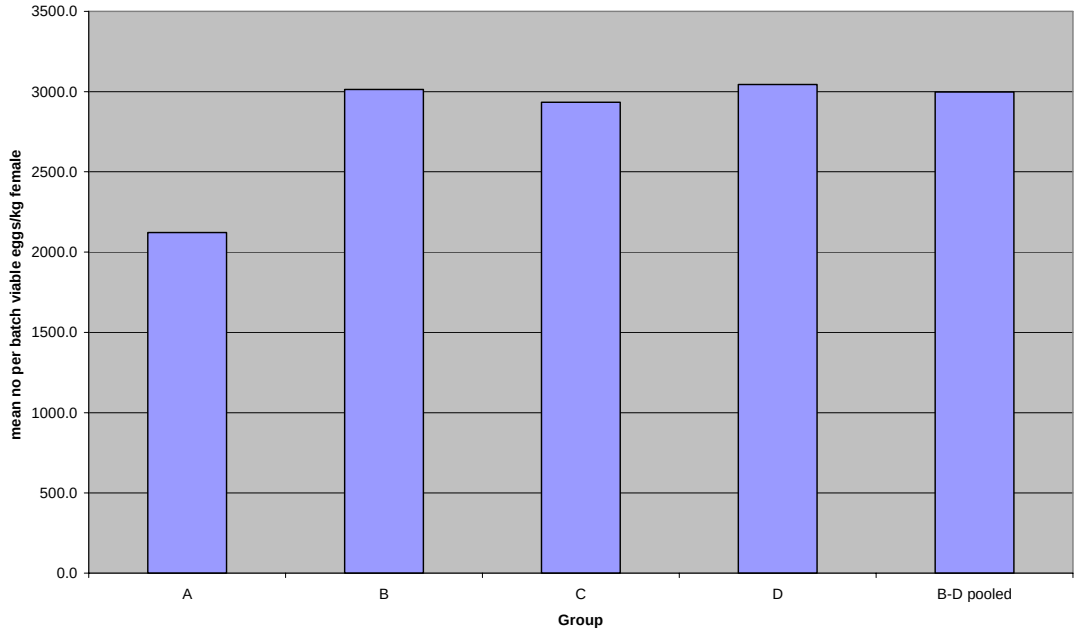


Figure 2. Mean number per batch of fertilised eggs produced per kg female in Experiment 1. Groups B-D were fed a diet supplemented with arachidonic acid. Differences between Group A and Groups B-D pooled are statistically significant.

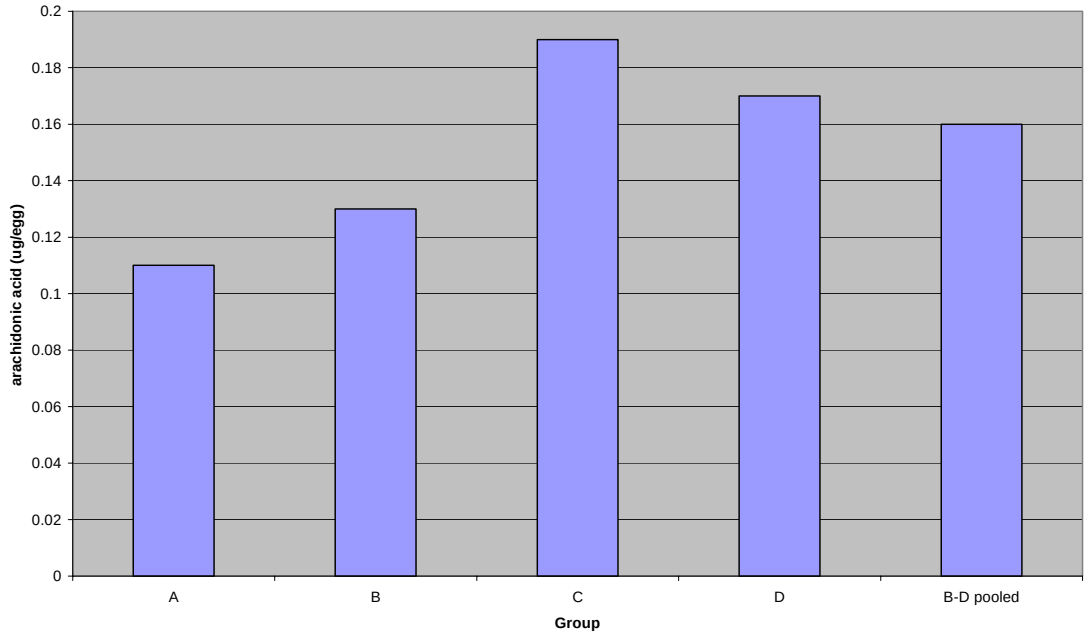


Figure 3. Mean arachidonic acid content in eggs in Experiment 1. Groups B-D were fed a diet supplemented with arachidonic acid for 1, 2 and 3 months respectively.

The results of fatty acid analysis of eggs are also summarised in Table 1. Lowest mean egg ARA concentrations were measured in Group A (control), and highest were measured in Group C fed the ARA supplemented feed for 2 months (Figure 3). Significant differences were detected between Group A and Groups C and D ($F=5.230$; $p<0.01$). The ratio of EPA/ARA in the eggs was highest in eggs from Group A and lowest in eggs from Group C. Significant differences in egg EPA/ARA ratio were detected between Group A and Groups B, C and D, Group B and C and Groups C and D ($F=18.938$; $p<0.001$).

A significant positive correlation was found between egg arachidonic acid content and date of collection in Group B (Spearman's $r=0.687$, $p<0.01$) suggesting that increasing amounts of ARA were retained in eggs in this group as the season progressed. No such correlation was evident in Groups C and D which may indicate that ARA was not selectively retained in the eggs of fish in these groups. Correlation analysis detected no relationships between egg ARA content, or EPA/ARA ratio, and egg quality in individual batches.

3.2 Evaluation of the effect of carotenoid supplementation on egg quality in hatchery reared cod.

Total carotenoid pigment concentrations were elevated from 14.8 mg/kg in the basal diet to 73.7 mg/kg in the astaxanthin-supplemented feed. Table 2, and Figure 4 show data on egg production and egg quality in the two treatment groups.

In the control group, Group A (tanks 3.1 and 3.3), total production was estimated to be 301032 eggs per kg female. Dropout within the tank was

approximately 7% and the number of eggs collected over the season was 280884 eggs per kg female. 123022 eggs per kg female (44 % of those collected) were floating eggs evaluated for incubation. The mean fertilisation rate of the floating eggs was 31% and the total number of viable eggs was 42573 eggs per kg female (15 % of eggs collected). The mean hatch rate was 11 % of floating eggs incubated, and the total number of hatched eggs was 13492 per kg female (5 % of collected eggs).

Group B, which received the astaxanthin supplement, produced fewer batches of eggs, but the average number per batch of eggs spawned per kg female was significantly larger ($F=4.03$; $p<0.05$). Fertilisation rates were similar but the weight per batch of floating eggs ($F=8.30$; $p<0.01$), number per batch of floating eggs per kg female ($F=8.57$; $p<0.01$), and number per batch of fertilised eggs/kg female ($F=7.30$; $p<0.01$) were all higher in the astaxanthin supplemented group than in the control group (Figure 4)

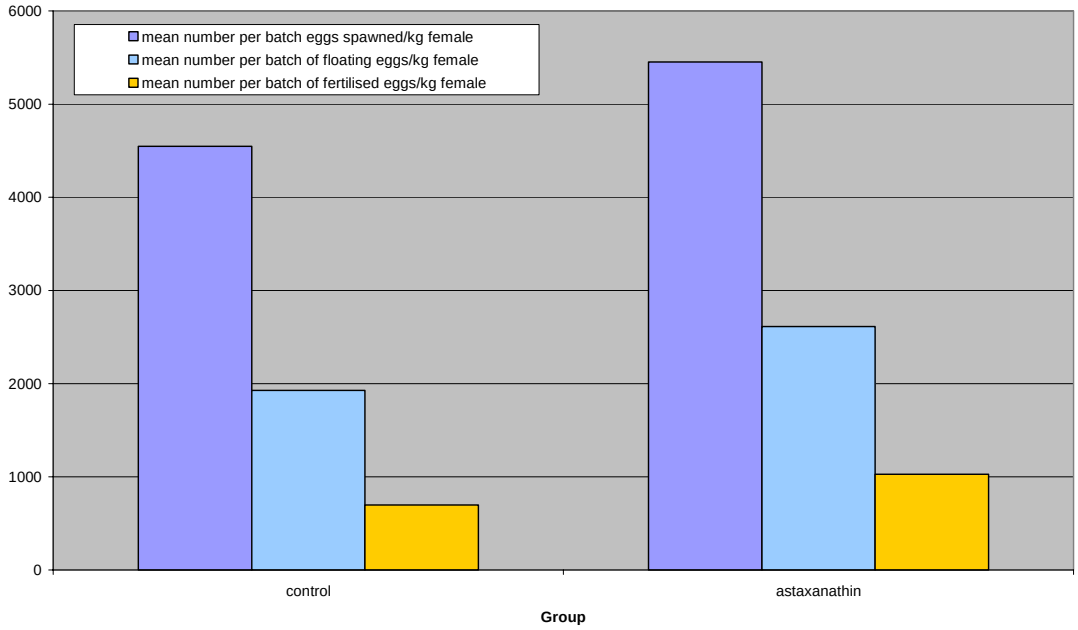


Figure 4. Egg production and egg quality parameters in Experiment 2 in groups fed a diet with and without added astaxanthin. Differences in the mean number of eggs spawned, mean number of floating eggs and mean number of fertilised eggs were statistically significant.

The mean egg astaxanthin content was 0.98 ng/egg in the control group, fed the unsupplemented diet, and 2.79 ng/egg in the group which received the astaxanthin supplemented diet. A significant correlation was detected between egg astaxanthin content and fertilisation rate (Spearman's $r=0.3061$, $p<0.01$) in individual egg batches.

Table 2. Egg production and egg quality indicators in Experiment 2. Egg numbers are expressed as numbers per kg female. Differences in mean weights or numbers per batch between the Control and Astaxanthin supplemented groups are shown as * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

	Control		Astaxanthin		Pooled	
	Tank 1	Tank 2	Tank 3	Tank 4	Control	Astaxanthin
total no of eggs produced/kg female	333725	268339	349900	321691	301032	335795
no of batches produced	69	63	58	66		
mean no per batch of eggs produced/kg female	4837	4259	6033	4874	4548*	5453*
total weight of eggs collected (g)	29487	24621	32338	27792	27054	30065
no of batches collected	69	63	58	66		
mean wt of collected egg batches (g)	427	391	558	421	409	489
total no of collected eggs/kg female	312248	249520	320792	301766	280884	311279
no of batches collected	69	63	58	66		
mean no per batch of eggs collected/kg female	4525	3960	5531	4572	4243	5052
total wt of floating eggs (g)	10677	13169	16421	13107	11923	14764
no of batches with floating eggs	67	60	53	63		
mean wt per batch of floating eggs (g)	159	219	310	208	189**	259**
total no of floating eggs/kg female	112678	133366	163046	142671	123022	152859
no of batches with floating eggs	69	60	55	63		
mean no per batch of floating eggs/kg female	1633	2223	2964	2265	1928**	2615**
mean fertilisation rate (% floating eggs)	35	28	32	34	31	33
total no of fertilised eggs/kg female	40923	44223	60479	54488	42573	57484
no of batches with fertilised eggs	66	57	52	61		
mean no per batch of fertilised eggs/kg female	620	776	1163	893	698**	1028**
mean hatch rate (% floating eggs)	10	12	14	13	11	13
Total no of hatched larvae/kg female	11437	15547	22978	18313	13492	20645
no batches	69	60	55	63	65	59
mean no per batch of hatched larvae/kg female	165.7516	259.1106	417.78	290.681	212	354
Astaxanthin (ug/egg)	1.313	0. 639	2.717	2.863	0. 98	2.79

3.3 Comparison of the relative effect of diets supplemented with ARA and astaxanthin on egg quality wild and farmed cod

Mean arachidonic acid concentrations in the diet used in Experiment 3 were 3.19 % fatty acids (equivalent to 0.41 g/kg finished feed) and astaxanthin concentrations were 80.2 mg/kg finished feed. The EPA concentration in this feed was 6.31% fatty acids (0.82 g/kg), and the EPA/ARA ratio was 2.0.

Table 4, and Figures 5 and 6, show data on egg production and egg viability in the farmed and wild stocks. The duration of the spawning period was 100 days. Farmed fish produced eggs more frequently than wild fish but there was no difference between the two groups in the mean number of eggs produced per batch,

The total number of eggs produced during the spawning season was 505584 eggs per kg female in Group A (farmed) and 584017 eggs per kg female in Group B (wild fish). No significant difference was detected in total egg production between wild and farmed fish. Total numbers collected were 243433 per kg female in Group A and 341114 in Group B. Significant differences were detected in the mean number per batch of eggs collected per kg female ($F=13.51$; $p<0.001$). The total number of floating eggs per kg female was 88678 per kg female in Group A and 131727 per kg female in Group B. Significant differences were detected in the mean weight per batch of floating eggs ($F=8.04$; $p<0.01$) and the mean number per batch of floating eggs per kg female ($F=210.65$; $p<0.001$). The total number per batch of fertilised eggs per kg female was 62991 in Group A and 94205 in Group B. Fertilisation rates were similar, but significant differences were detected in the number per batch of fertilised eggs per kg female ($F=136.55$; $p<0.001$) There was no difference in hatch rates which were again rather low.

Results of fatty acid analysis showed that ARA and DHA concentrations were similar in both groups, but EPA concentrations were higher in eggs from wild fish. As a result the DHA/EPA ratio was lower and the EPA/ARA ratio was higher in eggs from wild fish than in eggs from farmed fish. No difference was detected in astaxanthin concentrations, vitamin E concentrations were slightly higher in eggs from farmed fish, and concentrations of malondialdehyde from lipid oxidation (measured as TBARS) were similar in both groups.

No correlation between ARA content or EPA/ARA ratio and egg quality was detected, but asataxanthin concentrations showed a significant positive correlation with both number per batch of floating eggs/kg female (Spearman's $r=0.2953$, $p<0.05$) and number per batch of fertilised eggs/kg female ($r=0.2983$; $p<0.05$).

Table 3. Egg production and egg quality indicators in Experiment 3. Egg numbers are expressed as numbers per kg female. Differences in mean weights or numbers per batch between farmed and wild stocks are shown as * (p<0.05), ** (p<0.01) or *** (p<0.001).

	Farmed		Wild		Pooled	
	Tank 1	Tank 2	Tank 3	Tank 4	Farmed	Wild
total no of eggs produced/kg female	330447	680721	329592	838441	505584	584017
no of batches produced	77	72	64	74		
mean no per batch of eggs produced/kg female	4291	9454	5150	11330	6873	8240
total weight of eggs collected (g)	32269	20635	24099	46470	26452	35285
no of batches collected	77	72	64	74		
mean wt of collected egg batches (g)	419	287	376	628	353*	502*
total no of collected eggs/kg female	285932	200935	233737	448492	243433	341114
no of batches collected	77	72	64	74		
mean no per batch of eggs collected/kg female	3713	2791	3652	6061	3252***	4856***
total wt of floating eggs (g)	13898	5489	8454	18837	9694	13646
no of batches with floating eggs	73	63	58	69		
mean wt per batch of floating eggs (g)	190	87	146	273	139**	209**
total no of floating eggs/kg female	123539	53816	81462	181991	88678	131727
no of batches with floating eggs	73	63	58	69		
mean no per batch of floating eggs/kg female	1692	854	1404	2638	1273***	2021***
mean fertilisation rate (% floating eggs)	70	56	68	61	63	65
total no of fertilized eggs/kg female	96557	29425	59020	129390	62991	94205
no of batches with fertilized eggs	69	59	55	66		
mean no per batch of fertilized eggs/kg female	1399	499	1073	1960	949***	1517***
mean hatch rate (% floating eggs)	16	14	14	13	15	13
ARA	0.18	0.21	0.19	0.18	0.19	0.19
EPA	0.70	0.80	0.87	0.80	0.75*	0.83*
DHA	1.84	2.18	2.19	1.97	2.01	2.08
DHA/EPA	2.65	2.74	2.51	2.48	2.69***	2.50***
EPA/ARA	4.06	3.90	4.55	4.38	3.98**	4.47**
Astaxanthin (ng/egg)	4.6	2.7	1.6	3.6	3.7	2.6
vitamin E	3.79	3.72	3.19	3.51	3.75*	3.35*
TBARS (umol MDA/egg)	0.20	0.24	0.37	0.28	0.22	0.33

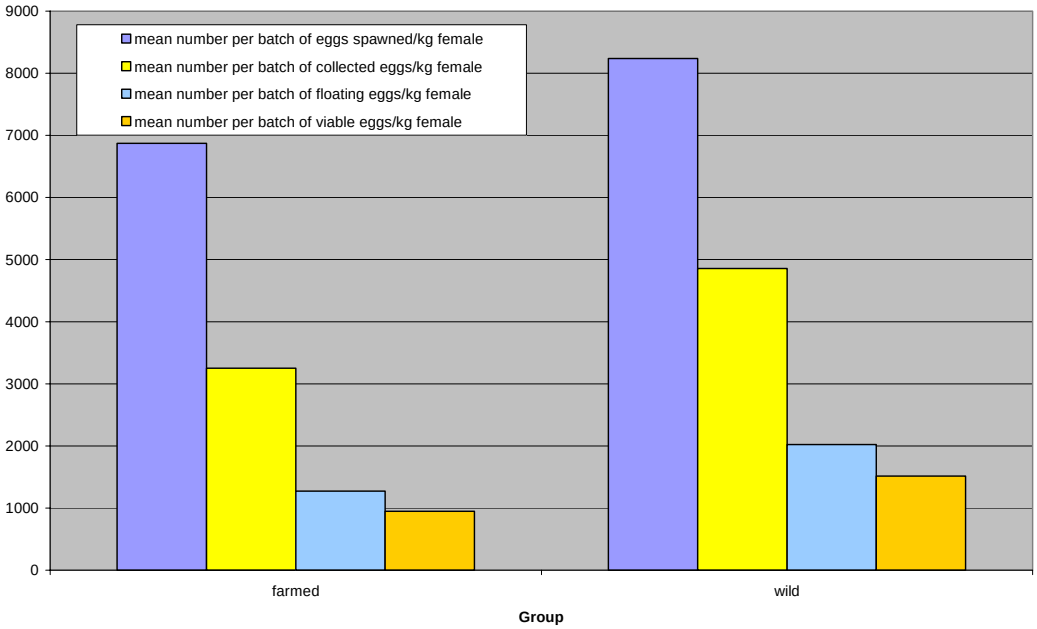


Figure 5. Egg production and egg quality parameters in Experiment 3 in farmed and wild fish fed a diet supplemented with arachidonic acid and astaxanthin. Differences in the mean number of eggs spawned, mean number of eggs collected, mean number of floating eggs and mean number of fertilised eggs were statistically significant.

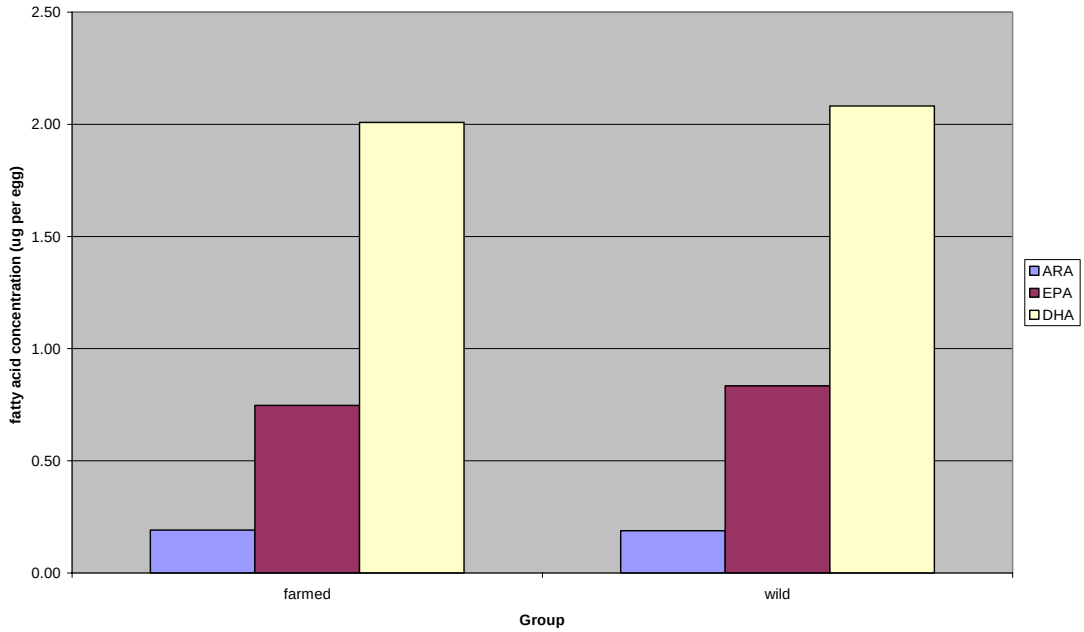


Figure 6. Concentrations of the fatty acids ARA, EPA and DHA in eggs from farmed an wild fish fed a diet supplemented with arachidonic acid and astaxanthin.

4. Discussion

Baseline egg quality data for wild and farmed cod broodstock at the start of the project was provided by data from the control groups in Experiments 1 and 2 (Table 5). In these groups, the total number of eggs spawned per kg female was higher in the wild broodstock than in the farmed broodstock. As may be expected, collection of eggs was less efficient in the $12m^3$ tanks used in Experiment 1 than in the $7m^3$ tanks used for Experiment 2 and this resulted in a relatively high percentage of eggs collected, but correspondingly low fertilisation rate, from the smaller tanks used in Experiment 2. Numbers of viable, fertilised eggs were higher in the wild fish, and hatch rates, whilst lower than expected in both groups, were also higher in the wild fish.

Table 5. Pre-study benchmark. Fate of eggs in wild and farmed broodstock fed unsupplemented feeds. Egg numbers are estimated total numbers per kg female biomass. Figures in parentheses are numbers relative to those spawned.

	Wild	Farmed	Comments
Spawned	590185 (100%)	301032 (100%)	Egg production/kg female 2.0 times higher in wild stock
Sank in tank	- 213846 (36%)	-20148 (7%)	Higher losses of sinking eggs in larger tanks used for wild fish
Collected	376339 (64%)	280884 (93%)	
Sank in collectors	-176144 (30%)	-157862 (52%)	
Floating	200195 (34%)	123022 (41%)	% floating eggs similar for both stocks
Unfertilised	-74973 (13%)	-80449 (27%)	Higher % of unfertilised eggs
Viable for incubation	125222 (21 %)	42573 (14%)	Wild fish produce 2.9 times more viable eggs than farm stock
Failed to hatch	-77175 (13%)	-29810 (10%)	Poor hatch rates, experimental hatching conditions sub-optimal
Survived to hatch	48047 (8%)	13492 (4%)	Wild fish produce 3.6 times more hatched larvae than farmed fish

In Experiment 1, mean arachidonic acid concentrations were lowest in the control group, higher in eggs from Group B fed the supplemented diet for 1 month and highest in eggs from fish in Group C fed the arachidonic acid supplement for 2 months prior to peak spawning. There was no further increase in the group fed the supplemented diet for 3 months. This suggests that two months prior to peak spawning is an adequate feeding period to enable manipulation of arachidonic acid concentrations in eggs just prior to spawning.

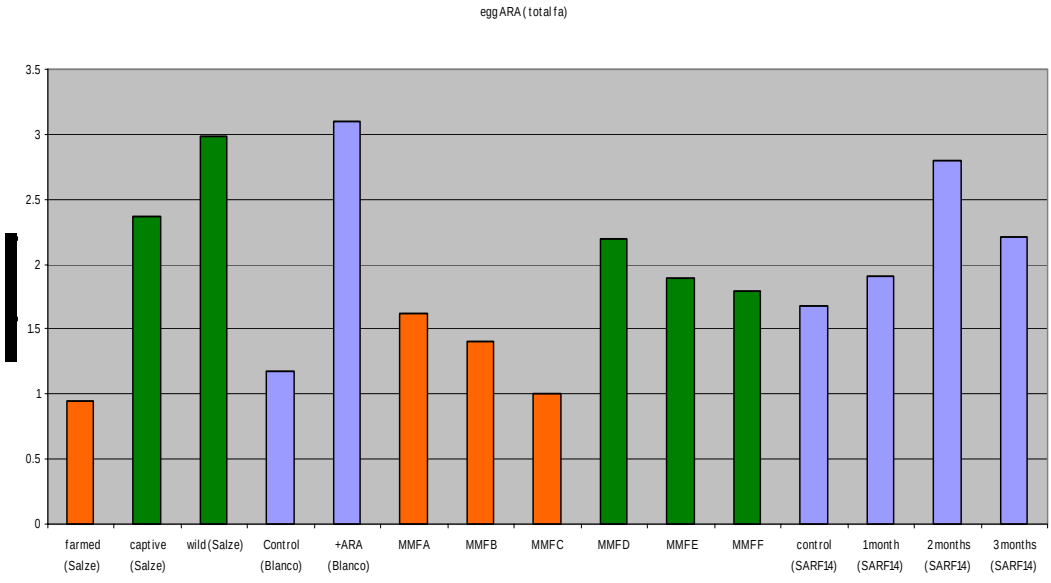
There was no consistent evidence that arachidonic acid supplementation increased the total number of eggs spawned, but larger numbers of eggs were collected in batches produced by fish fed the ARA supplement and these batches contained larger numbers of floating, fertilised eggs. In practical

terms, fewer, larger batches are preferred to more, smaller batches of eggs. Pooled data from Groups B-D indicate that, in fish fed the arachidonic acid supplement, the mean number per batch of floating eggs/kg female was 29% higher, and the mean number per batch of fertilised eggs was 41% higher than in the control group. However, the absence of tank replication in this experiment makes it difficult to distinguish the effects of treatment from the natural variability in broodstock populations in different tanks. Bell *et al* (2005) also reported inconsistent effects of arachidonic acid supplementation on F1 generation farmed cod broodstock.

Rosenlund (2006) recently reported a large scale study in which 3200 F1 generation cod were fed with diets containing 0.5, 1, 2 and 4% arachidonic acid (as % total fatty acids). The dietary arachidonic acid content had no effect on GSI (gonadosomatic index) in male or female fish, fecundity was higher in the group fed 1% arachidonic acid, but there was no difference in egg quality as indicated by the proportion of floating and sinking eggs.

There was no correlation between egg quality and duration of supplementation, and no correlation between mean arachidonic acid concentrations, or EPA/ARA ratios, and egg quality in individual batches. Performance of the control group was not severely compromised by the lack of ARA-supplementation. This indicates that, although some benefit of supplementation was identified in the present study, the arachidonic status of fish in the control group was not a major limiting factor in terms of reproductive performance.

A summary of available commercial and experimental data on arachidonic acid concentrations and egg quality in batches of cod eggs from various sources is shown in Figure 7. Poor quality batches generally show an arachidonic acid content of 1.6 % fatty acids or less, whereas good quality batches contain higher concentrations. Although this correlation may indicate that availability of arachidonic acid is a limiting factor in terms of egg quality, it must also be recognised that poor egg quality and low levels of arachidonic acid may also reflect the fact that some egg batches may have originated



from stock in a generally poor condition.

Figure 7. Arachidonic acid concentrations in cod eggs from a range of sources. Poor quality commercial batches are shown in red, good quality commercial batches are shown in green, and experimental data is shown in blue.

In Experiment 2, farmed fish fed the diet containing the added astaxanthin produced larger batches of eggs which contained more floating, fertilised eggs. In this experiment astaxanthin 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 astaxanthin supplementation of cod broodstock feeds.

The performance of wild and farmed fish in Experiment 3 fed the diet supplemented with arachidonic acid and astaxanthin is summarised in Table 6. Interesting comparisons can be made with the benchmark data, from the previous year, shown in Table 5. Farmed fish spawned 1.68 times more eggs in year 2 than in year 1 but fewer eggs were collected. This was due to the use of larger tanks in year 2 which resulted in a higher dropout of eggs inside the tanks. Fewer floating eggs were collected from farmed fish in year 2 which may indicate that systems for collecting eggs in larger tanks could be improved to reduce losses. However, fertilisation rates were improved markedly and the total number of fertilised eggs per kg female, a key indicator, was increased by a factor of 1.48.

Table 6. Post-study benchmark. Fate of eggs in wild and farmed broodstock fed the diet supplemented with arachidonic acid and astaxanthin. Egg numbers are total numbers per kg female biomass. Figures in parentheses are numbers relative to those spawned.

	Wild	Farmed	Comments
Spawned	584017 (100%)	505584 (100%)	Egg production/kg female 1.2 times higher in wild stock
Sank in tank	- 242903 (42%)	-262151 (52%)	Higher losses of sinking eggs in larger tanks
Collected	341114 (58%)	243433 (48%)	
Sank in collectors	-209387 (36%)	-154755 (31%)	
Floating	131727 (23%)	88678 (18%)	
Unfertilised	-37522 (6%)	-25687 (5%)	Higher % of unfertilised eggs
Viable for incubation	94205 (16 %)	62991 (12%)	Wild fish produce 1.3 times more viable eggs than farm stock
Failed to hatch	-76949 (13%)	-49689 (10%)	Poor hatch rates, experimental hatching conditions sub-optimal
Survived to	17256	13302	Wild fish produce 1.3 times more

hatch	(3%)	(3%)	hatched larvae than farmed fish

In contrast, wild fish performed relatively poorly. Numbers of eggs spawned were similar, but total numbers of eggs collected, numbers of floating eggs and numbers of fertilised eggs were all slightly lower than previously. Differences remained between farmed and wild fish which would be, at least in part, unrelated to nutritional status. Holding conditions, environmental conditions, stocking densities, and husbandry regimes were similar but some factors, such as mean weight, age and number of fish per tank, were unavoidably different.

5. Conclusions

- Baseline data was collected for both wild and farmed fish against which improvements in egg quality could be measured
- Short term supplementation with arachidonic acid and astaxanthin for a period of two months prior to peak spawning increased concentrations of these nutrients in the eggs of cod broodstock.
- Higher numbers per batch of floating eggs per kg female and fertilised eggs per kg female were measured in groups of wild –origin fish fed the arachidonic acid supplement
- No correlation between egg production or egg quality and the duration of ARA supplementation was identified
- 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 astaxanthin supplement.
- Fertilisation rate was correlated with the astaxanthin concentration measured in individual egg batches
- Farmed broodstock, fed a diet supplemented with arachidonic acid and astaxanthin, showed an increase in total egg production per kg female and numbers per batch of fertilised eggs per kg female when compared to baseline data from farmed fish, and data from wild fish fed a similar diet

6. Recommendations

1. The status of cod broodstock with regard to arachidonic acid, EPA/ARA ratio and astaxanthin should be considered and, if necessary, short term supplementation should be used to boost these nutrients prior to spawning. If the status of the broodstock is unknown, the farmer should consider sending samples of eggs for analysis at the start of each spawning period. Predictive tests should be developed to assess the nutritional status of eggs prior to spawning, thus allowing corrective action to be taken before spawning commences
2. More information on the arachidonic acid and astaxanthin status of eggs from commercial broodstock is required, and should be assessed in relation to egg quality. Records of egg quality in standard form (eg no of fertilised eggs

per kg female) are necessary to allow effective comparisons between eggs from different broodstock populations.
3. Future research should aim to establish the requirement of cod broodstock for arachidonic acid, experimentally, by using broodstock with a low arachidonic acid status. Experimental studies should also be conducted on the benefits of treatment with arachidonic acid at earlier stage in gonad maturation
4. Further work is necessary to determine the most efficient form and concentration of astaxanthin and alternatives for use in broodstock feeds
5. More information is required on the role of environmental conditions, husbandry and behavioural interactions in relation to spawning of cod broodstock.

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References

Alorend E.J., 2004. The effect of dietary arachidonic acid concentration on Atlantic halibut (*Hippoglossus hippoglossus*) broodstock performance. Assessment of egg, milt and larval quality. PhD thesis. Institute of Aquaculture, University of Stirling, 260p.

Ashton H.J., Farkvam, D.O. and March, B.E. (1993). Fatty acid composition of lipids in the eggs and alevins from wild and cultured Chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences*, **50**, 648-655.

Bell, G., Blanco, A., Roy, B., Robertson, D., Henderson, J. and Prickett, R (2005). Optimisation of diets for Atlantic cod (*Gadus morhua*) broodstock: effect of arachidonic acid on egg and larval quality. Presentation at Larvi'05, Ghent, Begium, September 2005.

Bruce, M., Oyen, F., Bell, G., Asturiano, J.F., Farndale, B., Carrillo, M., Zanuy, S., Ramos, J. and Bromage, N (1999). Development of broodstock diets for the European sea bass (*Dicentrarchus labrax*) with special emphasis on the importance of n-3 and n-6 highly unsaturated fatty acid to reproductive performance. *Aquaculture*, **177**, 85-97.

Bromage, N. R. (1995) Broodstock management and seed quality – general considerations. In Broodstock management and egg and larval quality (ed. By N.R. Bromage and R.J. Roberts) Blackwell, London pp 1-24.

Craik, J.C.A. (1985). Egg quality and egg pigment content in salmonids fishes. Aquaculture **47**, 61-88.

Czesny, S. and Dabrowski, K. (1998). The effect of fatty acid concentration on embryo viability in wild and domesticated walleye (*Stizostedion vitreum*). *Aquatic Living Resources* **11**, 371-378.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N. and Takeuchi, T. (2003). Effects of arachidonic acid levels in broodstock diet on egg and larval quality of Japanese flounder, *Paralichthys olivaceus. Aquaculture* **220**, 725-735.

Gallagher M.L., Paramore, L., Alvest, D., Rulifson, R.A. (1998). Comparison of phospholipids and fatty acid composition of wild and cultured striped bass eggs. *J. Fish biol*. **52**, 1218-1228.

Grung, M., Svendsen, Y.S. and Liaanen-Jensen, S. (1993). The carotenoids of eggs of wild and farmed cod (*Gadus morhua*). *Comp. Biochem. Physiol.* **106B**, 237-42.

Hardy, R (1985) Salmonid broodstock nutrition. In Salmonid reproduction (ed by R. Iwamoto and S. Sower). Washington Sea Grant Programme, University of Washington, Seattle, pp 98-108.

Mazorra, C., Bruce, M., Bell, J.G., Davie, A., Alorend, E., Jordan, N., Rees, J., Papanikos, N., Porter, M. and Bromage, N. (2003). Dietary lipid enhancement of broodstock reproductive performance and egg and larval quality in Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture* **227**, 21-33

Mikulin, A.E. (2000). Functional role of pigments and pigmentation in fish ontogeny p. 432. VNIRO, Moscow (In Russian).

Pavlov, D., Kjorsvik, E, Refsti T and Anderson O (2004) Brood stock and egg production. In Culture of Cold-Water marine Fish (ed by E. Moksness, E. Kjorsvik and Y. Olsen) Blackwell, Oxford pp 129-203.

Pickova, J., Dutta, P.C., Larson, P.O. and Kiessling, A. (1997). Early embryonic cleavage pattern hatching success and egg-lipid fatty acids composition: comparison between two cod stocks. *Can. J. Fish. Aquat. Sci*. **54**, 2410-2416.

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

Rosenlund, G. (2005). Broodstock nutrition – a key success factor in fish farming. Presentation at AQUA 2006, World Aquaculture Society, Florence, Italy, 11 May 2006.

Salze, G., Tocher, D.R., Roy, W. and Robertson, D.A. (2005) Egg quality determinants in cod (*Gadus morhua* L.): Egg performance and lipids in eggs from farmed and wild broodstock. Aquaculture Research, *36*: 1488-1499.

Sargent, J. (1995). Origins and functions of egg lipids: nutritional implication. In: *Broodstock Management and Egg and Larval Quality* (Bromage, N.R & Roberts, R.J eds). Blackwell Sciences Ltd., Oxford, pp. 353-372.

Sargent, J., Tocher, D.R. and Bell, J.G. (2002). The lipids. In: *Fish Nutrition* (third edition) (Halver J.E. and Hardy R.W. eds). Academic Press, San Diego, pp. 182-308.

Verakunpiriya, V., Mushiake, K., Hawano, K. and Watanabe, T. (1997). Supplemental effect of astaxanthin in broodstock diets on the quality of yellowtail eggs. *Fish. Sci*. **63**, 816-23.

Watanabe, T and Miki, W. (1993). Astaxanthin: an effective dietary component for red sea bream broodstock. In: *Fish Nutrition in Practice* (S.J.Kaushik and P. Luquet eds.) pp. 27-36. INRA, Paris.

Watanabe, T. (1985). Importance of the study of broodstock nutrition for further development in aquaculture. In: *Nutrition and Feeding in Fish* (Cowey C.B., Mackie A.M. & Bell J.G eds). Academic Press, London, pp. 281–232.