Final Report to NRAC

Grant # 2001-38500-10691 & # 2002-38500-12056

Development of broodstock diets for the Atlantic halibut *Hippoglossus hippoglossus*

Nick Brown, PhD

Director,

University of Maine

Center for Cooperative Aquaculture Research,

Franklin, Maine

February 2009

EXECUTIVE SUMMARY

A four year study was conducted at the Center for Cooperative Aquaculture Research (CCAR) to investigate the effect of diet on spawning performance and egg quality in the Atlantic halibut, *Hippoglossus hippoglossus*. The Northeastern Regional Aquaculture Center (NRAC) grant covered some of the costs of the study in 2004, 05 and 07.

A group of 38 wild caught mature fish, which had been introduced to captivity in 2001 were used for this study which began in 2004. Three diet types were tested, the "Control" diet which consisted of raw or frozen herring and frozen squid with added vitamins and two formulated feeds which were mixed into a paste and stuffed into collagen skins and fed as sausages. One of the feeds, the "Commercial" diet, was Breed M™, purchased from Inve Aquaculture and the second diet was formulated and manufactured in house and was fed to the fish in the "CCAR" treatment group. Initially the fish were distributed between nine 3.65 m diameter tanks, three replicate tanks per diet treatment, in a seawater recirculation system and then in 2006, more broodstock fish were added to the trial and then in early 2007, the fish were moved to three 6.4 m diameter tanks in a purpose built facility.

The fish were ultrasound scanned at the start of the study to identify sex since many of the fish had not yet spawned in captivity. The fish were also weighed and then distributed between the treatment groups into the replicate tanks. Throughout the study between 2004 and 2007, the feed intake and growth of the fish was monitored. During each spawning season which occurred between January and May, the spawning rhythms of the fish were followed as closely as possible. Egg batches were stripped manually from ovulating females and each egg batch was fertilized with milt from at least two males from the same treatment group. Fertilization rate for each batch was assessed at the 8 cell stage and hatching trials using microtitre well plates were attempted to assess hatch rate. Samples of eggs from each batch were analyzed for fatty acid composition. Samples of each diet were analyzed for proximate composition, fatty acid composition and amino acid composition.

The diets were all well accepted by the fish and feed rates up to 8% per month were achieved for fish which started at an average weight of just under 23kg and finished at

final average weights of between 25.8 kg (Control) and close to 30kg (Commercial and CCAR). In the first year only 7 fish spawned out of 23 females. By 2006, this number had increased to 15 fish. Total egg production increased between 2004 and 2006 from just fewer than 4 million eggs to nearly 8 million. In 2007, the fish were moved to a new facility and egg production that year fell back to just under 5 million eggs. There were no significant differences in terms of specific fecundity (egg/kg) between treatments.

Egg quality in terms of fertilization rates was highly variable and generally quite low on average. In 2005 no viable eggs were collected from the Commercial group whilst the fertilization rate in the Control and CCAR groups that year were 75.4% and 73.7% respectively. In 2006 significant differences in terms of egg quality were detected when the Commercial group had a lower average fertilization rate (59.3% vs 94.7% (Control) and 72.1% (CCAR)). This difference was not carried through 2007 however and high quality egg batches were collected and reared through to juvenile from all treatment during the study.

Differences in certain fatty acid composition were detected but levels of key essential fatty acids (EFAs): arachidonic acid (ARA), ecosapentaenoic acid (EPA)and docosahexaenoic acid were not different across treatment groups suggesting that Atlantic halibut broodstock conserve these EFA levels in the eggs.

The implications of the results are discussed in terms of diet effects, diet costs and in terms of Atlantic halibut broodstock management.

INTRODUCTION

Life History of the Atlantic Halibut

The Atlantic halibut is a large pleuronectid flatfish distinguishable from other right eyed flatfishes by its large mouth which opens as far back as the anterior half of its lower eye, its concave caudal fin and the distinctive arched lateral line. Dorsally, the adult fish is more or less uniformly chocolate brown or olive and the blind side is usually white, though in some cases may be partially brown (Collette and Klein-Macphee 2002). This species is among the commercially important groundfish of the Gulf of Maine where it has been harvested since the early part of the nineteenth century. The fishery was quickly depleted and has not been of economic importance since the 1940's. Annual catches after 1953 have been less than 100 metric tons on average. The Atlantic halibut is one of the largest fish in the region. The largest individual caught on record was 280 kg (head on gutted) and was estimated to weigh 318 kg (live weight).

The Atlantic halibut has a number of attributes that make it an excellent candidate for aquaculture and global production of this species continues to grow steadily (Brown, 2002). These characteristics include firm, white, mild tasting flesh with a good shelf life, a high fillet yield, efficient feed conversion rates and resistance to many common marine diseases.

Apart from the earliest trials (e.g. Rollefsen 1934), research into the techniques for the culture of halibut began in the 1980's and a few juveniles were reared past metamorphosis in the first attempts (Blaxter *et al*. 1983). Capture of early life stages in the wild is very rare, little is known about their distribution and for researchers attempting to close the life cycle in the hatchery, there has been a lot of trial and error. Atlantic halibut are known to spawn at great depths where temperatures are generally stable and between 5 to 7°C (Haug 1990; Neilson *et al*., 1993). The clear eggs are quite large for a marine fish (3 mm diameter) and they are bathypelagic during development floating close to the ocean floor and are neutrally buoyant at relatively high salinity of around 36 ppt. The spawning season occurs between November and April under natural photoperiod (Kjorsvik *et al*. 1987; Haug 1990; Neilson *et al*. 1993). Adult female halibut have large gonads and are highly fecund. Adult females of between 20 and 60kg fish are

capable of producing between 6 and 16 batches, each of 10 to 200 x 10^3 eggs in a spawning season (Haug and Gulliksen 1988; Brown *et al*. 2006). Following fertilization the embryo undergoes the typical developmental changes seen in most teleost eggs. The early blastomeres are easily seen in the translucent egg with the naked eye owing to the large size and peripheral displacement. After about two weeks at 6°C the egg hatches after enzymes released around the "hatching ring" create an opening for the larva to emerge through.

After hatching the larva hangs in a head down position exhibiting very little swimming activity (Pittman *et al*. 1990a). Halibut larvae hatch in a very primitive developmental state and organogenesis proceeds at a slow pace (Lonning *et al*. 1982; Blaxter *et al*. 1983; Pittman *et al*. 1990a). At around 150 °C.days, the eyes, mouth and intestine become functional and the eye takes on pigmentation (Blaxter *et al*. 1983; Pittman *et al*. 1990b; Kvenseth *et al*. 1996).

Exogenous feeding can begin from around 240 °C.days and metamorphosis occurs around 80 days post hatch. At this point, the stomach is formed, the left eye migrates to the right side of the head, and the fish becomes fully pigmented. For aquaculture purposes, this represents the end of the hatchery phase and coincides with the establishment onto formulated feeds which will continue until harvest.

Broodstock Management

Captive broodstock populations were first set up in Scotland and Norway in the early 1980s (Blaxter *et al*. 1983; Rabben *et al*. 1986; Smith 1987). Mature wild fish are caught using longlines or "tub trawls". A size 14/0 or larger circle hook is recommended to reduce injuries to the fish (Kanwit 2007). Fish for the University of Maine program, based at the Center for Cooperative Aquaculture Research (CCAR), were caught between 2000 and 2002. Around 60 fish were brought into the fishing ports of Jonesport, Stonington and Steuben by fishermen participating in an experimental tagging program run by the Maine Department of Marine Resources (DMR) (Kanwit 2007). The fish were transferred from holding tanks on the boats to live transport tanks supplied with oxygen and driven by truck overland to the facility. Additional fish from hatcheries in Canada were recruited to this founding population to result in a total population of 120 surviving mature fish. An additional 150 fish reared at the CCAR hatchery were selected from the

2006 production run for broodstock. Additional wild fish from a DMR tagging study were also added in 2007. All mature hatchery reared (F1) fish have been genotyped using microsatellite markers developed in Canada (Jackson *et al*. 2003) to establish pedigree for future breeding programs.

Halibut may take up to 3 years to acclimate sufficiently to spawn in captivity following capture. Weaning onto a non-living food item can be improved by using live fish such as mackerel as an intermediate step in the tanks. The use of large tanks, low light levels, good water quality and temperature regimes that follow the natural environment of the halibut all help to ensure successful acclimation.

Captive halibut are generally stripped by hand although natural spawning can occur (Holmefjord and Lein 1990). Year-round egg production is possible using altered photoperiod (Smith *et al*. 1991; Holmefjord *et al*. 1993; Naess *et al*. 1996). Manipulation of photoperiod is routinely used to influence natural spawning cycles enabling the production of out-of-season eggs and, when multiple broodstocks are used, all year round production (Smith *et al*. 1991; Holmefjord *et al*. 1993; Naess *et al*. 1996). Delays of up to 6 months can be achieved in a single year. Advancing spawning time is more difficult and more than 3 months per year is not recommended since the fish need to build up reserves over the summer months for the subsequent spawning season. Halibut are sensitive to changes in light levels and good light proofing around holding tanks is necessary to ensure clear photoperiod signals. With photoperiod shifted stocks attention must be paid to water temperature in out of season spawning groups to ensure good egg quality (Brown *et al*. 2006).

Broodstock Atlantic halibut are generally large fish that need to be housed in large tanks between 5 and 15m in diameter. The recommended stocking density for halibut is around 15 kg/m^2. Tanks bottoms should be textured to prevent the formation of papillomas which are common in halibut kept in smooth-bottomed tanks at low densities (Ottesen and Strand 1996; Ottesen *et al*. 2007). An essential piece of equipment for the halibut broodstock facility is a table on which fish can be handled for manual stripping. All facilities have this and there are as many designs as there are broodstock managers. Some tables are power assisted (hydraulic or pulley block) to help lift what can be very large fish out of the water. Most are covered with some sort of soft pad such as neoprene

rubber to help prevent injury to the valuable fish. The eyes of broodstock halibut are vulnerable and cataracts, gas bubbles or other types of eye traumas are seen in some facilities. The cause of these problems is not clear and may be related to handling, in tank injury, gas super-saturation or nutritional deficiencies.

Egg collectors installed on each tank to intercept egg releases are checked regularly during the spawning season, often many times per day. Fish are usually allowed to spawn in the tank for the first two ovulations to give an indication of spawning interval. A marked reduction in viability can occur if fertilization is delayed longer than 4-6 hours after ovulation (Bromage *et al*. 1994). It has been shown that close observation of individual female ovulatory cycles can help to pinpoint the timing of stripping and improve viability and fertilization rates for halibut (Norberg *et al*. 1991; Holmefjord 1991) though this can be very time consuming and potentially stressful for the fish. Egg quality can be highly variable in halibut and predicting the correct timing for manual stripping is one of the most difficult challenges remaining for halibut culture.

Ultrasound can be used to sex the fish and estimate the stage of development of the gonad (Shields *et al*. 1993; Martin Robichaud and Rommens 2001). Individual fish are marked by PIT tags, FLOY tags and/or sheep tags. The latter are easiest to use and are rarely lost.

To ensure good egg production it is necessary to feed a diet that provides the nutrients and energy for growth and health of the fish as well as for the developing oocytes. The natural diet of Atlantic halibut varies according to what is available in the region (McIntyre, 1953; Bowman, 2000). In the Gulf of Maine, the contents of 36 Atlantic halibut stomachs contained silver hake (24%), squid (24%), rock crab (17%) sand lance (9%), ocean pout (7 %), and alewife (3%) (Bowman, 2000). On the Georges Bank, 19 stomach samples contained longhorn sculpin (61%), squid (15.4%), ocean pout (12%), silver hake (7%), and Atlantic cod (3%) (Bowman, 2000). On the Scotian Shelf, 64 stomach samples contained pandalid shrimp (32.5%), American plaice (4%), yellowtail flounder (2%) unidentified fish (19%), octopus (4%), and unidentified Cephalopoda (19%) (Bowman, 2000).

Despite our knowledge of the diet in the wild, there is very little information about the nutritional requirements of broodstock halibut. The current lack of knowledge means that the practice of feeding raw fish and shellfish is still quite common. This carries serious

health risks for the broodstock and resulting eggs, larvae and juveniles. Diseases found in the wild components can be transmitted to the captive broodstocks. The feeding of raw fish has been implicated in the transmission of such viral diseases as nodavirus (VNN) and viral hemorrhagic septicemia (VHS) (Dannevig *et al*. 2000).

Studies on the nutritional requirements of broodstock halibut are rarely conducted on a meaningful scale because they are long term, expensive and complex. For these reasons, it is extremely difficult for commercial companies to commit sufficient resources for long periods to rigorously conduct this type of study. Further, owing to the expense of capturing, conditioning and caring for mature Atlantic halibut, there are few captive broodstock groups worldwide. The collection at the CCAR is one of the largest in the world and the only one in the USA.

This report describes a four year study to assess the effect of diet on spawning performance and egg quality in this population of broodstock halibut at the CCAR.

MATERIALS AND METHODS

Experimental set up

Thirty-eight wild caught Atlantic halibut (*Hippoglossus hippoglossus*) were caught during months of April and May of 2001 and 2002 and transported to the Center of Cooperative Aquaculture Research (CCAR) in Franklin, Maine. The fish were held in land based recirculating seawater systems for 2.5 to 3.5 years before the experiment began. Seawater was pumped continually from Taunton Bay, filtered by sand, and then sterilized with ultraviolet radiation before being introduced to the recirculating system with a rate of 10-20% per day turnover. The temperature and oxygen levels were measured daily. Water was tested weekly for ammonia, nitrite, nitrate, alkalinity, and pH using a Hach Odyssey DR/2500™ spectrophotometer. Dissolved CO_2 was measured using an Oxyguard CO_2 analyzer. The salinity was measured weekly in February of 2005 to September 2005 using a refractometer.

Nine tanks, 3.65 m in diameter and 1m deep were used from the start of this study until mid January 2007 (see Figure 1.). The tanks were located in a greenhouse structure; shade cloth was used during summer months to reduce light levels and solar heat input to the system. Chilling and heating systems maintained the water temperature at 6°C during the winter months through the spawning season and at 10 to 12° C during the summer months. Temperature changes among seasons were made gradually to prevent temperature shock to the fish and to promote healthy gamete growth during vitellogenesis

Figure 1. Facility used for the first three years of the study. The nine tanks on the right housed the halibut broodstock fish.

(Brown *et al*., 2006).

In January 2007, the fish were moved to a newly completed broodstock facility in the new 24,000 SF marine hatchery building. This designated facility which comprises two recirculation systems, each with three 6.5 m diameter tanks, 1.5 m deep (see Figure 2). The recirculation system includes a moving bed biofilter, a U.V. sterilizer, a submersible circulating pump and a drum filter (90 µm screen). The two systems are temperature controlled via titanium heat exchangers connected to oil fired heating and electrical chillers. The room temperature and humidity are controlled via a dedicated HVAC unit. Water exchange is relatively slow at around 0.5 exchanges per hour. To enable monitoring of egg releases during the spawning season, egg collectors are installed in the side box outlet where side and bottom drains meet before running to the treatment system. The light to each tank is controlled via PLC and can simulate dawn/dusk via programmable dimming. The light source is from a dimmable compact fluorescent lamp suspended above the water in the center of the tank.

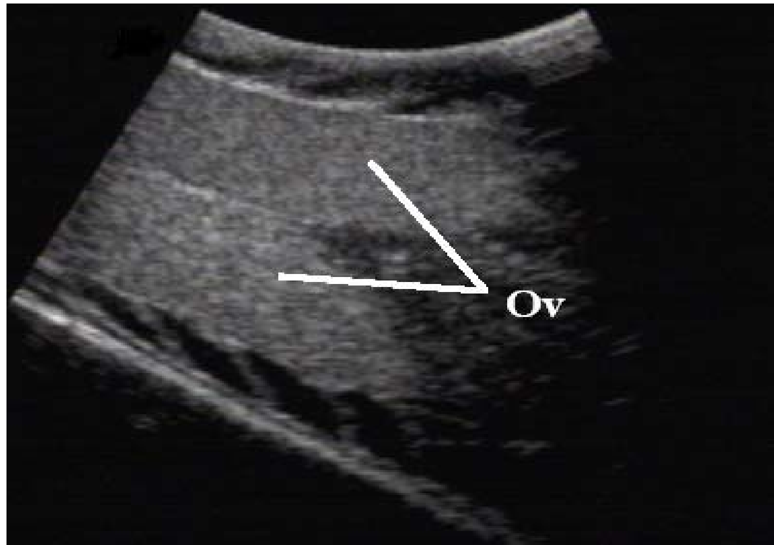
Figure 2. One of the six 6.5 m diameter halibut broodstock tanks at the CCAR (left) and hand feeding with sausage diet (right)

In September of 2003 the fish were sexed using ultrasound using a Sonosite 180 with a C60/S – 2 MHz transducer (Shields *et al*., 1993). Fish were removed from the water and a cross section of the gonads was recorded. Large gonads indicated female (Figure 3a), while smaller dense gonadal tissue indicated males (Figure 3b). At this time, the length

and weight for each fish was recorded. Passive Integrated Transponder tags (PIT) and Floy™ tags were inserted into each fish for identification throughout the experiment. The fish were distributed randomly among the nine fish tanks as shown in Table 1. Fish were weighed annually following their distribution into the tanks.

In May 2006, after the completion of the first phase of the project (NRAC funding initially covered 2004 and 2005), additional fish were recruited to the diet trial. The number and average weight of all fish in the trial in November 2006 is shown in Table 2. These fish had come from a commercial hatchery in New Brunswick, Canada and had previously spawned in captivity. Throughout the course of the trial, a total of 9 fish died. It was assumed that all fish had the same chance of spawning, since none had spawned in captivity up to the point of distribution. Three diets were then randomly assigned so that three tanks of fish were fed each diet treatment. Diets were fed two to three times per week depending on fish appetite and water quality. The only treatment that these fish received were formalin baths (250 ppm for 40 minutes) to remove *Entobdella hippoglossi*, an external parasite common to halibut.

(a)



(b)

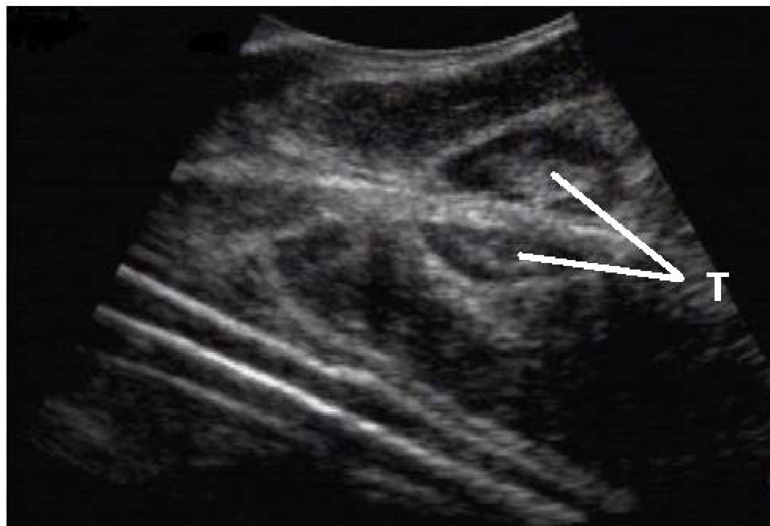


Figure 3. Ultrasound pictures of broodstock gametes for sex identification (a) females ovaries, OV, and (b) show male testis, T.

Table 1. Initial distribution of sex and size of broodstock halibut among tanks and treatments.

Control treatment			Commercial Treatment			CCAR treatment		
Tank	**Wt (Kg)**	**Sex**	**Tank**	**Wt (Kg)**	**Sex**	**Tank**	**Wt (Kg)**	**Sex**
1	21.5	F	3	21.5	F*	2	31.5	F
	34.5	F		15.0	F		15.5	F
	14.5	M		24.0	F		12.5	M*
	19.5	M		26.5	F		24.7	M
4	30.0	F*	5	29.2	F*	6	28.5	F
	12.5	F		30.0	F		18.5	F
	11.5	M*		21.5	F		14.0	M
	25.0	M		15.5	F		20.0	M
8	26.5	F		21.0	M	7	29.5	F*
	24.5	F	9	44.7	F		22.5	F*
	19.5	M		27.5	F		32.5	M
	23.5	M		13.5	F		18.5	M
				12.5	M		22.5	M

* - sex was not confirmed during trial period.

Table 2. Distribution of fish following introduction of new recruits to trial populations in May 2006. Weights were taken in November 2006.

Group	Females		Males	
	Number	Average weight (kg)	Number	Average Weight (kg)
CCAR	14	22.2	9	19.8
Commercial	16	26.5	5	14.9
Control	10	23.1	11	16.5

Dietary treatments and feeding

Three diets were used in this experiment; frozen herring and squid with vitamin supplements (Control), and two formulated diets. Fresh herring was obtained from Stinson/Bumble Bee cannery in Prospect Harbor, Maine. The head and gut was removed and the body was frozen before being fed to the broodstock. Frozen squid was purchased from Maine Shellfish Company, Incorporated, in Ellsworth, Maine. Three to four, approximately one gram, vitamin pellets (Table 3) were placed into herring and fed to each fish once per week.

A commercially available marine fish broodstock diet, Breed-M™, was obtained from INVE Aquaculture NV, Dendermonde, Belgium. This diet, used for the "Commercial" treatment group, was purchased as a dry powder. The powder was hydrated at a ratio of 1:1 (water: Breed-M™), pressed into a 1 1/8" collagen casing and sectioned to provide 6 inch long moist-sausage-like links. The links were frozen and stored up to a month prior to being fed. Although the ingredients of this diet were proprietary, it is known that it was formulated

Figure 4. Preparing the sausage diets

Table 3. Profile of vitamin supplement for control diet. Vitamins were added to herring and fed once per week at a rate of 0.9 to 1.2 g per fish.

Vitamin	mg/g
Vitamin D_3 (400000 IU/g)	0.75
Vitamin A (500000 IU/g)	1.33
Vitamin E (500 IU/g)	33.33
Ascorbic Acid (Stay C)	66.67
Folic acid	2.25
Vitamin K (menadione Na bisulphate)	3.00
Riboflavin	4.00
Vitamin B6, (pyridoxine HCl)	4.00
Vitamin B1 (thiamine HCl)	7.50
Vitamin B12 (0.1%)	6.67
Biotin (1%)	8.0
d-calcium panthothenate	12.00
Niacin	13.33
myo-Inositol	13.33
Ethoxyquin 75%	8.89
Carophyll Pink (8% Astaxanthin)	37.5
Wheat Shorts	777.45

with a high inclusion of squid meal and krill meal. Free amino acids, such as lysine, methionine, betaine, and taurine, were added to increase palatability of the diet. It was also formulated with specific fatty acid levels, added natural pigments, vitamins such as B1, B2, E, and C, and minerals. Increased levels of phospholipids (50% marine) were also added to the diet.

The second formulated feed (CCAR) was manufactured at the Center for Cooperative Aquaculture Research (Table 4). Marine meals such as herring meal, squid meal, and crab meal were added to the diet. In addition, fish hydrolysate (Special G, Zeigler Brothers Incorporated, Gardners, Pennsylvania) was added to improve palatability and amino acid availability. Sand worms were added as a bio-secure fresh/frozen component (Seabait LLC., Franklin, ME). Menhaden oil was the primary source of fat in this diet. The oil was supplemented with two oils extracted from single cell heterotroph organisms, DHAsco®, and ARAsco®, to supply DHA and ArA, respectively (Martek Biosciences Corporation, Columbia, Maryland). The oils were mixed to produce the ratio of essential fatty acids, DHA:EPA:ArA to equal 8:4:1, as suggested by Mazorra *et al*. (2003). A base level of 3 mg/g of ArA was used in the diet dry weight content (Alorend, 2004) so that the target levels of DHA, EPA, and ArA in the diet were 24, 12, and 3 mg / g respectively. Vitamins C, E, and choline were added in addition to a vitamin premix. Phospholipids were added to the diet as Enhanced '97™ (The Solae Company, Saint Louis, Missouri). Minerals were added to the diet as mineral premix. Ethoxyquin was added as a preservative. This diet was also pressed into 1 1/8" collagen casings and sectioned into 6" moist sausage-like links. The links were frozen and held for up to one month.

Table 4. Formulation of CCAR diet.

Ingredient	%, Dry Matter	%, As Fed
Herring Meal	35.67	21.96
Squid Meal	25.95	16.51
Fish Hydrolysate	12.44	7.27
Crab Meal	5.23	3.44
Sand worms	4.00	13.57
Menhaden Oil	2.30	1.29
ArAsco®	0.28	0.16
DHAsco®	2.47	1.38
Wheat Gluten	8.00	4.88
Sodium Phosphate	1.11	0.62
Vitamin E	0.11	0.12
Vitamin C	0.22	0.06
Vitamin Premix[1]	0.28	0.16
Mineral Premix[2]	0.14	0.08
Choline Chloride, 100%	0.11	0.06
Soy Lecithin	1.67	0.93
Ethoxyquin, 66%	0.015	0.009
Water	-	27.5

1 - vitamin premix supplied to the diet the following

	per Kg of dry diet	As fed/Kg
Vitamin A (IU)	7,268	4522
Vitamin D (IU)	485	301
Vitamin E (IU)	388	241
Vitamin B12 (mg)	0.024	0.015
Riboflavin B2 (mg)	58.1	36.2
Niacin (mg)	242.3	150.7
d- Pantothenic (mg)	116.3	72.4
Menadione (mg)	12.1	7.5
Folic Acid (mg)	9.7	6.0
Thiamine (mg)	38.8	24.1
Pyridoxine (mg)	33.9	21.1
d Biotin (mg)	0.39	0.24

2 - Mineral Premix supplied to the diet the following

	per Kg of dry diet	As fed/Kg
Mn (mg)	22.6	14.0
Zn (mg)	8.4	5.2
Cu (mg)	1.7	1.0
I (mg)	11.3	7.0

Prior to the start of the experiment, all fish were fed a diet of frozen herring and squid with weekly vitamin supplements as described for the Control diet. All fish being fed moist links were weaned onto the commercial diet during December 2003 and onto the CCAR diet during February of 2004. Freshly thawed herring and squid were fed separately by hand, allowing the fish to select their diet. Links were either offered by hand or they were dangled from a line before the fish. Feed was weighed prior to being fed and all uneaten or regurgitated food was removed from the tanks 1 hour after the feeding and weighed.

Feed consumption was calculated by dividing the feed consumed per tank by the average weight of the fish in the tank and expressed in terms of % body weight per month.

Egg collection and egg quality evaluation

Ovulation cycles were determined by allowing the female to naturally release two to three complete batches of eggs. Unfertilized eggs were collected from the tank effluent using an 800 µm filter bag. The date, time, volume, and number of eggs per 2 g sample were recorded for each egg batch. Eggs were stripped from the female during the estimated optimal time for fertilization based on her ovulation cycle.

For gamete collection, halibut were hoisted from the water on a stretcher and eggs or milt was collected into dry vessels by gently pressing the fish's abdomen (see Figure 5). This procedure did not require the fish to be anesthetized and the fish were handled with care. Ripe egg batches were determined by observing for optimum egg size and consistency of ovarian fluid. If an egg batch appeared to be under ripe the female was placed back in

Figure 5. Stripping a female Atlantic halibut

the tank and checked again at a later time. Milt from males within the same tank and/or treatment were used.

Prior to fertilization, motility of milt was checked to avoid using inactive milt. Seawater was adjusted to 32 ppt, filtered through 1 µm filter, and then sterilized with ultraviolet light. The prepared seawater at 6°C was combined with milt from two males at a ratio of 1 ml of milt per male per liter of water. The milt and eggs were immediately mixed together at a ratio of 2 parts milt water to 1 part eggs to fertilize the eggs. The eggs were allowed to sit for 20 minutes before being rinsed with fresh seawater to remove extra milt and ovarian fluid. A random sample of 50 ml of eggs was taken from each batch and then analyzed for fertilization rate, hatch rate, dry weight per egg, fatty acid profile, and egg diameter. Three sub-samples of 50 eggs were counted and held at -80° C in an eppendorph tube for dry weight calculations. Another subsample of 10 ml of eggs was rinsed with distilled water and held in an amber glass vial and held under nitrogen gas at -80° C until analyzed for fatty acids.

To assess fertilization and hatch rates, 30 ml of the egg sample was place in a 500 ml beaker filled with prepared seawater. The beakers were held constant at 5.5 ± 1°C for 12-16 hours (at 4-8 cell) in an incubator controlled with a Standard Intellus Environmental Controller™ (Percival Scientific Incorporated, Perry, Iowa). A random sample of eggs was selected by mixing the contents of the beaker to homogenize the sample before transferring 200-300 random eggs to a Petri dish. Using a dissecting scope in a dark room, fertilized eggs were examined by counting random trisects of the dish until 200 egg were counted. Mean egg diameter was determined by measuring the length of 10 eggs using the vernier scale. Hatch rates were determined by stocking 192 randomly selected fertilized eggs at the 4-8 cell stage into 2-96 count microtiter plates (Greiner, No. 655180) filled with 200 µl of filtered and sterilized seawater at one egg per well (Shields *et al*., 1997; Unuma *et al*., 2004). The trays were held in the dark at 5.5° C until hatch, approximately 15-16 days, at which point the tray was examined for hatched yolk-sac larvae.

Diet and egg chemical analyses

Every batch of prepared diet, herring, squid, and each new ingredient was sampled and analyzed for dry matter, percent crude protein, percent fat, and ash. In addition, fatty acid

profiles were obtained for each new ingredient and batch of herring, squid, and worms. Composite samples of all batches of herring, squid, commercial diet, and the CCAR diet were sent to Eurofins Scientific Incorporated (Memphis, Tennessee). for a complete amino acid profile.

Dry weight per egg was obtained by counting three replicates of 50 eggs per batch. The egg samples were stored at -80° C in eppendorf tubes. To obtain dry weight per sample, the eggs were removed from the eppendorf tube and placed in a pre-weighed, dry, weighing tin and held in an oven at 105° C for 24 hours. The tins with the dry eggs were weighed and the dried egg weight was calculated. Dry weight per egg was calculated as the average of 50 eggs.

Diet dry matter content was determined by drying a sample of the feed for 24 hours at 105° C. Prior to the analyses of protein, fat, and ash, the feed was dried as above, ground by mortar and pestle and redried for an additional 2 hours, and stored in a desiccator until analyzed. Crude protein was analyzed according to the AOAC method (1965) as modified by Tecator™ (Application Note 1981.10.05, 1981), using a Kjeltec Auto 1030 Analyzer. A copper catalyst was used during digestion and a mixture of boric acid (1%) with bromocresol green/methyl red indicator was used as the receiving solution. Ash content was measured after heating a feed sample at 600° C for 12 hours in a Sybron/Thermolyne™ muffle furnace. Crude lipid was determined gravimetrically following extraction in dichloromethane using a Soxtec HT2 apparatus.

A composite sample from each diet was analyzed for gross energy content using a Parr 1108 Oxygen Combustion Bomb. Standard operating procedure (No. 205M) was used.

A sub sample of lyophilized eggs from each egg batch were sent in dry ice to the Center of Marine Biotechnology (COMB), University of Maryland Biotechnology, Baltimore, Maryland, were they were analyzed for fatty acid composition. All organic solvents used were of pesticide grade and were from Fisher Scientific (Pittsburg, PA). All egg samples and diet samples were freeze-dried, weighed (± 0.1 mg), and ground. Lipids were extracted from ca. 100 mg of sample using a modified version of Folch *et al*. (1957). Briefly, samples were homogenized with 3.0 ml dichloromethane:methanol (2:1 CH_2Cl_2:MeOH), centrifuged for 15 minutes at 2000 RPM and the supernatant transferred to a large test tube. This procedure was repeated with 1:1 CH_2Cl_2:MeOH and 2:1

CH_2Cl_2:MeOH. The lipid extract was first washed with 0.88% potassium chloride water solution, and then with CH_2Cl_2:MeOH:H_2O (3/48/47). Samples were dried under nitrogen, weighed (±0.1mg), resuspended in 500 µl of 1:1 methylene chloride:methanol, and capped under nitrogen.

Quantification of fatty acid methyl esters was achieved by hydrolyzing ca. 500 µg of extracted lipid with methanolic HCl, adding 25 µg of internal standard mixture of equal amounts of nonadecanoic acid (C19:0) and heinecosanoic acid (C21:0) (Nu-chek Prep Inc.) to each sample, and extracting the methyl esters into methylene chloride. An aliquot of the methylene chloride extract was subjected to gas chromatography directly on a Hewlett-Packard model 5890A instrument equipped with a flame ionization detector using a J&W DBWAX fused silica capillary column (30 m x 0.25 mm i.d., J. & W. Scientific Inc., Folsom, California). The oven temperature was programmed from an initial temperature of 50° C for 2 min to 200° C in 16 min, from 200° C to 210° C in 11 min, and from 210° C to 220° C in 18 min. Helium was used as the carrier gas with a flow rate of 1.0 ml min^{-1}. Peaks were identified by comparison with retention times of known standards and expressed as percentages of fatty acid methyl esters.

Fatty acids per gram of egg were calculated by multiplying the dry weight per egg by the percent lipid per egg by the percent fatty acid of total fat.

Statistical Methods

Differences in egg quality (fertilization and hatch rates), egg characteristics (dry weight per egg, percent lipid per egg), and egg biochemistry (fatty acid of total lipid, and fatty acid mass per egg) among dietary treatments were analyzed as a one way analysis of variance using SYSTAT 12. Data were first tested for normality using the Kolmogorov-Smirnov test. *Post hoc* separation of means among treatments was done using Tukeys HSD. Multiple forward stepwise regression was used to detect functional relationships between fatty acid levels in the eggs, total % lipid and fertilization rates. Since percentage data have a tendency to form a binomial distribution rather than a normal distribution, all percent data (p) were transformed prior to statistical analyses using an arcsine transformation using the following equation:

$$\text{Trans}(p) = 180/\Pi \times \text{arcsine}((p / 100)^{0.5}) \qquad \text{(Zar, 1980)}$$

RESULTS

Composition of diets

Proximate analysis of the diets showed similar levels of protein among treatments ranging from 60 to 64% (Table 5).

Table 5. Proximate analysis, gross energy, and selected fatty acids compositions of experimental diets from Dec 2003 to Sept. 2005*.

	Control			Commercial	CCAR
	Herring	Squid	COMP**		
Proximate (% of Dry Weight)					
Dry Weight	27.0 ± 6.5	19.2 ± 2.8	25.4	47.9 ± 2.6	56.5 ± 2.0
Protein	57.1 ± 5.1	70.5 ± 4.4	59.8	60.8 ± 4.6	63.8 ± 2.3
Fat	31.4 ± 7.2	7.5 ± 4.9	26.6	13.5 ± 1.7	16.1 ± 1.0
Ash	7.5 ± 1.2	8.0 ± 2.0	7.6	13.8 ± 0.7	10.3 ± 0.5
Gross energy (MJ / Kg)	24.8 ± 0.1	21.6 ± 0.6	24.2	21.6 ± 0.5	22.6 ± 0.4
Selected fatty acid (mg per gram DW of diet)					
ArA	0.6 ± 0.5	0.7 ± 0.1	0.6	1.5 ± 0.3	2.8 ± 0.4
EPA	15.2 ± 8.1	6.7 ± 1.0	13.5	9.1 ± 0.9	13.1 ± 0.2
DHA	20.0 ± 8.8	15.9 ± 3.6	19.1	24.0 ± 2.1	27.3 ± 2.7
DHA:EPA	1.3	2.4	1.4	2.6	2.1
EPA:ArA	26.2	9.5	22.3	6.1	4.7
∑n-3	42.7	22.7	38.7	37.9	46.7
∑n-6	4.0	0.8	3.4	17.8	14.2
∑n-3 / ∑n-6	10.6	28.2	11.4	2.1	3.3

* - Mean ± SD

** - Composite (COMP) based on an average consumption of herring to squid of 4 to 1.

Gross energy values of the three diets are also presented in Table 5. Digestibility of the three diets was not obtained in this experiment.

Fat content was highest in the herring (31.5 ± 7.2%) and lowest in the squid (7.5 ± 4.9). The fat content of the combined Control diet was higher (26.5%) than either of the formulated feeds; CCAR (16.1 ± 1.0%) and Commercial (13.5 ± 1.7%) diets fell in-between. Protein was highest in the Squid (70.5 ± 7.2%) and was similar in the herring (57.1 ± 5.1%), commercial (60.8 ± 4.6%), and CCAR (63.8 ± 2.3%). Ash levels were highest in the Commercial diet (13.8 ± 0.7%), followed by the CCAR diet (10.3 ± 0.5%), while herring (7.5 ± 1.2%) and squid (8.0 ± 2.0%) were similar. The gross energy level was highest in the herring (24.8 ± 0.1 MJ/Kg), followed by the CCAR diet (22.6 ± 0.4 MJ/Kg), while the squid (21.6 ± 0.6 MJ/Kg) and Commercial diet were similar (21.6 ± 0.5 MJ/Kg).

Amino acid profiles of the composite samples of the three diets are presented in Table 6. Fatty acid profiles of the diets are presented as a percentage (percent fatty acid of total fat) in Table 7 and as mass (mg fatty acid per gram diet dry weight) in Table 8. The control diet contained lower levels of ARA and higher levels of EPA compared to the other two diets. The EPA to ARA ratio is much lower in the two formulated diets along with the total omega three fatty acid to the total omega six fatty acid ratio. DHA was highest in the CCAR diet while the DHA to EPA ratios were 2 or above in the Commercial and CCAR diet. Other differences in fatty acids are noticeable; 20:1 and a 22 carbon fatty acid are present is high amounts in the Control diet compared to the two formulated diets.

Table 6. Amino acid composition (gm / 100gm protein) of experimental diets from December 2003 to September 2005.

	Control			Commercial	CCAR
	Herring	Squid	Composite*		
trp	1.34	1.14	1.30	1.14	1.05
asp	11.44	10.79	11.31	9.48	9.92
thr	5.19	4.92	5.14	4.88	4.68
ser	4.13	3.88	4.08	4.68	3.92
glu	15.63	16.15	15.74	20.28	18.47
pro	5.31	4.31	5.11	6.01	6.33
gly	6.12	6.65	6.22	6.16	7.19
ala	6.53	7.33	6.69	6.42	6.62
val	5.24	5.83	5.36	4.53	5.34
isl	5.17	4.85	5.11	4.05	4.82
leu	8.01	8.37	8.08	8.21	7.98
lys	7.54	9.07	7.85	7.46	6.91
his	2.29	2.77	2.38	2.07	2.22
arg	7.71	6.44	7.46	6.33	6.73
tyr	4.08	3.31	3.92	3.72	3.53
phe	4.28	4.17	4.26	4.58	4.27
Cys	1.44	0.77	1.31	1.14	1.08
Met	2.83	2.97	2.86	2.88	2.61

* - based on an average consumption of herring to squid of 4 to 1.

Table 7. Average fatty acids composition of the experimental diets as percent of total lipid

Fatty Acid	Control			Commercial	CCAR
	Herring	Squid	COMP*		
14:0	6.6 ± 1.8	2.2 ± 0.7	5.8	3.6 ± 0.1	6.4 ± 1.4
14:1	2.5 ± 6.0	0.0 ± 0.0	2.0	0.2 ± 0.2	0.4 ± 0.3
16:0	11.0 ± 5.4	22.1 ± 2.7	13.2	19.1 ± 1.1	18.2 ± 1.4
16:1	5.7 ± 1.1	0.3 ± 0.6	4.6	3.3 ± 2.7	6.2 ± 1.0
17:0	0.1 ± 0.1	0.2 ± 0.3	0.1	0.3 ± 0.3	0.2 ± 0.3
18:0	1.3 ± 0.2	4.6 ± 0.5	2.0	4.5 ± 0.4	4.0 ± 0.6
18:1n9	6.0 ± 3.3	2.8 ± 1.2	5.3	8.6 ± 5.4	8.4 ± 8.0
18:1n7	1.4 ± 0.8	0.7 ± 0.9	1.3	1.8 ± 1.4	1.6 ± 2.0
18:2n6 (LA)	1.6 ± 0.5	0.2 ± 0.4	1.3	11.5 ± 1.8	6.9 ± 1.1
18:3n3	1.0 ± 0.5	0.1 ± 0.1	0.8	1.7 ± 0.1	1.2 ± 0.1
18:4n3	2.5 ± 1.3	0.1 ± 0.1	2.0	1.1 ± 0.1	1.4 ± 0.0
20:0	0.1 ± 0.2	0.1 ± 0.2	0.1	0.2 ± 0.2	0.2 ± 0.2
20:1 ab	7.8 ± 8.8	4.6 ± 1.0	7.1	1.2 ± 2.0	2.8 ± 4.0
20:1 c	7.9 ± 8.5	0.1 ± 0.1	6.3	1.7 ± 2.6	1.9 ± 2.1
20:4n6 (ArA)	0.3 ± 0.2	1.8 ± 0.6	0.6	1.0 ± 0.1	1.7 ± 0.3
20:4n3	0.1 ± 0.1	0.1 ± 0.2	0.1	0.2 ± 0.2	0.3 ± 0.4
20:5n3 (EPA)	7.3 ± 1.8	16.8 ± 2.9	9.2	6.5 ± 0.8	7.9 ± 0.2
22:?	13.4 ± 14.8	0.1 ± 0.2	10.7	1.6 ± 2.6	0.3 ± 0.0
22:5n3	0.2 ± 0.3	0.2 ± 0.3	0.2	0.6 ± 0.6	1.5 ± 0.3
22:6n3 (DHA)	9.7 ± 2.2	39.2 ± 1.4	15.6	17.0 ± 1.1	16.3 ± 1.4
24:1	0.3 ± 0.4	0.1 ± 0.2	0.2	0.4 ± 0.4	0.4 ± 0.1
DHA:EPA	1.3	2.3	1.7	2.6	2.1
EPA:ArA	24.0	9.2	15.2	6.2	4.7
∑n-3	20.8	56.4	27.9	27.1	28.6
∑n-6	1.9	2.0	1.9	12.6	8.5
∑n-3 / ∑n-6	10.8	27.8	14.3	2.2	3.4

* - Composite (COMP) based on a average consumption of herring to squid of 4 to 1.

Mean ± S.D.

Table 8. Average fatty acid composition of the experimental diets as amount in diet, expressed as mg of fatty acid per gram of dry diet (mg per g diet).

Fatty Acid	Control			Commercial	CCAR
	Herring	Squid	COMP*		
14:0	14.0 ± 7.8	0.8 ± 0.1	11.4	5.1 ± 0.1	10.6 ± 2.3
14:1	5.2 ± 12.4	0.0 ± 0.0	4.1	0.2 ± 0.2	0.5 ± 0.5
16:0	23.7 ± 18.1	8.8 ± 1.4	20.7	26.9 ± 2.5	30.4 ± 2.1
16:1	12.5 ± 7.5	0.2 ± 0.3	10.0	4.7 ± 3.8	10.3 ± 1.6
17:0	0.1 ± 0.1	0.1 ± 0.2	0.1	0.4 ± 0.4	0.3 ± 0.5
18:0	2.3 ± 1.6	1.8 ± 0.3	2.2	6.3 ± 0.8	6.7 ± 0.9
18:1n9	13.5 ± 12.1	1.1 ± 0.2	11.0	12.3 ± 8.0	13.9 ± 13.
18:1n7	3.1 ± 2.8	0.3 ± 0.3	2.5	2.6 ± 2.2	2.6 ± 3.5
18:2n6 (LA)	3.5 ± 2.0	0.1 ± 0.2	2.8	16.3 ± 3.1	11.5 ± 1.7
18:3n3	2.0 ± 1.5	0.0 ± 0.1	1.6	2.4 ± 0.3	2.0 ± 0.2
18:4n3	4.9 ± 3.1	0.0 ± 0.1	4.0	1.6 ± 0.2	2.3 ± 0.0
20:0	0.3 ± 0.4	0.0 ± 0.1	0.2	0.3 ± 0.3	0.3 ± 0.4
20:1 ab	18.9 ± 23.2	1.9 ± 0.9	15.5	1.7 ± 2.7	4.7 ± 6.7
20:1 c	15.8 ± 23.7	0.0 ± 0.1	12.6	2.4 ± 3.8	3.1 ± 3.5
20:4n6 (ArA)	0.6 ± 0.5	0.7 ± 0.1	0.6	1.5 ± 0.3	2.8 ± 0.4
20:4n3	0.2 ± 0.3	0.1 ± 0.1	0.2	0.3 ± 0.3	0.5 ± 0.7
20:5n3 (EPA)	15.2 ± 8.1	6.7 ± 1.0	13.5	9.1 ± 0.9	13.1 ± 0.2
22:?	31.6 ± 45.7	0.0 ± 0.0	25.3	2.3 ± 3.9	0.4 ± 0.2
22:5n3	0.5 ± 0.7	0.1 ± 0.1	0.4	0.9 ± 0.8	2.0 ± 1.3
22:6n3 (DHA)	20.0 ± 8.8	15.9 ± 3.6	19.1	24.0 ± 2.1	27.3 ± 2.7
24:1	0.5 ± 0.9	0.1 ± 0.1	0.4	0.5 ± 0.5	0.7 ± 0.1
DHA:EPA	1.3	2.4	1.4	2.6	2.1
EPA:ArA	26.2	9.5	22.3	6.1	4.7
∑n-3	42.7	22.7	38.7	37.9	46.7
∑n-6	4.0	0.8	3.4	17.8	14.2
∑n-3 / ∑n-6	10.6	28.2	11.4	2.1	3.3

* - Composite (COMP) based on a average consumption of herring to squid of 4 to 1.

Variance expressed as ± S.D.

Feed consumption and growth rates

From the introduction of the diets in January 2004, all groups accepted feed well. Feed intake was tracked on a weekly basis and then summarized monthly for each group. This data is presented in Figure 6. Monthly feed intake increased following spawning and peaked during the fall. Feeding rate tended to fall off during spawning and in the colder months. Weekly temperature data are presented in Figure 7. During the summers of 2004 and 2005, high average temperatures and inadequate chilling capacity resulted in water temperatures which were above the optimum for this species and this also caused a depression of appetite.

In terms of dry weight feed intake, the consumption of the two formulated feeds was generally always higher than for the wet fish and squid diet. Summer feed intake for the CCAR and Commercial feeds reached between 5 to 8% per month and fell to around 1 to 3% per month in the winter months during spawning. The consumption of fish and squid in the Control group ranged between 1 and 5% winter to summer. There was a general trend downward in feed consumption as a percentage of body weight as the fish grew.

The increase in average weight in the three groups is shown in Figure 8. The average size of the fish in the two groups fed formulated feeds increased to near 30kg whilst the Control group average weight reached only 26kg. Fish weight was not significantly different amongst the groups (ANOVA, $p>0.05$) due to the large coefficient of variation at the start and end of the trial.

Though the sausage diets are presented in large pieces (30 mm diameter, 15 cm long), the larger broodstock will eat several pieces in a meal. Initial concerns about the fines that would be released from the formulated feeds proved unfounded. Apparently binders in the formulation were very effective and though some feed particles are released through the operculum during swallowing and some feed links remain in the tank uneaten for several hours this did not seem to have a large impact on suspended solids within the recirculation systems.

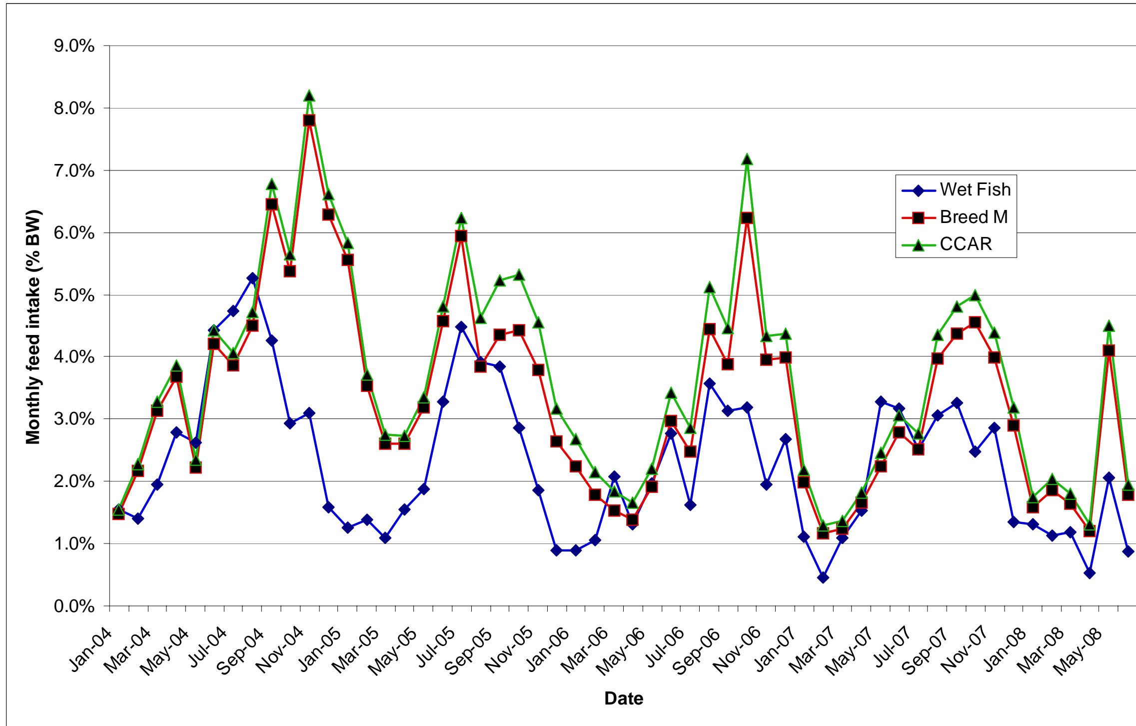


Figure 6 Monthly feed intake - dry weight as % of wet body weight

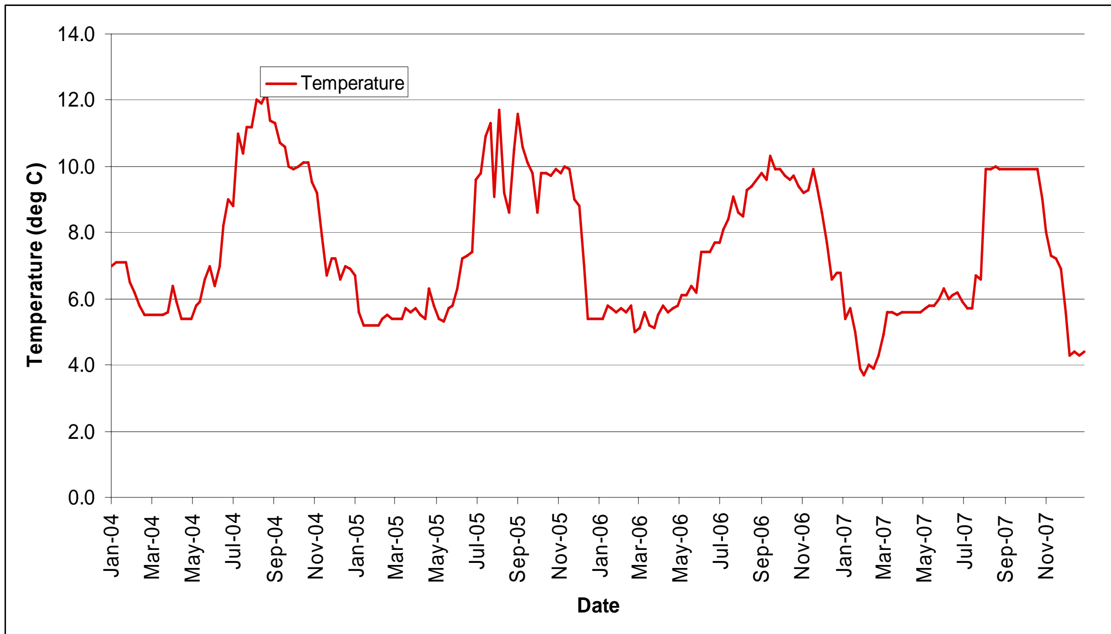


Figure 7. Weekly average temperature.

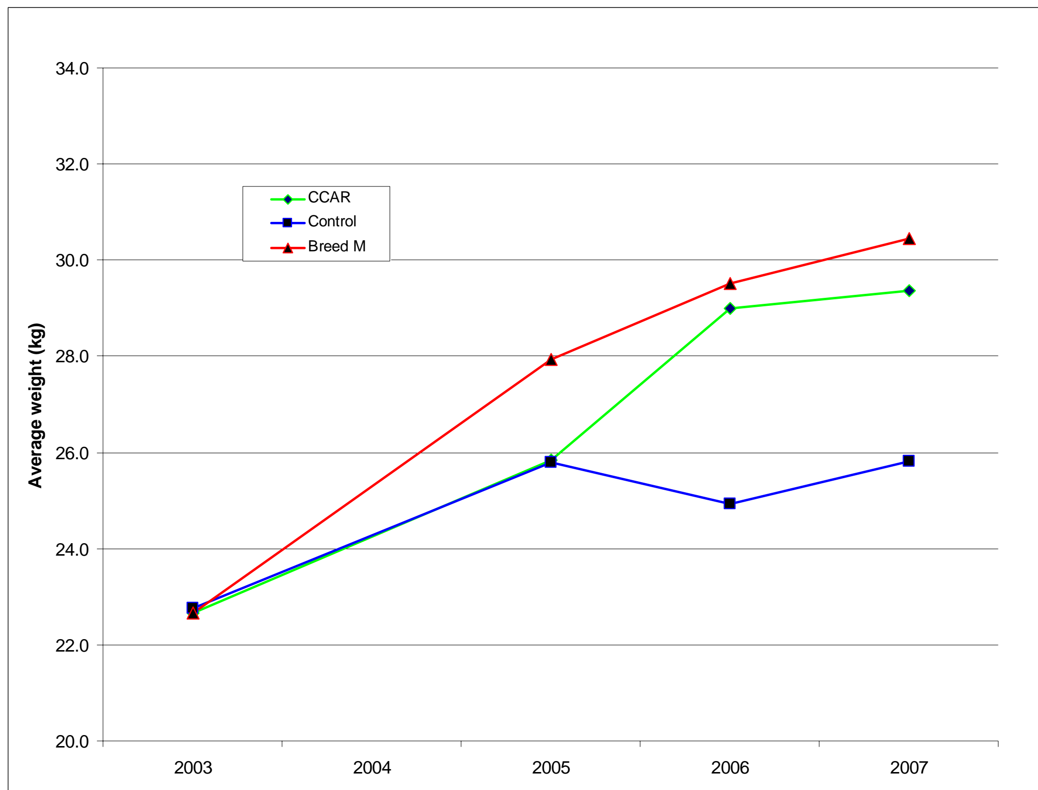


Figure 8. Change in average weight of broodstock fish in original population during project.

Spawning performance

The data on spawning performance is summarized in Table 9. Only 7 females spawned in 2004 and 8 females in 2005. By 2006 and again in 2007, 15 females were producing eggs. To compare fertilization rates between females and between treatments, the top 3 fertilization rates above 10% from each female were used. There was a large variation in fertilization rates in all years. In 2005, no viable egg batches were collected from female spawning in the Commercial group and so differences in fertilization rate were not analyzed statistically. In 2006, fertilization rates were significantly lower in the Commercial group than the Control group (ANOVA, $p<0.05$). In 2007 there were no significant differences between groups with respect to fertilization rates.

Fecundity generally increased from 2004 to 2006; just under 4 million eggs total were produced in 2004 and nearly 8 million eggs released or collected in 2006. In 2007

however, there was a sharp decline following the move to a new system. Assessment of specific fecundity was possible between 2004 and 2006. Egg batches released naturally could be accurately attributed to specific fish since there were only 2 or 3 females per tank which were generally not spawning at the same time. There was no significant difference in this parameter in 2005 or 2006 (ANOVA, $p>0.05$). In 2007, the grouping of all fish in each group into a single tank made the origin of naturally released eggs unclear since very often more than one fish would be spawning at a given time. Therefore, only total fecundity by group is given in Table 9.

Table 9 (next page). Spawning performance data: Number of females spawning in each group, mean fecundity in terms of total egg stripped or released naturally per female, specific fecundity (eggs per kg body weight), numbert of batches collected (stripped) manually, mean fertilization rate (%) of top 3 egg batches per female, excluding those fertilization rates <10%, and average fish weight each season (kg).

		CCAR		Control		Commercial	
		Value	S.E.	Value	S.E.	Value	S.E.
2004	# females spawning	2 of 6		3 of 6		2 of 11	
	Mean fecundity (eggs/female)	624,718	373,854	493,477	113,524	483,825	6,728
	Specific Fecundity (eggs/kg)	23,979	9643.4	15,916	3795.3	17,358	676.7
	# Batches collected manually	14		13		12	
	Fecundity (Total eggs spawned)	1,249,436		1,480,432		967,650	
	Mean fertilization rate (%)	52.0	6.13	67.0	14.07	69.7	10.74
	Average fish weight (kg)	22.7	1.9	22.8	2.0	22.7	2.8
2005	# females spawning	3 of 6		3 of 6		2 of 11	
	Mean fecundity (eggs/female)	567,898	249,996	567,898	249,996	198,547	116,712
	Specific Fecundity (eggs/kg)	20,723[a]	7948.0	20,723[a]	7948.0	7,777[a]	5304.5
	# Batches collected manually	14		16		5	
	Fecundity (Total eggs spawned)	1,703,694		1,997,849		397,094	
	Mean fertilization rate (%)	73.7	6.4	75.4	13.7	0.0	0.0
	Average fish weight (kg)	25.9	2.0	25.8	2.3	27.9	3.0
2006	# females spawning	4 of 6		4 of 6		7 of 11	
	Mean fecundity (eggs/female)	699,435	290,454	427,089	73,451	472,968	110,101
	Specific Fecundity (eggs/kg)	20,074[a]	6463.0	13,451[a]	2554.1	17,689[a]	3956.5
	# Batches collected manually	15		5		24	
	Fecundity (Total eggs spawned)	2,797,739		1,708,356		3,310,777	
	Mean fertilization rate (%)	72.1[ab]	8.8	94.7[a]	0.7	59.3[b]	6.0
	Average fish weight (kg)	29.0	2.4	24.9	2.5	29.5	3.0
2007	# females spawning	4 of 14		4 of 10		8 of 16	
	Mean fecundity (eggs/female)	477,049	NA	232,670	NA	240,034	NA
	# Batches collected manually	18		15		17	
	Fecundity (Total eggs spawned)	1,908,194		930,680		1,920,270	
	Mean fertilization rate (%)	56.7[a]	7.0	76.3[a]	7.2	58.9[a]	8.42
	Average fish weight (kg)	29.4[a]	2.4	25.8[a]	2.1	30.4[a]	2.7

Egg fatty acid composition

Stepwise regression was performed on the 2005 data with fertilization rates as the dependant variable and fatty acid values and lipid content as the independent variables. This final model is shown in Table 10. (a to c).

Table 10. (a to c). Stepwise regression of 2005 fertilization rates (arcsine transformed), egg fatty acid content (in mg/g dry wt) and lipid content (% arcsine transformed).

Table 10.a. Regression Coefficients $B = (X'X)^{-1}X'Y$

Effect	Coefficient	Standard Error	Std. Coefficient	Tolerance	t	p-value
CONSTANT	-34.697	32.253	0.000	.	-1.076	0.293
C16:1	6.299	2.166	0.471	0.711	2.908	0.008
C16:3 n4	-63.465	16.521	-0.606	0.748	-3.842	0.001
C18:4 n3	-80.560	34.947	-0.319	0.973	-2.305	0.030
Lipid %	1.861	0.959	0.327	0.657	1.940	0.064

Table 10.b. Confidence Interval for Regression Coefficients

Effect	Coefficient	95.0% Confidence Interval		
		Lower	Upper	VIF
C16:1	-34.697	-101.264	31.870	.
C16:3 n4	6.299	1.829	10.769	1.406
C18:4 n3	-63.465	-97.562	-29.368	1.337
Lipid %	-80.560	-152.688	-8.432	1.027
C16:1	1.861	-0.119	3.841	1.522

Table 10.c. Analysis of Variance

Source	SS	df	Mean Squares	F-ratio	p-value
Regression	12727.287	4	3181.822	7.411	0.000
Residual	10304.152	24	429.340		

Similar analysis was performed on the 2007 data which revealed a different model:

Table 11. (a to c). Stepwise regression of 2007 fertilization rates (arcsine transformed) vs egg fatty acid content (in mg/g dry wt) and lipid content (% arcsine transformed).

Table 11. a. Regression Coefficients $B = (X'X)^{-1}X'Y$

Effect	Coefficient	Standard Error	Std. Coefficient	Tolerance	t	p-value
CONSTANT	65.210	4.763	0.000	.	13.690	0.000
Unknown (1)	-2.186	0.916	-0.293	0.787	-2.388	0.021
C17:0	37.491	10.766	0.553	0.469	3.482	0.001
C18:2 n6	-1.765	0.305	-0.852	0.544	-5.782	0.000

Table 11. b. Confidence Interval for Regression Coefficients

Effect	Coefficient	95.0% Confidence Interval		
		Lower	Upper	VIF
CONSTANT	65.210	55.610	74.811	.
Unknown (1)	-2.186	-4.032	-0.341	1.271
C17:0	37.491	15.793	59.188	2.130
C18:2 n6	-1.765	-2.380	-1.150	1.838

Table 11. c. Analysis of Variance

Source	SS	df	Mean Squares	F-ratio	p-value
Regression	12681.292	3	4227.097	13.533	0.000
Residual	13743.846	44	312.360		

Egg fatty acid values in terms of mg/g dry egg weight and % lipid as dry weight are given in Table 12 for 2004, 2005 and 2007. These data were analyzed with ANOVA and any significant differences in specific fatty acid levels between treatments within each year are shown for 2005 and 2007.

Diet	2004						2005						2007					
	Commercial		**CCAR**		**Control**		**Commercial**		**CCAR**		**Control**		**Commercial**		**CCAR**		**Control**	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
% Total lipid	18.33	2.82	19.50	2.51	17.24	3.63	16.91[a]	20.90	19.46[a]	1.82	22.72[a]	2.61	16.27[a]	0.92	15.41[a]	0.42	17.76[a]	0.85
C14:0	6.1	0.6	7.9	2.2	5.6	0.6	3.21[a]	0.57	3.13[a]	0.55	3.82[a]	0.49	2.90[a]	0.57	6.53[a]	0.57	6.86[a]	1.99
C16:0	34.1	3.3	42.4	3.4	29.1	2.3	56.47[a]	7.49	40.0[a]	4.55	39.72[a]	4.41	76.82[a]	11.42	91.40[a]	5.82	59.80[a]	13.45
Unknown (1)	2.2	0.3	2.6	0.4	2.2	0.1	3.75[a]	0.72	2.3[a]	0.36	2.64[a]	0.38	3.44[a]	0.80	3.54[a]	0.62	3.46[a]	1.03
C16:1	5.3	0.7	6.1	0.5	3.7	0.3	5.68[a]	0.75	5.0[a]	0.66	4.85[a]	0.66	6.82[a]	1.05	12.02[b]	0.97	5.42[a]	1.32
C16:2n4	nd	nd	nd	nd	nd	nd	0.68[a]	0.18	0.2[b]	0.08	0.15[b]	0.10	2.87[a]	0.48	1.61[b]	0.12	0.99[b]	0.26
C17:0	nd	nd	nd	nd	nd	nd	0.98[a]	0.28	0.3[b]	0.08	0.17[b]	0.13	0.52[a]	0.12	0.26[b]	0.05	0.12[b]	0.07
C16:3n4	nd	nd	nd	nd	nd	nd	0.53[a]	0.14	0.1[b]	0.05	0.07[b]	0.07	0.06[a]	0.04	0.00[a]	0.00	0.00[a]	0.00
C16:4n1	nd	nd	nd	nd	nd	nd	0.29[a]	0.21	0.0[b]	0.02	0.04[ab]	0.03	nd		nd		nd	
C18:0	6.4	0.5	8.7	0.9	6.0	0.5	14.86[a]	2.64	9.7[a]	1.10	9.28[a]	1.20	16.26[ab]	2.43	19.53[a]	1.35	10.73[b]	2.40
C18:1n7	3.4	0.3	4.4	0.3	2.7	0.2	6.51[a]	0.90	5.2[a]	0.67	4.15[a]	0.57	7.58[a]	1.17	11.65[b]	0.88	5.04[a]	1.20
C18:1n9	10.6	1.2	14.5	1.2	9.0	0.7	21.59	2.45	13.0	1.49	13.72	1.58	24.70[ab]	3.73	36.33[a]	3.05	14.79[b]	3.98
Unknown (2)	nd	nd	nd	nd	nd	nd	nd		nd		nd		1.07[a]	0.64	1.64[a]	0.62	0.13[a]	0.08
C18:2n6	0.2	0.2	1.7	0.7	0.6	0.2	18.37[a]	2.98	6.7[b]	0.95	3.26[b]	2.04	19.74[a]	3.15	21.51[a]	1.55	3.15[b]	0.83
C18:3n3	nd	nd	nd	nd	nd	nd	0.87[a]	0.22	0.1[b]	0.05	0.11[b]	0.11	0.64[a]	0.12	0.58[a]	0.08	0.38[a]	0.16
C18:4n3	nd	nd	nd	nd	nd	nd	nd		nd		nd		0.20[a]	0.13	0.01[a]	0.01	0.30[a]	0.18
C20:1n11	6.2	0.5	8.2	0.6	5.8	0.4	4.71[ab]	0.64	3.0[a]	0.72	6.67[b]	0.73	6.15[a]	1.08	7.76[a]	0.66	10.67[a]	2.54
Unknown (3)	nd	nd	nd	nd	nd	nd	nd		nd		nd		1.83[a]	0.33	0.42[b]	0.06	0.18[b]	0.08
C20:2n6	nd	nd	nd	nd	nd	nd	1.91[a]	0.55	0.7[b]	0.19	0.32[b]	0.23	1.54[a]	0.29	1.89[a]	0.19	0.13[b]	0.08
C20:4n6 ARA	0.0	0.0	0.4	0.2	0.2	0.1	5.48[a]	1.02	4.7[a]	0.66	1.80[b]	0.62	5.53[a]	0.88	10.90[a]	0.74	2.18[a]	0.62
C20:4n3	nd	nd	nd	nd	nd	nd	0.32[a]	0.14	0.2[a]	0.07	0.16[a]	0.09	0.34[a]	0.10	0.26[a]	0.06	0.50[a]	0.27
C20:5n3 EPA	18.6	2.4	28.3	2.1	3.6	2.0	29.03[a]	4.71	23.3[a]	3.02	27.55[a]	2.83	37.97[a]	5.57	48.62[a]	3.22	40.40[a]	10.22
C21:0	23.9	3.8	18.3	3.9	17.4	4.7	0.24[a]	0.16	0.1[a]	0.05	0.11[a]	0.08	nd		nd		nd	
C22:0	2.0	2.0	0.0	0.0	15.5	2.8	0.24[a]	0.10	0.3[a]	0.26	2.37[a]	0.34	1.94[a]	0.55	0.97[a]	0.14	3.48[a]	1.41
Unknown (3)	1.4	0.3	3.0	0.2	0.5	0.3	nd		nd		nd		0.11[a]	0.06	0.02[a]	0.01	0.22[a]	0.12
C22:1	0.0	0.0	0.0	0.0	1.5	0.3	0.06[a]	0.06	0.0[a]	0.01	0.10[a]	0.05	nd		nd		nd	
C22:5n3	2.4	0.3	3.0	0.2	0.5	0.3	2.82[a]	0.30	2.9[a]	0.38	2.38[a]	0.27	4.17[a]	0.70	5.54[a]	0.41	3.93[a]	1.04
C24:0	0.3	0.3	0.0	0.0	4.7	2.7	nd		nd		nd		nd		nd		nd	
C22:6n3 DHA	49.0	6.7	65.2	5.1	8.7	4.7	93.60[a]	12.04	67.4[a]	8.03	67.66[a]	7.97	114.32[a]	17.06	150.22[a]	10.20	105.03[a]	26.13
C24:1	6.2	6.2	0.3	0.2	37.3	6.7	1.14[a]	0.36	1.0[a]	0.26	1.18[a]	0.31	0.03[a]	0.03	0.10[a]	0.06	0.24[a]	0.13
n	12		13		13		5		13		11		15		18		15	

Table12. Fatty acid values as mg/g dry egg weight and % lipid as dry weight. Values are means of *n* samples. Significant differences between treatments are indicated by superscripts (ANOVA, $p<0.05$)

DISCUSSION

After more than 4 years feeding Atlantic halibut broodstock fish on the three different diets, we can say with certainty that all the diets seemed to fulfill the nutritional requirements in terms of health and growth. The two formulated feeds actually resulted in faster growth on average than the wet fish diet, probably due to the nutrient density of the feeds, which is due in turn to the moisture content. Although there were some mortalities during the trial, these did not seem to be due to feed type. In most cases, mortality was related to reproductive dysfunction whereby females became what is termed "egg bound".

Differences in egg fatty acid composition were caused by diet type. The few significant differences came in the levels of C:16:1, C16:2 n4, C:17:0, C16:3 n4, C16:4 n1, C18:0, C18:1 n7, C18:1 n9, C18:2 n6, C18:3 n3, C20:2 n6. In these cases, the formulated feeds (one or both) were higher in each case than in the eggs from the Control group. In the essential fatty acids that are of common interest to researchers in this area, namely Arachidonic acid –ARA- (C20:4 n6), eicosapentaenoic acid –EPA- (C20:5 n3) and docosahexaenoic acid –DHA- (C22:6 n3), there are no significant differences between groups. The levels of these fatty acids in the diets were quite different in some cases (e.g. DHA much higher both formulated feeds and ARA much higher in the CCAR diet). The fact that these levels did not differ significantly in the eggs suggests that the nutrients are incorporated into the egg independent of dietary levels.

It has been shown previously that broodstock Atlantic halibut can be conservative in the levels of nutrients, in particular essential fatty acids, that it sequesters to the eggs (Bruce *et al*., 1993). In this and other studies on the species it has taken months or years for deficiencies to emerge following a change in diet. These fish can survive for up to a year without feed after capture (CCAR unpublished data).

Several marine fish broodstock and egg quality studies have demonstrated that manipulation of various dietary components can have a direct effect on egg viability (Watanabe, 1985; Kjorsvik *et al.*, 1990, Izquierdo *et al.*, 2001). Lipids and fatty acids have historically been one of the main focuses of marine teleost broodstock nutrition. Polyunsaturated fatty acids are important for the formation and function of membranes

and as precursors in the synthesis of certain molecules involved in a variety of biochemical pathways (Bell *et al*., 1986). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are incorporated into the phospholipid fraction of marine fish eggs (Rainuzzo, 1993) and are an important source of energy during embryonic development (Tocher *et al*., 1985; Rainuzzo *et al*., 1992). Lipid and carbohydrate are the dominant sources for energetic requirements during early embryonic development, the emphasis switching to free amino acids as hatching approaches (Finn *et al*., 1991; Zhu *et al*., 2003). Tandler *et al*. (1995) demonstrated an improvement in egg quality in broodstock sea bream fed an n -3 HUFA (highly unsaturated fatty acid) enriched diet. The resulting larvae grew faster and were less likely to develop problems associated with swim bladder inflation. Harel *et al*. (1994) showed a very rapid incorporation of essential fatty acids in the reproductive organs of gilthead sea bream, reaching equilibrium with the diet within 15 days. A decrease in egg viability reflected the switch to a diet deficient in n-3 HUFA in 10 days. Similar results using essential fatty acid deficient diets have been obtained for other fish species (Watanabe, 1985). Navas *et al*. (2001) determined that the total lipid content of the broodstock diet in sea bass did not affect the fatty acid composition and quality of eggs, though variances in dietary fatty acid ratios did.

The most recent related studies on Atlantic halibut broodstock nutrition (Mazorra *et al*., 2003; Alorend, 2004) both looked at dietary levels of arachidonic acid (ARA) and its effect on egg quality. Mazorra *et al.* (2003) showed an improvement in egg quality when the dietary DHA/EPA/ARA ratio was 6:4.6:1. The authors further suggest a dietary ratio of around 8:4:1. Alorend (2004) demonstrated that dietary levels of ARA at 4-12 mg/g have an immediate positive impact on egg quality. However long-term effects of ARA at these levels showed a decrease in egg quality and ARA levels in the egg suggesting an optimum level of 3 mg/g of ARA.

Interest in dietary ARA in aquaculture feeds is relatively recent but it has a proven importance in broodstock nutrition (Bell, 2003). ARA is an important precursor for prostaglandins which are used for egg development and maturation (Sorbera *et al*., 2001; Patiňo *et al*., 2003). Dietary ARA was shown to concentrate in the eggs and sperm and increases egg quality of sea bass (Navas *et al*., 1997; Bell *et al*., 1997), Japanese flounder

(Furuita *et al*., 2002), spotted wolf-fish (Tveiten *et al*., 2004), and Atlantic halibut (Mazorra *et al.*, 2003). There is evidence that the effectiveness of ARA is dependant on the dietary ARA/EPA ratio due to the competition of these two fatty acids for the same enzyme system in hormone production (Tocher, 2003).

Our study provides little evidence for differences in egg quality caused by diet type. However, we did not set out to vary the level of one specific nutrient to one extreme or another to induce an effect on egg quality. This project aimed to find a suitable alternative to raw fish ingredients that would perform the same function but without the biosecurity risk of using untreated marine ingredients. In one of the years, 2006, there seemed to be a significantly lower fertilization rate overall in the Commercial group but this was not repeated in 2006 or 2007. When all the egg batches were pooled, there were no significant differences detected between the groups. The stepwise regression analysis suggested that there were functional relationships between certain fatty acids and fertilization rate but these were different fatty acids in 2005 and 2007 and so it is hard to draw conclusions from these results. However, we might conclude that both formulated feeds seem to perform as well the Control and from this point of view, the project was a success. Some of the egg batches from the formulated diet groups had very high fertilization rates and this indicates a potential for good quality eggs in broodstock halibut halibut fed formulated feeds and this was the major purpose of the study. Indeed, high quality juvenile halibut were successfully reared with high survival rates, from eggs spawned by fish that had fed on formulated feed.

If effects of diet on egg quality do exist, it is very difficult to determine them in this species due to the large variability in fertilization rates. Even when only the top 3 batches, with over 10% fertilized, from each fish were considered in the analyses (similar to the criteria used by Mazorra *et al*. 2003) the variability in the data was high. The cause of this variability is almost certainly the timing of stripping. It is likely that the stage of final maturation is at least as important as the potential quality of an egg batch and there is a relatively short window of time during which Atlantic halibut eggs can be successfully fertilized as shown by Bromage *et al*. (1994). Halibut are determinate batch spawners ovulating at intervals of 70 to 90 hours over the spawning season (Holmefjord 1991; Norberg et al. 1991). The ovary of a spawning female consists of eggs in all stages

of oocyte development (Riple, 2000; Finn *et al*., 2002): oogonia, primary oocyte, and secondary oocyte. During the maturation process batches of oocytes are sequentially hydrated. As with other marine teleosts that produce pelagic eggs, the secondary oocytes are hydrated after the onset of maturation. Prehydrated secondary oocytes increase in volume four times their original size before they are fully hydrated (Finn *et al*., 2002). The driving force for the uptake of water is an increase in osmolality of the yolk compared to the blood and ovarian fluid (Finn *et al*., 2002). This increase in osmolality is primarily due to the rapid hydrolysis of yolk proteins to create high concentrations of free amino acids (FAA) (Finn *et al*., 2002). The FAA are also utilized by the developing embryo after fertilization as a primary energy source and to continue to osmoregulate the egg while in the ocean (Fyhn *et al*., 1999).

The duration of the hydration cycle is 33 to 54 hours, and when it is complete the egg emerges from the follicle and is ovulated into the oviduct (Finn *et al*., 2002). Given the optimal conditions, the female then releases her eggs, as secondary oocytes, into the water column where milt is waiting and the eggs are fertilized.

As yet there is no useful indicator of stage of maturity that can be used to determine whether the timing of stripping was accurate. In turbot (*Scophthalmus maximus*), changes in pH were used as an indicator for overripe eggs (Fauvel *et al*., 1993). However, this indicator is not reliable in halibut (Martin-Robichaud unpublished data). In halibut, the ovarian fluid may contain indicators such as hormone levels, enzymes, or changes in water content. Further research is needed in this area and also to look at methods for the control of spawning using hormone implant technology.

Unfortunately, there were large variations in egg quality in all treatments and some environmental stressors caused by activities on site outside our control may have played a role. During 2005 there was a great deal of construction activity near the broodstock facilities and this included rock blasting in the near vicinity of the tanks. Also, the move to the new facilities in early 2007 seemed to result in a reduction in overall fecundity. Spawning in 2008, not included in this study, was delayed in the Control group, possibly due to light leakage from the room into the Control group tank. It is apparent from this study that environmental conditions are of the utmost importance in this species.

The CCAR has a large population of hatchery reared F1 broodstock fish that have now matured. It is hoped that these fish which have spent their entire life on formulated feeds and adapted to tank conditions will spawn more successfully in captivity.

An attempt was made to hatch eggs in microtitre plates in this study to obtain a second very important measure of egg quality, namely hatch rate. This technique can be very successful (Shields *et al*., 1997; Brown *et al*., 2006). However there were large inconsistencies in this project with the results from microtitre plate incubations. The technique is difficult to master and really needs to be done by the same individual for every batch and by someone well practiced. Stocking small fish eggs into well plates late at night in a cold dark room is a significant challenge for anyone and the eggs are quite delicate at this stage. For these reasons, the hatch data was not used.

The basis of many marine fish diets, including broodstock feeds, is fishmeal and fish oil. The use of these components is attracting increasing criticism (Naylor *et al*. 2000) and this combined with the rising cost of these resources has meant that the search for sustainable sources of replacement raw materials is intensifying. The investigation of alternative protein and lipid sources in this study is partly an attempt to address those concerns. Highly enriched fatty acids derived from heterotrophic algal culture are used in the CCAR diet. Farmed seaworms (*Nereis virens*) are used to provide proteins, lipids and attractants. Underutilized fishery products such as squid and crab meal are also included.

It is generally thought important to ensure that broodstock feeds are formulated with the highest quality ingredients. Our inclusion of farmed polychaete worms was in part due to the desire to have unprocessed, fresh marine ingredients that could be considered biosecure. The use of polychaete worms as a finfish broodstock feed component is a recent development and the opportunity arose to use farmed worms produced at the CCAR for our diet. Apart from broodstock studies in sole (Baynes *et al.*, 1993) there is little published information on their use in broodstock feeds. Significant improvements in shrimp broodstock performance have been attributed to the addition of polychaete worms to feeds (Naessens *et al.*, 1997) and this practice is now widespread. The "polychaete effect" as it is referred to, might be explained by the fact that they are a good source of essential fatty acids or a number of other characteristics yet to be investigated. Given their popularity as bait for angling, it is certain that they are highly attractive and may be

used to enhance palatability, perhaps during the acclimation stage for newly captured broodstocks. However, the worms and certain other components such as the purified fatty acids that were used to trim the relative proportions of DHA, EPA and ARA were very expensive components of the CCAR diet. The cost of the Control diet was approximately $1.10 per Kg as fed (Herring at $0.10 per Kg and squid at $4.66 per Kg). The Commercial diet (Breed–M) cost $5.14 per Kg as fed, while the CCAR diet cost $9.33 per Kg as fed. Additional costs of the diets include labor and equipment. Removing the heads and guts from herring is a labor intensive activity. However, the Control diet required no extra equipment or supplies as did the formulated feeds which require a batch mixer and a sausage maker. The price of the formulated diets includes collagen casings which have a cost of $0.59 per Kg of feed (6.3% of the cost of the CCAR diet). The use of hog intestine may reduce the cost, but may not be digestible by fish. Other primary sources of cost in the CCAR diet (Table 13) are DHAsco® at 43.0% of the cost followed by sandworms at 23.1% of the cost. The use of DHAsco® to increase DHA content was deemed necessary for the CCAR diet to follow recommendations of other researchers. The cost of DHAsco® could be reduced with the use of fish oil with a DHA to EPA ratio greater than 1.5 or at 2.

Table 13. Cost analyses of the CCAR diet. Each ingredient is expressed as percent of the total cost.

Ingredient	% Cost
Herring Meal*	4.51%
Squid Meal*	8.28%
Fish Hydrolysate	5.45%
Crab Meal*	0.31%
Sand worms*	23.10%
Menhaden Oil	0.27%
ArAsco®	4.77%
DHAsco®	42.03%
Wheat Gluten	3.39%
Sodium Phosphate	0.08%
Vitamin E	0.08%
Vitamin C	0.27%
Vitamin Premix	0.28%
Mineral Premix	0.04%
Choline Chloride 100%	0.43%
Soy Lecithin*	0.36%
Ethoxyquin, 66%	0.01%
Collagen Casings	6.32%

* - cost depends on volume

Such oils are mackerel or capelin oil; however neither of these oils was available during the study.

Since the study did not reveal significant benefit from using the CCAR formulation over and above the Commercial feed it was concluded that the CCAR diet was not worth pursuing as a broodstock diet for Atlantic halibut beyond this study. In addition to the cost of the diet. It is not convenient for a farm or research facility to carry an extensive inventory of feed ingredients. One of the main goals of the study was to seek an off the shelf formulated feed with a reliable composition that can be conveniently purchased,

stored, mixed and fed. Following the completion of this study, a second commercially available feed was introduced and that will be compared over the 2009 spawning season and beyond with the diet used in the Commercial treatment.

CONCLUSIONS

This is probably the longest running broodstock nutrition trial of its kind where diet comparisons were maintained for 4 years. In fact the diets were maintained for yet another year beyond the scope of this report. Despite the considerable resources that were committed to this project however, it still remains unclear whether formulated feeds adequately replace wet fish components for broodstock Atlantic halibut. The most important egg quality parameter which was monitored in this study on a regular basis, fertilization rate, was very inconsistent even from the same fish during any given spawning season. The reason for this is almost certainly the timing of egg collection which remains the most challenging aspect of Atlantic halibut broodstock management. While statistically lower fertilization rates were obtained in one of the years in the Commercial group, this is not conclusive. The fact that at least some good quality batches were obtained from fish which have been exclusively fed formulated feeds for a number of years indicates that these formulated feeds have the potential to replace raw fish. Given the biosecurity risks associated with feeding raw fish components to these valuable fish, it is concluded that this goal is still worth pursuing.

Given the high cost of the CCAR diet and the fact that it did not seem to result in improved egg quality or fecundity over and above the Commercial feed, this use of this diet has now been dropped. The Commercial diet used for the entire project, Breed M™ from Inve Aquaculture, is still in use though we are now combining this with another commercially available feed, Vitalis from Skretting. The broodstock fish are now held in much improved facilities with good environmental control. The new hatchery for Atlantic halibut is almost completed and large scale juvenile production is planned for 2010.

REFERENCES

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

Baynes, S.M., Howell, B.R. and Beard, T.W. 1993. A review of egg production by captive sole, *Solea solea* (L.). Aquaculture and Fisheries Management 24, 171-180.

Bell J.G, Farndale BM, Bruce MP, Navas JM, Carrillo M. 1997. Effects of broodstock dietary lipid on fatty acid compositions of egg from sea bass (*Dicentrarchus labrax*). Aquaculture 149: 107-119.

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

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

Blaxter, J.H.S., Danielssen, D., Moksness, E. and Oiestad, V. 1983. Description of the early development of halibut (*Hippoglossus hippoglossus*) and attempts to rear the larvae past first feeding. Marine Biology 73, 99-107.

Bowman, R., E., Stillwell, C., E., Michaels, W., L., Grosslein, M., D., 2000. Food of Northwest Atlantic fishes and two common species of squid. NOAA Technical Memorandum NMFS-NE 155. Woods Hole, MA: U.S. Department of Commerce.

Bromage, N., Bruce, M., Basavaraja, N., Rana, K., Shields, R., Young, C., Dye, J., Smith, P., Gillespie, M. and Gamble, J. 1994. Egg quality determinants in finfish: The role of overripening with special reference to the timing of stripping in the Atlantic halibut (*Hippoglossus hippoglossus*). Journal of the World Aquaculture Society 25, 13-21.

Brown, N. 2002. Flatfish farming systems in the Atlantic Region. Reviews in Fisheries Science. 10, 403-419.

Brown, N., Shields, R.J. and Bromage, N.R. 2006. The influence of water temperature on spawning patterns and egg quality in the Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture. 261, (3), 993-1002

Bruce M.P., Shields R.J., Bell M.V., Bromage N.R., 1993. Lipid class and fatty acid composition of eggs of Atlantic halibut, *Hippoglossus hippoglossus* (L.), in relation to egg quality in captive broodstock. Aquaculture and Fisheries Management 24, 417-422

Collette, B.B and Klein-MacPhee, G. 2002. Fishes of the Gulf of Maine. Smithsonian Institution Press, Washington DC. 748 pp.

Dannevig B.H., Nilsen R., Modahl I., Jankowska M., Taksdal T., Press C. McL., 2000. Isolation in cell culture of nodavirus from farmed Atlantic halibut *Hippoglossus hippoglossus* in Norway. Diseases of Aquatic Organisms 43, 183-189.

Fauvel, C., Omnes, M., Suquet, M., Normant, Y., 1993. Reliable assessment of overripening in turbot (*Scophthalmus maximus*) by a simple pH measurement. Aquaculture 117, 107-113.

Finn, R.N., Fyhn, H.J. and Evjen, M.S. 1991. Respiration and nitrogen metabolism of Atlantic halibut eggs (*Hippoglossus hippoglossus*). Marine Biology 108, 11-19.

Finn, R., N., Østby, G, Norberg, B., Fyhn, H., J., 2002. In vivo oocyte hydration in Atlantic halibut (*Hippoglossus hippoglossus*); proteolytic liberation of free amino acids, and ion transport, are driving forces for osmotic water influx. The Journal of Experimental Biology 205, 211-224.

Folch, J., Less, M. and Sloane Stanley G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry 226, 497-509.

Fyhn, H., J., Finn, R., N., Reith, M., Norberg, B, 1999. Yolk protein hydrolysis and oocyte free amino acids as key features in the adaptive evolution of teleost fishes to seawater. Sarsia 84, 451-456.

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

Harel, M., Tandler, A., Kissil, G.W. and Appelbaum, S.W. 1994. The kinetics of nutrient incorporation into body tissues of gilthead seabream (*Sparus aurata*) females and the subsequent effects on egg composition and egg quality. British Journal of Nutrition 72, 45-58.

Haug, T. 1990. The biology of the Atlantic halibut, *Hippoglossus hippoglossus* L., 1758). Advances in Marine Biology 26, 1-70.

Haug, T. and Gulliksen, B. 1988. Fecundity and oocyte sizes in ovaries of female Atlantic halibut, *Hippoglossus hippoglossus* (L.). Sarsia 73, 259-261.

Holmefjord, I and Lein,I. 1990. Natural spawning of Atlantic halibut (*Hippoglossus hippoglossus* L.). in captivity. International Council for the Exploration of the Seas, C.M. 1990/F:74, 5pp.

Holmefjord, I. 1991. Timing of stripping relative to spawning rhythms of individual females of Atlantic halibut (*Hippoglossus hippoglossus* L.). In: P. Lavens, P. Sorgeloos, E. Jaspers, and F. Ollevier, (Editors). Larvi '91. Fish and Crustacean Larviculture Symposium. European Aquaculture Society Special Publication No. 15, Gent, Belgium, pp. 203-204.

Holmefjord, I., Gulbrandsen, J., Lein, I., Reftsie, T., Lèger, P, Harboe, T., Huse, I., Sorgeloos, P., Bolla, S., Reitan, K.I., Vadstein, O., Øie, G. and Danielsberg, A. 1993. An intensive approach to Atlantic halibut fry production. Journal of the World Aquaculture Society 24, 275-284.

Izquierdo, M., Fernández-Palacios, H. and Tacon, A.G.J. 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquacukture 197, 25-42.

Jackson, T.R., Martin-Robichaud D. J. and Reith, M.E. 2003. Application of DNA markers to the management of Atlantic halibut (*Hippoglossus hippoglossus*) broodstock. Aquaculture 220, 245-259.

Kanwit, J.K. 2007. Tagging results from the 2000-2004 Federal experimental fishery for Atlantic halibut (*Hippoglossus hippoglossus*) in the Eastern Gulf of Maine. Journal of Northwest Atlantic Fisheries Science 38, 37-42.

Kjorsvik, E, Haug, T. and Tjelmsland, J. 1987. Spawning season of the Atlantic halibut (*Hippoglossus hippoglossus*) in northern Norway. Journal du Conseil Internationale pour Exploration de la Mer 43, 285-293.

Kjorsvik, E., Mangor Jensen, A. and Holmefjord, I. 1990. Egg quality in fishes. Advances in Marine Biology 23, 71-113.

Kvenseth, A. M. Pittman, K. and Helvik, J.V., 1996. Eye development in Atlantic halibut (*Hippoglossus hippoglossus*): Differentiation and development of the retina from early yolk sac stages through metamorphosis. Canadian Journal of Fisheries and Aquatic Sciences. 53: 2524-2532.

Lonning, S., Kjorsvik, E., Haug, T. and Gulliksen, B. 1982. The early development of the halibut, *Hippoglossus hippoglossus* (L.), compared with other marine teleosts. Sarsia 67, 85-91.

MacIntyre, A.D. 1953. The food of halibut from north Atlantic fishing grounds. Marine Research 3, 1-20.

Martin-Robichaud, D. J. and Rommens, M. 2001. Assessment of sex and evaluation of ovarian maturation of fish using ultrasonography. Aquaculture Research 32, 113 - 120

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.

Naess, T., Harboe, T., Mangor-Jensen, A., Naas, K. E. and Norberg, 1996. Successful first feeding of Atlantic halibut larvae from photoperiod-manipulated broodstock. The Progressive Fish Culturist 58, 212-214.

Naessens, E., Lavens, P., Gomez, L., Browdy, C. L., McGovern-Hopkins, K., Spencer, A.W., Kawahigashi, D. and Sorgeloos, P. 1997. Maturation performance of *Penaeus vannamei* co-fed *Artemia* biomass preparations. Aquaculture 155, 87-101.

Navas JM, Thrush M, Zanuy S, Ramos J, Bromage N, Carrillo M. 2001. Total lipid in the broodstock diet did not affect fatty acid composition and quality of eggs from sea bass (*Dicentrarchus labrax L.*). Scientia Marina 65(1): 11-19.

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

Naylor, R.L., Goldberg, R.J., Jurgenne, H.P., Kautasky, N., Beveridge, M.C., Clay, J., Folkes, C., Lubchenko, J., Mooney, H. and Troell, M. 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.

Neilson, J.D., Kearney, J.F., Perley, P. and Sampson, H. 1993. Reproductive biology of Atlantic halibut (*Hippglossus hippoglossus*) in Canadian waters. Canadian Journal of Fisheries and Aquatic Sciences 50, 551-563.

Norberg, B., Valkner, V., Huse, J., Karlsen, I. and Lerøy Grung, G. 1991. Ovulatory rhythms and egg viability in the Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 97, 365-371.

Ottesen, O.H. and Strand, H.K. 1996. Growth, development, and skin abnormailities of halibut (*Hippoglossus hippoglossus* L.) juveniles kept on different substrates. Aquaculture 146, 17-25.

Ottesen, O.H., Noga, E.J. and Sanda, W. 2007. Effect of substrate on progression and healing of skin erosions and epidermal papillomas of Atlantic halibut, *Hippoglossus hippoglossus* (L.). Journal of Fish Diseases 30, 43-53.

Patiño, R., Yoshizaki, G., Bolamba, D., Thomas, P., 2003. Role of archidonic acid and protein kinase C during maturation-inducing hormone-dependent meiotic resumption and ovulation in ovarian follicles of Atlantic croaker. Biology of Reproduction 68, 516-523.

Pittman, K., Bergh, O., Opstad, I., Skiftesvik, A.B., Skjoldal, L. and Strand, H. 1990a. Development of eggs and yolk sac larvae of halibut (*Hippoglossus hippoglossus* L.). Journal of Applied Icthyology 6, 142-160.

Pittman, K., Skiftesvik, A.B. and Berg, L. 1990b. Morphological and behavioural development of halibut, *Hippoglossus hippoglossus* (L.) larvae. Journal of Fish Biology 37, 455-472.

Rabben, H., Nilsen, T.O., Huse, I. and Jelmert, A. 1986. Production experiment of halibut fry in large enclosed water columns. Council Meeting of the International Council for the Exploration of the Sea F19, 10 pp.

Rainuzzo, J.R. 1993. Fatty acid and lipid composition of fish eggs and larvae. In: H. Reinertsen, L.A. Dahle, L. Jorgensen and K. Tvinnereim (Editors), Fish Farming Technology. Proceedings of the First International Conference on Fish Farming Technology, Trondheim, Norway. 9-12 August 1993. pp 43-49.

Rainuzzo, J.R., Reitan, K.I. and Joergensen, L. 1992. Comparative study on the fatty acid and lipid composition of four marine fish larvae. Comparative Biochemistry and Physiology 103B (1), 21-26.

Riple, G., L., 2000. Ovarian development and egg viability aspects in turbot (Scophthalmus maximus) and Atlantic halibut (*Hippoglossus hippoglossus* L.). PhD. Thesis. University of Bergen, Bergen, Norway.

Rollefsen, G. 1934. The eggs and larvae of halibut (*Hippoglossus vulgaris*). Det Kongelige Norske Videnskabers Selskab Forhandlinger 7 (7), 20-23.

Shields, R.J., Brown, N.P., Bromage, N.R., 1997. Blastomere morphology as a predictive measure of fish egg viability. Aquaculture 155, 1–12.

Shields, R.J., Davenport, J., Young, C. and Smith, P.L. 1993. Oocyte maturation and ovulation in the Atlantic halibut, *Hippoglossus hippoglossus* (L.), examined using ultrasonography. Aquaculture and Fisheries Management 24, 181-186.

Smith, P., Bromage, N., Shields, R., Ford, L., Gamble, J., Gillespie, M., Dye, J., Young, C. and Bruce, M. 1991. Photoperiod controls spawning time in the Atlantic halibut (*Hippoglossus hippoglossus*, L.). In: A.P. Scott, J.P. Sumpter, D.E. Klime and M.S. Rolfe (Editors), Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish, University of East Anglia, Norwich. Fishsymp 91, Sheffield. p 172

Smith, P.L. 1987. The establishment of a potential broodstock at Ardtoe 1983-1987. Seafish Industry Authority Technical Report 137, 31pp.

Sorbera, L., A., Asturiano, J., F., Carrillo, M., Zanuy, S., 2001. Effects of polyunsaturated fatty acids and prostaglandins on oocyte maturation in a marine teleost, the European sea bass (*Dicentrarchus labrax*). Biology of Reproduction 64, 382-389.

Tandler A, Harel M, Koven WM, Kolkovski S. 1995. Broodstock and larvae nutrition in gilthead seabream *Sparus aurata*- new findings on its mode of involvement in improving growth, survival and swim bladder inflation. Israeli Journal of Aquaculture, Bamidgeh 47 (3-4), 95-111.

Tocher D.R. 2003. Metabolism and Functions of Lipids and Fatty Acids in Teleost Fish. Reviews in Fisheries Science 11(2): 107-184.

Tocher, D.R., Fraser, A.J., Sargent, J.R. and Gamble, J.C. 1985. Fatty acid composition of phospholipids and neutral lipids during embryonic and early larval development in Atlantic herring (*Clupea harengus* L.). Lipids 20 (2), 69-74.

Tveiten H, Jobling M, Andreassen. 2004. Influence of egg lipids and fatty acids on egg viability, and their utilization during embryonic development of spotted wolf-fish, *Anarhichas minor* Olafsen. Aquaculture Research 35:152-161.

Unuma, T., Kondo, S., Tanaka, H., Kagawa, H., Nomura, K., Ohta, O, 2004. Determination of the rates of fertilization, hatching and larval survival in the Japanese eel, *Anguilla japonica*, using tissue culture microplates. Aquaculture 241, 345-356.

Watanabe, T. 1985. Importance of the study of broodstock nutrition for further development of aquaculture. In: C.B. Cowey, A.M. Mackie and S.G. Bell (Editors), Nutrition and Feeding of Fish. Academic Press, London. pp 395-414.

Zar, J.H. 1980. Biostatistical Analysis, 2nd Edition. Prentice Hall International, London, UK. 718 pp.

Zhu P, Parrish CC, and Brown JA. 2003. Lipid and amino acid metabolism during early development of Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture International 11: 43-52.

ACKNOWLEDGEMENTS

This study would not have been possible without the help of the following individuals:

University of Maine: James Cameron, Dr Linda Kling, Stefanie Plaud, Stephen Eddy, Peter Harvey, Scott Ford, Marcela Hincapie, Jaqueline Hunter

DFO Biological Station, St Andrews, New Brunswick, Canada: Debbie Martin-Robichaud

Center for Marine Biotechnology, Maryland: Dr Allen Place