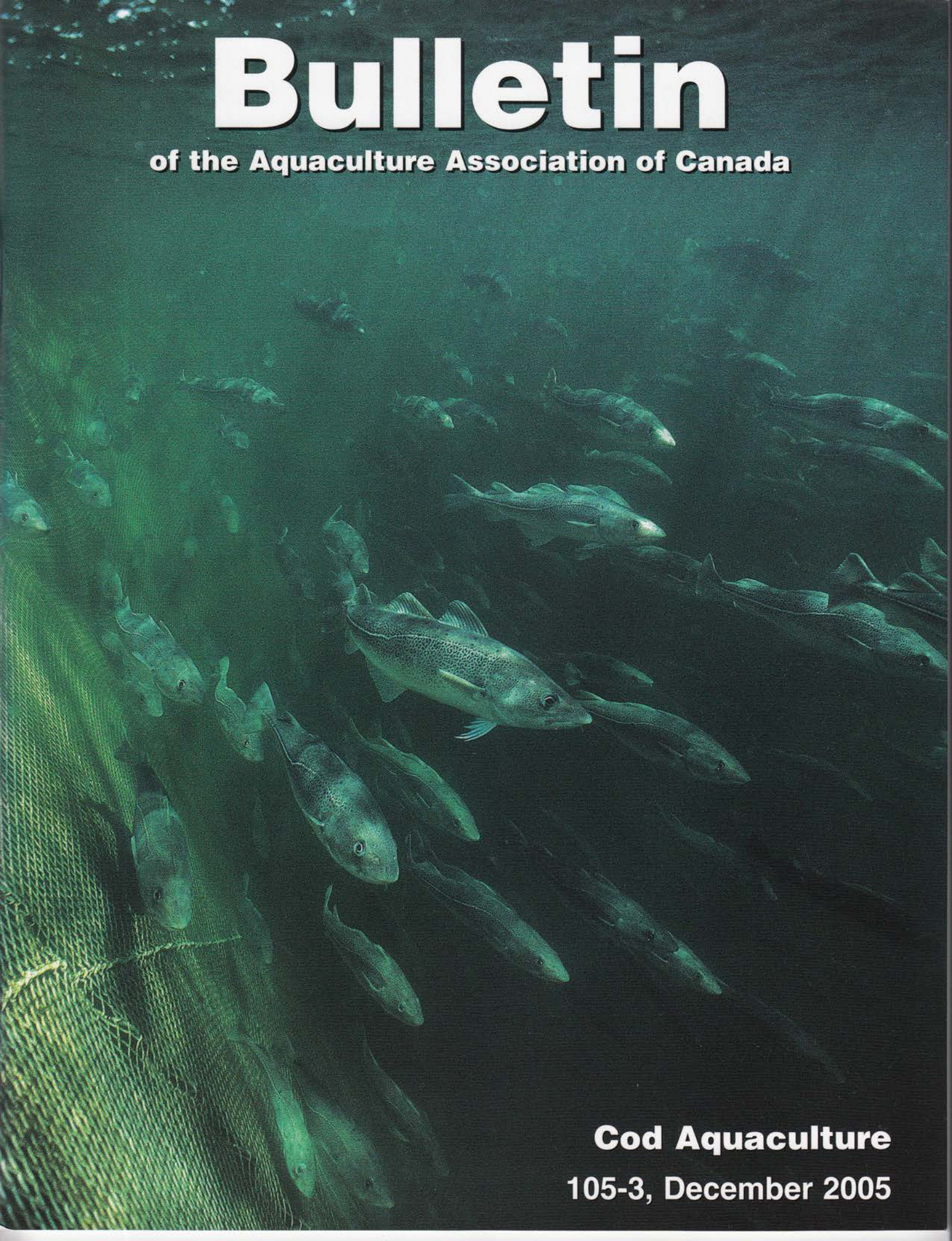
Bulletin

of the Aquaculture Association of Canada



Cod Aquaculture

105-3, December 2005

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décembre 2005 (105-3)

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Front cover: Atlantic cod swim in a cage in Bay Bulls, Newfoundland. Photo by Gilbert van Ryckevorsel.

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Possible Causes of Vertebral Deformity in Atlantic Cod Larvae (*Gadus morhua*)

V. Puvanendran

Velmurugu Puvanendran, Colleen Calder-Crewe, and Joseph A. Brown

Skeletal deformities have become a major concern in several cultured marine finfish species. Environmental conditions and husbandry during embryonic stages could have a negative impact in skeletal formation which could result in skeletal deformities. To test this hypothesis, Atlantic cod eggs were incubated at four different densities: 3, 6, 12, and 48 ml eggs per L of water. Water quality parameters such as oxygen, ammonia, carbon dioxide and temperature were monitored throughout the incubation period. Once hatched, larvae were transferred to 30-L glass aquaria and reared using standard larval rearing protocols. Larval samples were taken at various intervals from hatching to 56 days post-hatch (dph). To document skeletal deformities, we used bone and cartilage staining methods. Incubation density did not have a significant effect on skeletal abnormalities in Atlantic cod larvae. However, our results indicate that during the larval stage husbandry, genetics and/or nutrition could play a major role in vertebral deformities of Atlantic cod.

Introduction

In the past decade, the aquaculture potential for Atlantic cod has been pursued by Norway, Scotland, and more recently by Canada and the United States.[1,2] In order to maximize production, several intensive culture methods have been adopted[1] and attempts are being made to domesticate broodstock.[2] However, despite the improved production levels intensive culture practices provide, they also result in a high incidence of fish health problems.[1,3] One such health problem is the incidence of skeletal abnormalities.[2,3]

Skeletal abnormalities, as with any fish health problem, are an economic issue in aquaculture due to decreased survival, reduced market value of the product, and increased labour costs by manual sorting procedures to remove affected individuals.[4,5] Skeletal abnormalities have been reported in several hatchery-reared species including Atlantic salmon (*Salmo salar*), gilthead seabream (*Sparus aurata*), red seabream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*), sea bass (*Dicentracus labrax*), Senegal sole (*Solea senegalensis*), rainbow trout (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*).[3-8]

In recent years with the expansion finfish aquaculture, deformities in juveniles have become a significant problem with vertebral deformities found in 5-10% of the total aquaculture production.[3] Recent reports from Norway indicate a deformity rate of 60% in the rearing of Atlantic cod.[2] However, reports from Canada and the United States indicate a much lower incidence of juvenile deformity (less than 10%). Two significant differences between the culture methods used in North America and Norway are egg stocking density and larval rearing tempera-

tures.[9] In North America the egg stocking density during incubation is 6ml of eggs per liter of water and the larval rearing temperatures are kept below 12°C.[9] In most Norwegian hatcheries an egg stocking density of 48 ml/L of water is utilised and the larval rearing temperature ranges from 14-16°C.[9] Thus the purpose of this study was to examine the effect of egg density during incubation on the incidence of skeletal abnormalities in Atlantic cod larvae at hatching through larval development (0-56 dph).

Materials and Methods

Egg Incubation

Eggs were collected from photo-manipulated captive Atlantic cod broodstock held at the Aquaculture Research and Development Facility (ARDF), Ocean Sciences Center. The broodstock were held in 20-m^3 tanks with flow-through water and were fed baitfish enriched with vitamins. Eggs were collected through spontaneous spawning. Two and a half litres of eggs were disinfected using Perosan® before transferring to incubators. Eight 18-L fiberglass conical shaped incubators were used in this experiment. Eggs were stocked at 54, 108, 216, and 864 ml (3, 6, 12 and 48 ml L^{-1} respectively) of eggs per incubator, with two replicates per treatment. All incubators were provided with continuous air supply and water flows of 1 L min^{-1} for two low egg densities and 2 L min^{-1} for two high egg densities. The water temperature was maintained between 5 and 6°C, oxygen content and temperature was measured daily and ammonia, nitrate, CO_2 and pH levels were measured weekly. Hatching occurred at approximately 90 degree days (average daily temperature * number of days).

Larval Rearing

Cod larvae were reared according to the protocol described by Brown et al.[1]. Larvae were reared in 30-L aquaria set up in a raceway surrounded by a water bath. Larvae were grouped based on the same four treatments utilized in egg incubation, (54, 108, 216, and 864 ml of eggs per incubator), with three replicates for each treatment. Larvae were stocked at a density of 1500 larvae per tank (50 larvae L^{-1}). Water temperature was maintained between 9.5-12 °C. Light intensity was maintained at 2000 lux with a 24L:0D photoperiod. Initial water flows into the tanks were set at 100 ml min^{-1} and increased to 250ml min^{-1} as the larvae grew. Larvae were fed rotifers until 42 dph. After 42 dph the larvae were fed *Artemia*. Prey levels were maintained at 4000 prey L^{-1} with the larvae fed 3 times per day: 9am, 3pm and 9pm. Before each feeding prey numbers were enumerated and adjusted accordingly. Daily temperature and oxygen measurements were taken and ammonia, nitrate, CO_2, and pH levels were measured on a weekly basis.

Sampling, Histological Procedures, Data Collection, and Data Analysis

Larvae were sampled for both skeletal analysis and growth measurements. Ten larvae were sampled from each tank and each incubator at hatch (0dph). For growth analysis, bi-weekly five larvae per tank were sampled at 0, 14, 28, 42, and 56 dph. Larval samples for skeletal analyses were taken at 0, 14, and 42 dph (10 per tank) and at 56 dph (5 per tank). All samples were preserved in buffered formalin until further analysis.

Skeletal development was examined by staining the cartilaginous structures through a previously described staining protocol.[10] Briefly this method involved

staining the calcified cartilage with Alcian Blue for 24-48 hours, buffering in borax for 24 hours, clearing of the tissue with the enzyme trypsin for 8 to 48 hours. For the 56 dph samples bone staining was performed along with the cartilage by buffering the samples in a series of KOH dilutions for 24 hours and then staining with Alizarin red for 24 hours.[10] Post staining all samples were put through a series of glycerol dilutions (40%, 70%, 100%), before being preserved in 100% glycerol.[10]

For growth measurements, larvae were photographed using a Pixera digital camera and later analyzed using the Matrox inspector® image analysis software. Skeletal abnormalities of stained larval samples were assessed post staining using an Olympus stereo microscope with a Pixera digital camera pack attachment and an Optem macro video zoom lens. Skeletal abnormalities were classified based on density, age class, type of abnormality, and the location of the abnormality on the skeleton. The vertebral column was subdivided into four distinct categories; cephalic, pre-haemal, haemal and caudal region.[11]

One-way ANOVA analysis was performed on observed skeletal abnormalities at hatch and for each age class sampled with a significant value of $P<0.05$.

Results and Discussion

Water quality parameters were satisfactory throughout the experiment both during egg incubation and larval rearing. Oxygen ranged from 100-105% and 98-109% and temperature ranged from 5.7-5.9°C and 10.0-10.9°C during egg and larval stages respectively. CO_2, ammonia, and nitrite levels were negligible throughout the experiment..

Vertebral deformity in newly hatched cod larvae ranged from 18-25% (Fig. 1). However, stocking density during incubation did not affect the vertebral deformity of newly hatch cod larvae ($F_{3,77}=0.335$; $p=0.8$). Few other studies indicated that hypoxia and higher temperature during egg incubation may cause higher incidence of deformities in fish and shellfish.[8,12] However, the water quality parameters during the incubation, including oxygen levels and temperature, were

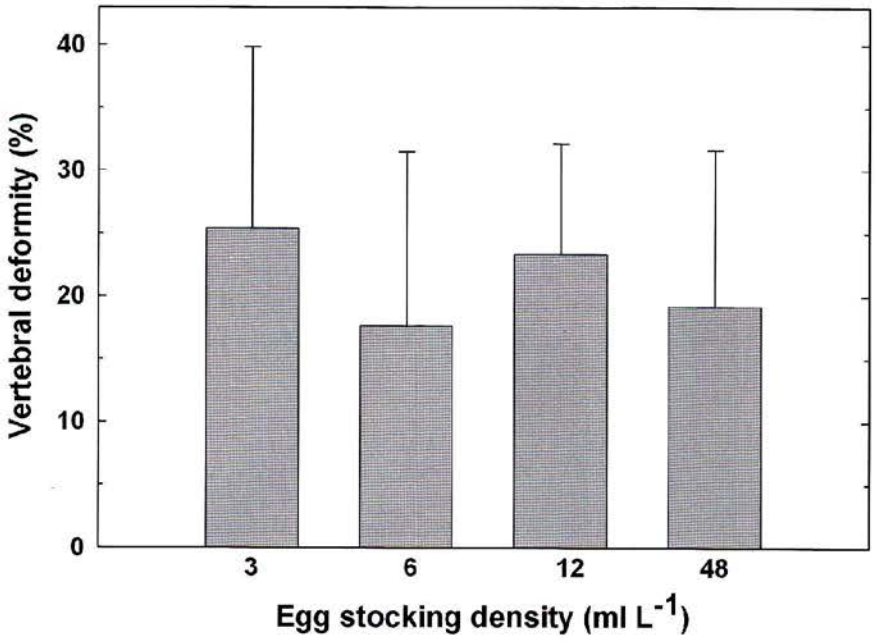

Figure 1
Incidence of vertebral deformity (%) in newly hatched Atlantic cod larvae incubated at different egg stocking densities. Values are ± SD.

within the acceptable levels and therefore may not be the reason for the relatively higher vertebral deformity at hatch observed in our experiment. Kolstad et al.[13] found differences in the incidence of spinal deformity among Atlantic cod from different geographic location and also within a population among different families which indicates that spinal deformity could be related to genetics.

During the larval stage, incidence of vertebral deformity initially decreased in all treatments until the larvae were 42 dph (Fig. 2A), however, no significant difference in incidence of vertebral deformity was found between any of the treatments ($F_{3, 290}$ =0.035; p=0.733). At 56 dph, incidence of vertebral deformity increased from 42 dph in all treatments except in 48 ml egg L^{-1} treatment, however, this increase was not significant (p=0.862; 0.848; 1.0 for 3 ml egg L^{-1}; 6 ml egg L^{-1}; 12 ml egg L^{-1} respectively). Our results indicate that the egg stocking density may not be the cause for the differences deformity levels that has been reported

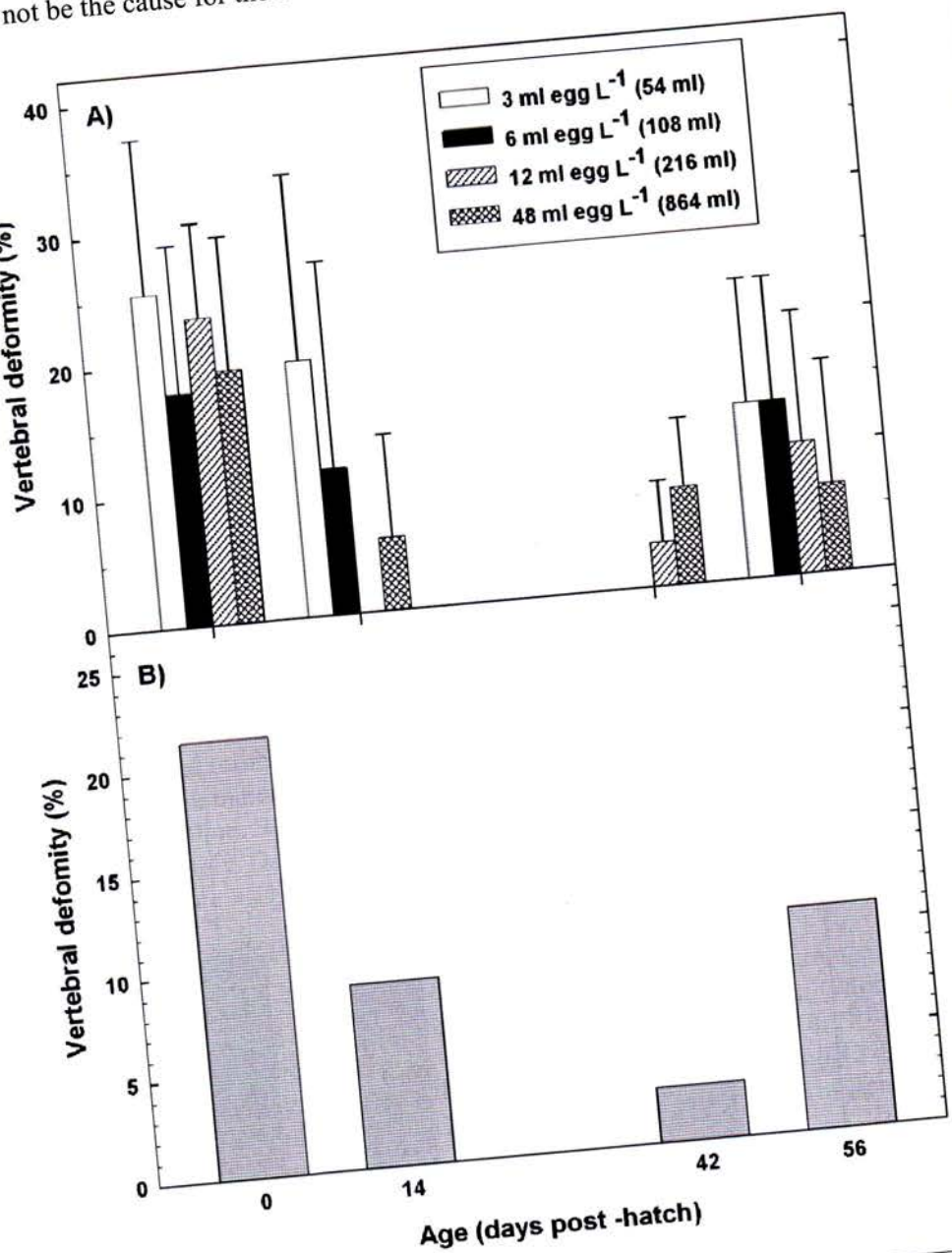

Figure 2
A) Incidence of vertebral deformity (%) in Atlantic cod larvae from 0 to 56 dph that were incubated at four different egg stocking densities. and B) Combined incidence of vertebral deformity (%) in Atlantic cod larvae at different larval stages. Values are ± SD.

between cod hatcheries in Norway and North America. As discussed before, the higher incidence of spinal deformity at hatch could be related to genetics or even to the husbandry or nutrition of the broodstock or to the possible inbreeding due to relatedness among the group of captive broodstock.

Since no significant differences were found, all the deformity data within an age were combined (Fig. 2B). Combined data showed that age had significant effect on the incidence of deformity in larval cod ($F_{3, 77}$=0.314; p=0.009). Larvae at 42 dph had significantly lower incidence of vertebral deformity compared to larvae at 0 dph (p = 0.005), however the increase at 56 dph was not significantly different fro 42 dph (p = 0.469). The pattern of incidence of deformity shown in Fig. 2B was interesting as it showed a decline in spinal deformity from hatch to 42 dph and an increase at 56 dph. Although the spinal deformity was high as 21% at hatch, it was reduced to 8% at the end of yolk-sac period (14 dph). Langan[14] indicated that tadpole larvae with deformities may not swim and forage efficiently compared to normal tadpole. Thus, it is possible, in our experiment, that most cod larvae with spinal deformity could not have foraged as efficient as larvae without deformity due to impaired swimming which might have lead to a selective mortality of deformed larvae at the end yolk-sac period. Further reduction of incidence of vertebral deformity at 42 dph indicates a possible selective mortality of deformed larvae, however, an increase in deformity at 56 dph could indicate some other factor may have involved. In our experiment, we started feeding the larvae with *Artemia* at 42 dph and continued until 56 dph. Shields et al.[15] suggested that cod larvae fed with *Artemia* before metamorphosis could experience an over-inflation of swimbladder consequently which may cause some skeletal deformity. Further, nutritional deficiency especially Vitamin A and C, in *Artemia* could also cause skeletal deformity in fish larvae.[6,16] We did not evaluate the swimbladder inflation or nutritional quality of the *Artemia* during this experiment. However, results from other studies indicate that prey type and/or nutrition may play a role in the increase in spinal deformity incidence observed in our experiment.

Conclusion

Our results indicated that incubation density may not be the cause for skeletal abnormalities observed in larval cod in this study. However, variable incidences of vertebral deformity at different larval stages indicate that genetics, environment, prey type and/or nutrition could be involved in the skeletal abnormalities observed during larval stages in this study.

Acknowledgements

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Influence of Lighting Regime on the Sexual Maturation of Cage-Cultured Atlantic Cod (*Gadus morhua* L.)

Anne Kellett, Velmurugu Puvanendran, Atef Mansour, and Cyr Couturier

Anne Kellett

The early maturation of cage cultured Atlantic cod (*Gadus morhua*, L.) is a concern in the development of commercial aquaculture. Photoperiod manipulation has been shown to have significant effects on delaying maturation in cod in tanks, however the results in sea cages are not clear. We are investigating the effects of continuous light on early maturation of Atlantic cod in sea cages on the south coast of Newfoundland. Two experimental groups of 2000 cod, each in two cages, were exposed to continuous light (LL; 24 hrs a day) from two 900 lux submerged bulbs. Control fish in two other cages received only the natural photoperiod (NL). This experiment was started at the summer solstice when the cod juveniles were 16 months old. Samples of 15-30 fish per cage were taken 5 times between June 2005 and May 2006. Experimental fish exposed to LL had lower gonadosomatic and higher hepatosomatic indices which could be an indication of a difference in their level of maturity. Growth was significantly different for females ($p=0.027$), but not for males ($p=0.474$), between LL and NL treatments for May 2006 sample. A swimbladder abnormality found predominantly in LL fish may explain a lower overall growth rate. Our results indicate that supplying continuous light could be effective in delaying early maturation in Atlantic cod in sea cages.

Introduction

A significant obstacle to commercial scale cod production is early maturation of the fish.[1] Farmed Atlantic cod (*Gadus morhua*) usually spawn for the first time around two years of age.[2] Early maturation causes the fish to use energy stores to develop their gonads instead of muscle. This increases the length of culture time and the cost of production.[3] Thus, the maturation of cod needs to be delayed to develop viable cod aquaculture.

Norberg et al.[4] cite photoperiod as an environmental cue that has been shown to play an important role in the onset of reproduction. The technique of continuous light has successfully changed photoperiod to control spawning time in broodstock.[4] This strategy has been expanded to delay maturation in cultured fish. Periods of continuous light have successfully prevented early maturation of cod in indoor tanks.[3]

Translating this knowledge to culture in sea cages has presented some complications. The level of light needed and the diel rhythms created by natural photoperiod are not well understood. Different theories regarding light intensity,

the placement of the lights in or above the cage, the timing of light exposure, and the amount of natural light to be allowed into the cage, have all been investigated as strategies of photoperiod manipulation. Moreover, the differences in latitude resulting in different day lengths, biological differences between species and genetic factors within species also need consideration. Thus, we conducted an experiment to investigate the effect of continuous light as a strategy to prevent early maturation of cod raised in sea cages.

Materials and Methods

Cod juveniles were reared at the Aquaculture and Research Development Facility (ARDF) of Memorial University of Newfoundland and transferred to sea cages located in McGrath's Cove, Belle Bay, Newfoundland at approximately 5 g in size. Just after the summer solstice in 2005, eight thousand Atlantic cod juveniles were placed in four cages each measuring 7m*7m*15m (2000 juveniles per cage). Two of the cages received only natural light (NL) while the other two received continuous light (LL) supplemented through two submerged bulbs at 2 m and 8 m. The light intensity was 900 lux. All cages were equipped with sun shades and reared under the same conditions besides light intensity and photoperiod. Juveniles were 16 months old and weighed an average of 230 g at the start of the experiment. Lights were turned off on the experimental cages on November 20, 2005, due to technical difficulties.

Sampling, data collection, and analysis

The initial sample was taken on June 29 and 30, 2005. Ten fish from each cage were sampled to provide baseline data prior to experimental conditions. During the experiment, fifteen fish from each cage (30 per treatment) were sampled on July 19 and 20, 2005 (two weeks after lights were turned on in the experimental cages); September 24, 2005; November 22 and 24, 2005; May 9 and 10, 2006. During each sampling date, individual fish length & weight and gonad & liver weight were recorded and samples of muscle, skin, liver and gonad were preserved in 10% formalin for histology. Blood samples were also collected from each fish in heparinised vacutainers for hormone analysis and frozen at -80°C. Gonad samples were dehydrated, embedded, sectioned, and stained with H&E. Stage of maturity for both male and female samples will be determined for each sampling date.

From the data collected, gonadosomatic Index (GSI) was calculated as:

$$GSI = 100\, WG * W^{-1} \quad (1)$$

Where WG and W represent gonad weight (g) and wet body weight (g), respectively.

The HSI is similarly calculated as:

$$HSI = 100\, WL * W^{-1} \quad (2)$$

Where WL and W represent liver weight (g) and wet body weight (g), respectively.

The initial sample was not used in the analysis as it was a common sample. The effects of photoperiod on growth, GSI and HSI were analysed using one-way ANOVA and the significance level (α) was set at 0.05. When a significant differ-

ence was found, a Bonferroni post-hoc test was carried out to determine which mean differed.

Results and Discussion

Overall, our results showed that the extra lighting had a significant effect on GSI ($F_{1,259}$ =27.8; p=0.000), however, it had no significant effect on the body weight ($F_{1,259}$ =0.131; p=0.718) and a marginal effect on HSI ($F_{1,259}$ =2.92; p=0.089) of Atlantic cod.

Atlantic cod in LL and NL cages averaged 988 g and 952 g (wet weight) respectively, in May 2006. However, LL female fish weighed 1084 g while in NL treatment the female weighed 979 g. Conversely, males in LL weighed 891 g and 925 g in NL (Fig. 1). When comparing the weight of the females between sampling dates from both treatments, fish from LL were significantly heavier compared to fish from NL in May 2006 (p=0.027), while no significant difference was found between the males during the same period (p=0.474). Similar studies from Norway indicated that both male and female Atlantic cod from LL have gained significant weight compared to fish from NL.[3,5]

Female cod from LL treatment had significantly lower GSI than females from NL treatments in May 2006 sample (p=0.000) and no significant differences were found on any of the previous samples. Females from LL treatment had a lower GSI of 6.7 while female cod from NL had 12.6 in May 2006 sample. However, the males from LL had significantly lower GSI compared to male cod from NL in November 2005 and May 2006 samples (p=0.000 and 0.006, respectively). The GSI of males from LL treatment was 0.65 in November 2005 and 7.25 in May 2006 while males from NL treatments had GSI values of 1.85 in November 2005 and 8.16 in May 2006 (Fig. 2A). Our results indicate a differential gonad development between males and females from the LL treatment. Taranger et al.[5] indicated that a GSI value above 8 in juvenile cod during spawning season could be used as an indicator that these fish ready to spawn. In our experiment, GSI was averaging below 8 for both males and females from LL treatment. In comparison, the GSI of juveniles from NL treatments were averag-

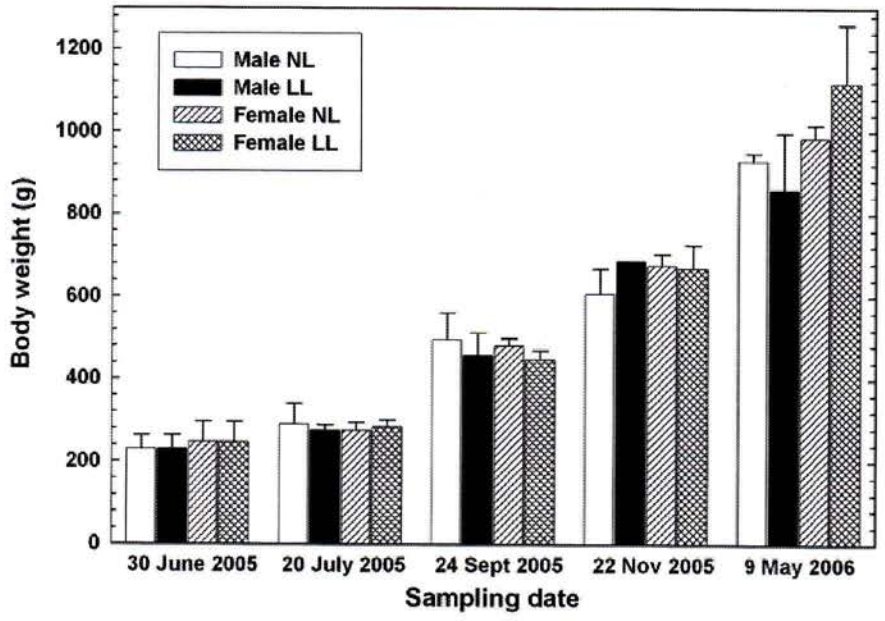

Figure 1
Body weight (g) of male and female Atlantic cod from continuous light and natural light treatments at different sampling dates. Values are ±SD. N=30 per treatment.

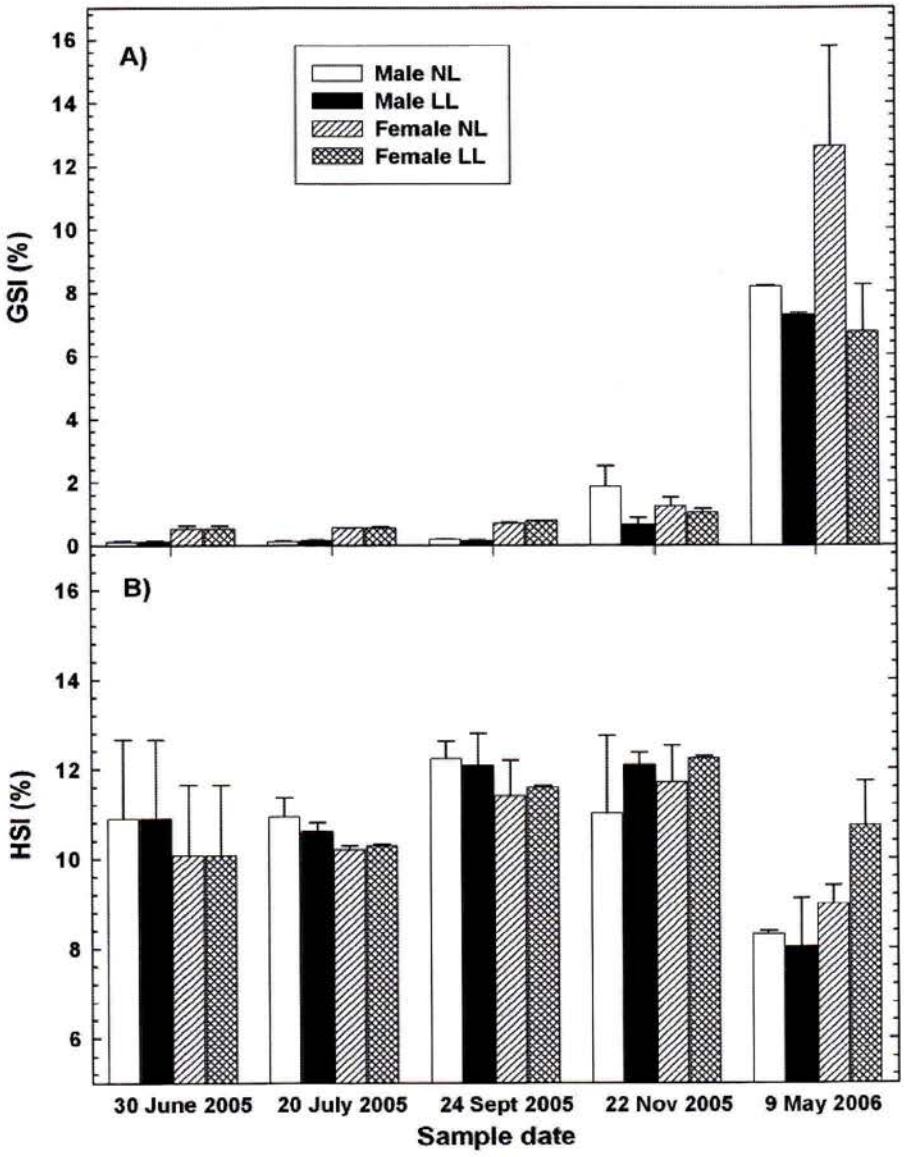

Figure 2
A) gonadosomatic index (%) and B) hepatosomatic index (%) of male and female Atlantic cod from continuous light and natural light treatments at different sampling dates. Values are ±SD. N=30 per treatment.

ing above 8. This indicates that a higher number of the males and females from NL treatments could have been ready to spawn compared to those from LL treatment groups. Further analysis of gonads through histology is expected to confirm these results.

Although no significant differences were found in HSI of female cod between LL and NL treatments from the first four samples (up to November 2005), female cod from LL treatments had significantly higher HSI values than female cod from NL treatment in the final sample ($p=0.012$). The HSI was not significantly different among males from LL and NL treatments. Interestingly, both male and female cod from NL and LL had significantly lower HSI values ($F_{1,131}=5.367$; $p=0.022$; $F_{1,131}=34.38$; $p=0.000$; $F_{1,131}=16.82$; $p=0.000$; $F_{1,131}=12.87$; $p=0.000$ between LL female, LL male, NL female, and NL male respectively) in May 2006 compared to November 2005 (Fig. 2B). Taranger et al.[5] reported that the HSI increased in female and males from LL, but a decreased in males and females from NL. Nevertheless, a decreasing trend seen in our experiment in the last sample compared to

the previous sample may be an indication of energy reserves from the liver being converted for gonad development. However, a greater reduction in HSI in NL treatments indicates that the fish not receiving additional light appear to use more of its liver energy reserves than those who received continuous photoperiod. Thus, a significantly higher weight and HSI values and significantly lower GSI values in females from LL treatment compared to NL treatment indicate that continuous light in the sea cages appears to have delayed the maturation in female Atlantic cod.

The insignificant differences in body weight of males between the treatments at the end, and even a small significant difference among females, are interesting given that they had highly significant differences in GSI values. If the energy was not invested in gonad development, then it should have been invested in growth (muscle). During our last sampling, we observed that 97% of the fish from LL had over-inflated swim bladders while only 3.3% of fish from NL had this abnormality. The fish from LL may have spent most of their energy in counter balancing the buoyancy created by the over-inflated swim bladders rather than investing it into growth. This could account for similar growth rates in males from both the LL and NL groups.

Conclusion

The results thus far analysed indicate that exposure of 16-month-old Atlantic cod in sea cages to continuous light (LL) from midsummer to late fall (4 months) successfully delayed maturation in cod beyond spring spawning season. GSI values were significantly lower in the continuous light treatment, however differences in growth were smaller than expected. Female cod were significantly larger when reared under continuous light, whereas growth in male cod was similar between LL and NL groups. The abnormal swim bladder inflation observed in fish from continuous light cages could have had a negative impact on growth. Further investigation is needed to determine the cause of swim bladder abnormalities in Atlantic cod.

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Lipids and Fatty Acids as Indicators of Egg and Larval Viability in Atlantic Cod (*Gadus morhua*)

Marsha Clarke

M.J. Clarke, C.C. Parrish, and R.W. Penney

An understanding of the factors affecting egg and larval quality can serve as a means to separate poor eggs from high-success eggs, thus allowing producers to select viable eggs that will result in maximum rates of fertilization, hatch, and larval survival. Biochemical composition is examined as an indicator of egg and larval viability in cod, correlating fertilization rates, hatch rates, and survival with profiles of lipids and fatty acids. Batches of eggs spawned from cultured cod broodstock at the Ocean Sciences Centre were used, with fertilization success ranging from 45-99%, and hatching success ranging from 4.5-96%. Samples were taken at six stages from spawn to yolk-sac absorption (10 dph). Lipid analysis was carried out using an Iatroscan Mark V TLC/FID analyzer. The fatty acid composition of the sample was analyzed by Hewlett-Packard gas chromatograph with a flame ionization detector. Preliminary results show no significant differences between batches. However, examination of blastomere morphology shows that symmetry correlates with egg success and may be useful as an indicator.

Introduction

With the decline of wild stocks, there has been a rising interest in the cultivation of Atlantic cod to meet international market demands. In the last year there have been both private and government investments in cod aquaculture in Newfoundland. In order to make this industry a success, it is necessary that intensive research go into developing protocols for the rearing of these fish at all stages of their life history.

Research has been successful in developing effective protocols to deal with environmental factors such as temperature, lighting regimes, salinity levels, and nutrition. However, further research is always necessary so that the growth and quality of stocks can be improved. Of particular interest is the egg and larval stage of cultured cod. The growth and survival of fish in early life stages affects the recruitment and final product.[1] By developing methods to increase growth and survival rates at this stage, total production can be maximized.

An understanding of the factors affecting egg and larval quality can serve as a means to separate poor eggs from high-success eggs, thus allowing producers to select viable eggs that will result in maximum rates of fertilization, hatch, and larval survival. The viability of fish eggs – the success of fertilization and hatch – has been found to be dependent on a number of factors. These include genetic, environmental, and biochemical factors.

The present study examines batches of eggs spawned from cultured cod broodstock at the OSC facilities, from spawning to first-feeding. The objective is

to evaluate use of biochemical factors as indicators of egg and larval viability in cod, correlating fertilization rates, hatch rates, and yolk-sac larval survival with profiles of lipids and fatty acids. We are establishing the particular lipid compounds with the most influence on egg and larval viability by correlating their levels with fertilization, hatch, and survival rates in each group. We are focusing particularly on phospholipids and triaclyglycerols (TAG) and their acyl moieties, the fatty acids – especially DHA and EPA – and as well as their ratios.

With regard to indicators of egg and larval viability, there has been limited research. There exists a need for specific indicators that can help predict the success (or quality) of spawns in culture. With a controlled environment and selected stock being a fixed variable it is likely that the best indicator is biochemical – specifically, lipid classes, and fatty acid content.

From egg to first-feeding larval stage, cod depend on an endogenous supply of nutrients. These come from a supply of fatty acids (FAs) and lipids within the egg and yolk. Lipid profiling has shown that there is a relation between the levels of these lipids and FAs and the success of the eggs and larvae. Of particular importance are the polar phospholipids (PL) phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and neutral lipid triacylglycerol (TAG), which serve as the main sources of stored energy in eggs.[2] Phospholipids are also important in the formation of cells and tissues.[3] Of great importance are the ω3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosoaexaenoic acid (DHA), which are required at some level by most fish species.[2] Studies have shown that by profiling these in early life stages of marine fish, one may be able to predict the nutrient requirements for larval diets.[4] It has also been demonstrated that there is a relation between the levels of these lipids and FAs with fertilization success, hatch rate, and mortality of the larvae.[5,6]

Sufficient levels of these lipids and FAs are no doubt needed to support a developing embryo and the newly hatched larvae. However, research has shown that it is not only the base level of these, but also the relations between them. It has been found that ratios of DHA:EPA affect hatching, growth, and neural development of both the common and spotted wolfish,[5,6] yellowtail flounder,[7] and cod.[2] These studies have all found that DHA:EPA has a positive correlation with growth, survival and neural function. There are a variety of reasons that may explain this, including the fact that there is competition between DHA and EPA for some enzymes, in order to esterify FAs into phospholipids, and also that there are high levels of DHA found in neural membranes.[7] Thus a low DHA:EPA may compromise neural development. Rainuzzo et al.[8] state that high amounts of EPA in relation to DHA can create an imbalance in the structure of PL. Pickova et al.[2] found that the arachidonic acid (AA) content of the PL fraction also influences egg viability and symmetry in cod, and is involved in prostaglandin formation. AA and EPA are both used as a substrate for eicosanoids.[7]

By being able to sort eggs as being poor eggs or successful eggs and analyzing lipid and fatty acid levels, the above factors may be correlated with the viability of eggs and larvae in culture, thus being useful as indicators of their success. This is the purpose of this study. Similar studies have been done with sea bass,[4] common wolffish,[6] and Atlantic halibut.[9,10]

Nocillado et al.[4] found by sorting eggs into two groups (zero fertilization vs. fertilization and hatch) there were differences in total lipids, saturated fatty acids, EPA, and DHA. Their results show that egg components may serve as quality measures in spawns of sea bass. Pickova et al.[2] suggest that the DHA:EPA ratio serves as an important factor influencing egg viability in cod. Halfyard and Parrish[6]

Group	Fertilization (%)	Symmetry (%)	Uniformity (%)	Adhesions (%)	Margins (%)	Clarity (%)	Cell number (%)	Degree days to hatch (%)	Hatch rate (%)
1	97	97	92	99	89	95	100	82.6	88
2	97	94	96	99	88	100	99	88.4	91
3	66	88	92	93	98	99	94	90.3	40
4	52	80	84	94	74	100	90	100.0	23
5	69	90	95	99	96	97	99	91.8	66

Table 1
Fertilization success and blastomere morphology. Cell symmetry correlates with fertilization success ($r = 0.94$, $p < 0.02$)

found that in both eggs and larvae, the total and specific fatty acid and lipid class content (DHA, EPA, DHA:EPA, TAG, and PL) were positively correlated with survival and growth in the common wolfish, as well as the levels of taurine.

Materials and Methods

Eggs were collected from the cod broodstock in the Aquaculture Research and Development Facility at the Ocean Sciences Center, Logy Bay, Newfoundland. The eggs were reared in 300-L flow-through incubators at 6°C, at a density of approximately 50 eggs/L. Eggs were sampled immediately after spawning, at fertilization, half-way to hatch, at hatch, yolk-sac stage, and post-yolk-sac absorption stage. The batches were separated into three sampling groups (tanks): high fertilization success (>95% of eggs fertilized), medium fertlization success (~85%) and low fertilization success (40%-50%). Each group was sampled in triplicate. Triplicate samples were taken from each tank for each sampling stage, and wet and dry weights taken. Unfertilized eggs were collected along with fertilized eggs at Day 0, and analyses run on these as well. The tanks were monitored daily for mortalities and for hatched larvae.

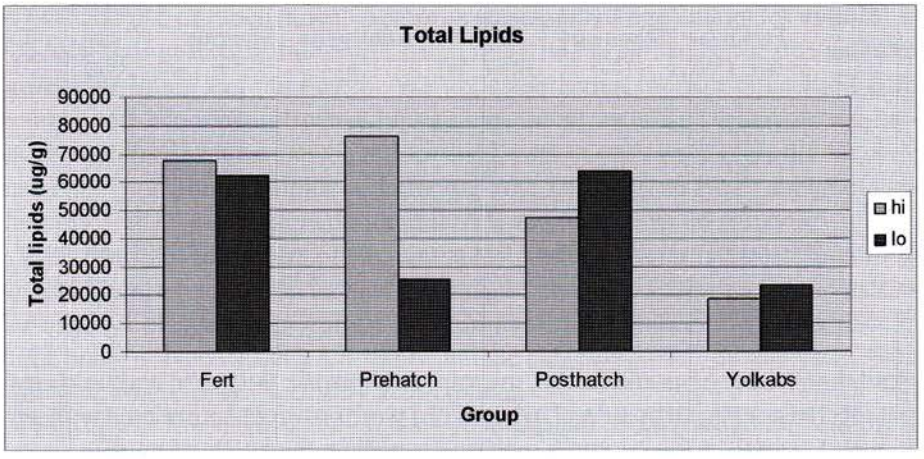
Egg and larval samples were rinsed and dried, and then placed in 10-mL vials with 2 mL of $CHCl_3$. Samples were then be filled with N_2 (g), sealed with Teflon® tape, and stored at -20°C until lipid and fatty acid analysis took place. Lipids were extracted from the samples using a modified Folch method. The lipid extract was separated into classes using a silica gel-coated Chromarods-SIII in four solvent systems, and lipid analysis was carried out using an Iatroscan Mark V TLC/FID analyzer.

The fatty acid component of the sample was analyzed using a Hewlett Packard 6890 Series II gas chromatograph with a flame ionization detector. Fatty acid methyl ester derivatives were made using hexane and BF_3/CH_3OH.

Samples were sorted according to fertilized vs. unfertilized eggs, hatching success, and mortality vs. survival to see if there are statistical relationships between lipid and FA profiles with the egg viability, looking both at each individual stage, and at the changes over time.

Results

The following are preliminary results, using a subset of five batches of eggs from the total twelve. The batches are divided into two groups based on fertilization success – the first group with high success (>80%) and the second with fertilization success <70%.



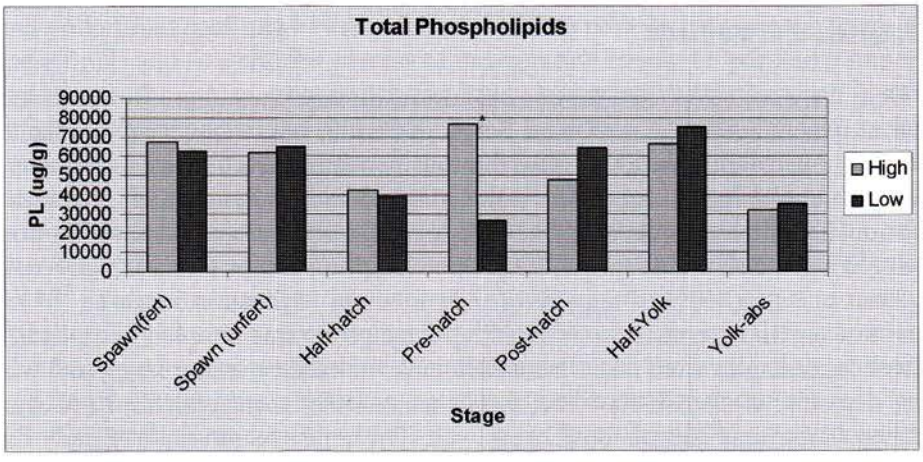


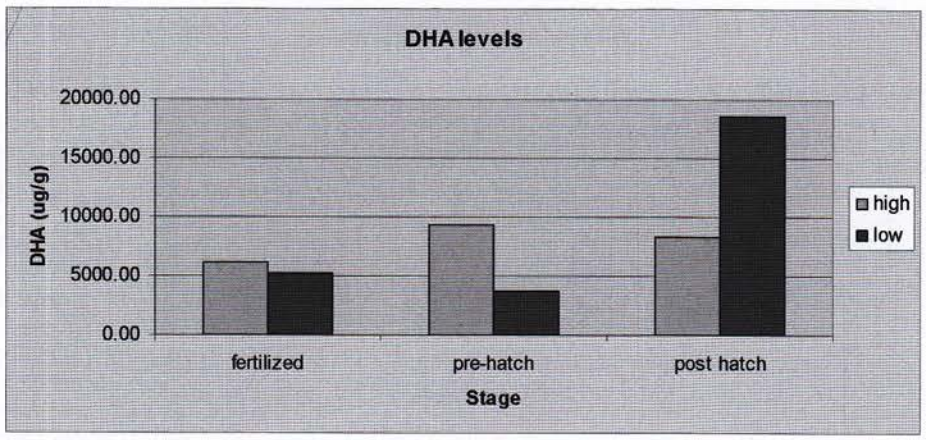
Figure 1
Comparison of total lipids (µg/g) and total phospholipids (µg/g) in eggs and larvae, between groups of high and low fertilization success. No significant differences appear at any stage from Day 0 to yolk sac absorption. The * indicates where differences between the groups are significant.

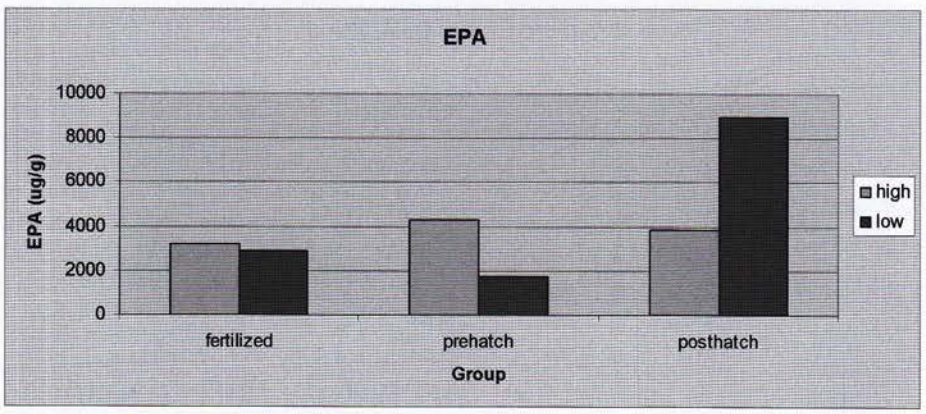
Discussion

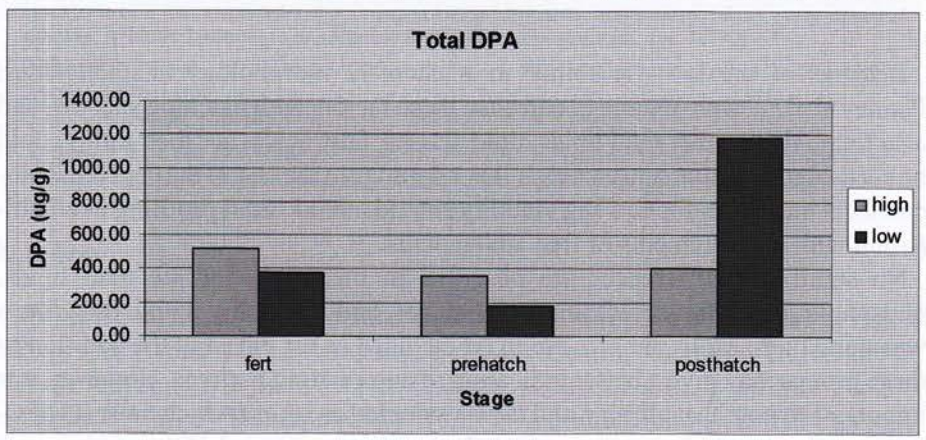
The cell morphology shown in Table 1 indicates a strong correlation between cell symmetry and fertilization ($r = 0.94$, $p < 0.02$). While no patterns exist between fertilization success and other cell morphologies, this correlation indicates that symmetry may be an effective indicator of success at spawn.

Few differences were seen between high and low success groups in the level of total lipids and phospholipids (Figure 1) or fatty acids (Figure 2), and no significant difference seen between the groups of fertilized and unfertilized eggs from the same batches. In Figure 3 there is no obvious pattern shown in the DHA or EPA levels of fertilized and unfertilized eggs.

A recent paper published by Penney et al.[11] looked at the morphology and the lipid biochemistry of Atlantic cod eggs. This paper compared eggs from three different groupings (according to broodstock). Factors measured included egg size and dry weights, as well as blastomere morphology, total lipids and lipid classes, and fatty acids and ratios. Similar results to those reported in Table 1 were found, in that fertilization rates were generally high (averaging 97.8%, 98.7%, and 93.7% for the three groups), and morphology characteristics averaged above 90% for all groups. Also, the mean hatching rates were relatively high (>75%), with individual hatch rates ranging from 31.0% to 98.4%. This is similar to the data in Table 1, where for the five reported batches the hatching success ranged from 23.0% to 91.3%.







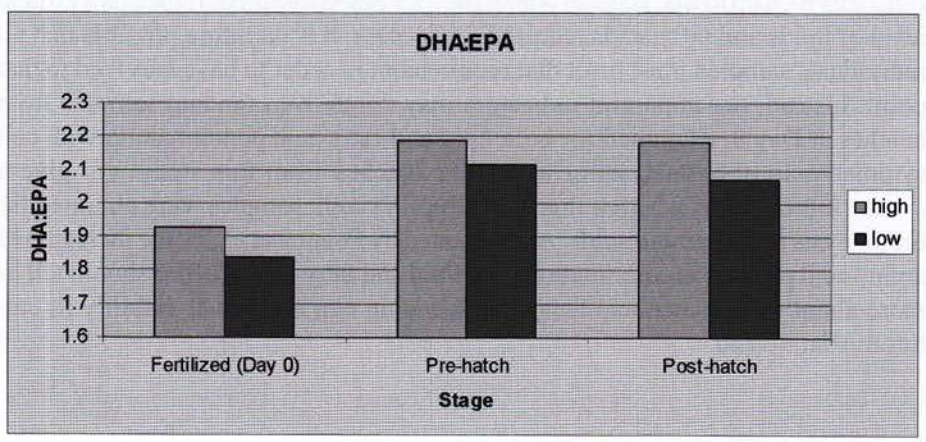
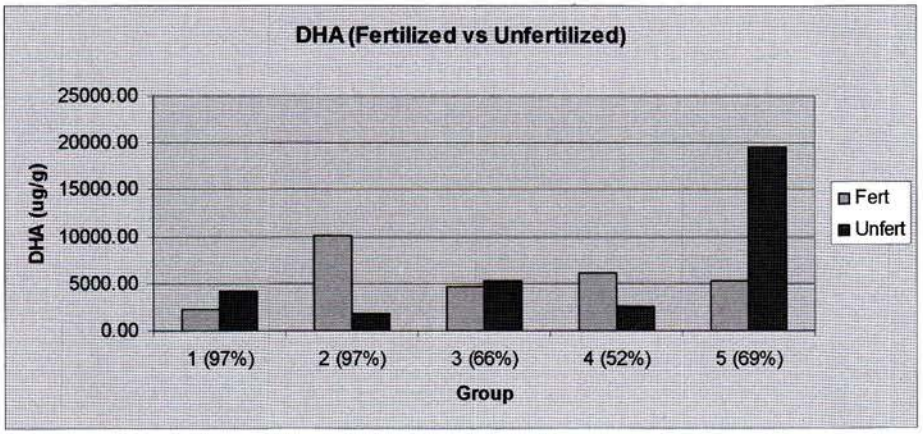
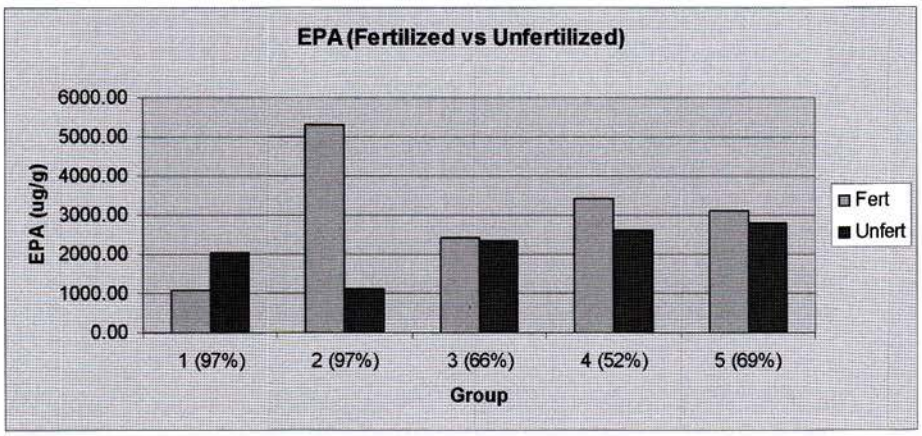


Figure 2
Comparison of DHA, EPA, and DHA levels (µg/g) and DHA:EPA ratios between groups of high and low fertilization success. Differences not significant.

Figure 3
Levels of DHA (µg/g) and EPA (µg/g) in fertilized and unfertilized eggs from the same egg batch. Differences were not significant.





Because of the low variability in blastomere morphology characteristics, Penney et al.[11] were unable to correlate these factors with hatching success, and also found that neither total lipids, lipid classes, fatty acids, nor their ratios could be correlated with hatching success. It is suggested at the end of the paper that biochemical indicators for cod egg viability should include lipid profiling in combination with measurements of free amino acids.

No significant differences are observed in this study for the total lipids, phospholipids, or fatty acids amongst batches, and variability within the groups was quite high. More samples are to be included in the data and the addition of these groups may give a better indication of any existing patterns. However, it is important to note that all eggs and larvae sampled are those which have successfully survived up to that stage in which they are being sampled. If there is a critical level of lipids and fatty acids required for success, it is therefore possible that all individuals sampled must meet that requirement in order to have survived to that point. Instead, the important indicators may come from random samples from batches at Day 0, examined for both lipid and fatty acid content along with cell symmetry. Further analysis of all twelve batches from this study will give a clearer indication of whether this holds true. As well, samples were obtained for the analysis of free amino acids. As suggested by Penney et al.,[11] the inclusion of amino acid profiles in combination with lipid and fatty acid profiles should tell us more about the biochemical factors influencing egg and larval viability in cultured Atlantic cod.

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Sharen Bowman

Generation of Genomics Resources for Atlantic Cod (*Gadus morhua*): Progress and Plans

Sharen Bowman, Brent Higgins, Cynthia Stone, Cathy Kozera, Bruce A. Curtis, Jillian Tarrant Bussey, Jennifer Kimball, Jane Symonds, and Stewart C. Johnson

Atlantic cod is poorly represented in current sequence databanks. A major aim of the "Atlantic Cod Genomics and Broodstock Development" project (CGP: *www.codgene.ca*) is to generate an extensive set of molecular tools for cod, including a large expressed sequence tag (EST) component. To date, approximately 25 000 EST sequences have been processed from a set of normalised cDNA libraries. These libraries were produced from various tissues (brain, heart, ovary, gill) sampled from fish collected in New Brunswick and Newfoundland. Individuals collected at the same time as sampled fish have been used to generate cod families for selective breeding.

The sequences generated are currently being mined for marker development. Three different methods of generating markers within specific genes have been assessed. These include identification of gene-linked microsatellites, development of exon-primed intron-flanking (EPIC) markers, and analysis of single nucleotide polymorphisms (SNPs) within EST clusters. Ultimately, a set of markers will be used to generate a high-resolution genetic map for Atlantic cod, and markers will also be used for analysis of quantitative trait loci (QTL) in the CGP cod family programs. In addition, sets of genes will be selected for inclusion on a "cod chip" microarray.

Introduction

Little genomic sequence is currently available for Atlantic cod. To date, only five sequences greater than 10 kb in length have been deposited in GenBank; three represent immunoglobulin gene clusters[1] and the remainder are cod mitochondrial genome sequences.[2,3] Other sequence data available for cod include a number of EST sequences, a few genes of interest to researchers in this species, and a limited number of microsatellite sequences used in population studies.[4,5] Thus, genomics for this species is in its infancy, but with the onset of large-scale sequencing of ESTs for cod as part of the "Atlantic Cod Genomics and Broodstock Development" initiative (CGP) and other international projects focusing on the development of molecular tools for cod, rapid progress should be achieved within this field in the forthcoming years.

The CGP is a large, multi-partner project based in Atlantic Canada, which integrates two family-based cod breeding programs (based in Newfoundland and New Brunswick) with a large-scale genomics initiative (based at the Atlantic Ge-

nome Centre, Nova Scotia). This is a three- to four-year project which will generate substantial molecular tools for Atlantic cod. The genomics component of the CGP has already made substantial progress in its first year. Below, we describe the current status of genomics research within the CGP, and our plans for completion of CGP genomics milestones.

Materials and Methods

Tissue collection

Tissue samples were extracted immediately after fish were euthanized with a lethal dose of anaesthetic. A small amount of material (~0.5 cm^3) was removed for each tissue selected, and preserved in RNAlater (Ambion®) according to the manufacturer's instructions.

EST library generation

Tissue samples were suspended in Trizol and homogenised using a Polytron generator, or alternatively frozen in liquid nitrogen followed by crushing using a pestle and mortar. After extracting the resulting homogenate with chloroform, RNA was precipitated from the aqueous phase using isopropanol. Integrity of the RNA produced was checked using both denaturing and native agarose gel electrophoresis.

A total of 2 µg of the extracted 100-200 µg RNA (total RNA) was taken forward for EST library production. First strand cDNA was prepared using the Creator SMART cDNA library construction kit method (Clontech) and PowerScript reverse transcriptase (Clontech). The cDNA was then amplified by LD-PCR according to the Creator SMART method. Amplified cDNA was purified using the QIAquick PCR purification kit, quantified using a NanoDrop spectrophotometer, and normalized using the TRIMMER-DIRECT protocol. After digestion with SfiI, products smaller than 500 bp were removed using the Chroma Spin-400 DEPC column, as described in the Creator SMART protocol. The resulting cDNAs were directionally cloned into the SfiI sites of pDNR-LIB (Clontech) and transformed into ElectroMAX DH10B T1 phage-resistant cells (Invitrogen) by electroporation. Randomly picked clones (96 from each amplified library) were screened for insert size by protoplasting, or by PCR using the M13 forward and reverse primers flanking the multiple cloning site of the vector.

Sequencing

Individual bacterial clones were inoculated into LB/glycerol in 384-well format and incubated overnight at 37°C. DNA from each clone was amplified using Templiphi™ DNA polymerase (GE Healthcare) according to manufacturer's instructions. Sequencing reactions were carried out using ET terminator chemistry (GE Healthcare) and, after removal of excess fluorescent terminators, samples were loaded onto MegaBACE capillary sequencers. The resulting sequence was basecalled using Phred, trimmed to remove vector sequence, with masking applied to low quality sequence. Sequence reads originating from *E. coli* or other contaminating material were removed where possible.

EST clustering and annotation

Sequences generated were clustered using Paracel Transcript Assembler, with the cluster threshold parameter increased to a value of 100, for relatively stringent

clustering. Both individual sequence reads, and contig consensi generated by the clustering procedure, were processed using an automatic annotation pipeline based on AutoFACT.[6] This annotation tool can be customised to run a variety of similarity comparisons, and to filter the results of informative searches to generate a functional assignment for each sequence.[6] Databases searched include PFAM, the protein domain database, and the pathways database KEGG.

Marker identification and genotyping

The program tandem repeats finder[7] was used to find microsatellite sequences within the set of unique sequences (contigs + singletons). Primer3 was used to design sets of primers flanking repetitive areas. For EPIC genotyping,[8,9] the sequence for each selected cod EST was aligned with its predicted orthologues in *Tetraodon nigroviridis* and *Danio rerio* (identified through reciprocal BLAST analysis) and primers designed within blocks of similarity between these sequences. For both microsatellite and EPIC genotyping, a 5′- tailed primer method was employed where a primer extension sequence (generally T7 sequence 5′GTAATACGACTCACTATAGGGC) was added to the 5′ end of one primer in each pair,[10] and PCR was carried out with the addition of a third primer to each reaction, in most cases a fluorescently-labelled T7. This amplifies from the extended primer to generate a fluorescent product.[10] Phusion high-fidelity DNA polymerase was used in preference to Taq to avoid non-templated additions of a terminal base.[11] Products for both microsatellite and EPIC genotyping were size-fractionated by electrophoresis on a Beckman CEQ8000 capillary sequencer.

Results and Discussion

The "Atlantic Cod Genomics and Broodstock Development" project (CGP) aims to develop large scale genomics resources for Atlantic cod. Genomics activities for the CGP began in January 2006 with the isolation of tissue samples from fish collected by the two cod breeding programs based at the Ocean Sciences Centre in Newfoundland (NL), and the St Andrews Biological Station/Huntsman Marine Sciences Centre in New Brunswick (NB). The fish from which samples were taken were not enrolled as parents within the breeding programs, but were captured at the same location and time, and therefore were considered to originate from the same populations as the parental fish within the two programs. These fish were collected from Bay Bulls for the NL program and in the waters surrounding Cape Sable Island off the southern tip of Nova Scotia for the NB program.

The first molecular resource to be developed by the CGP is a large set of expressed sequence tags (ESTs). As EST sequencing focuses on sequencing the genes within an organism, with different genes being expressed in discrete tissues and under different conditions, a variety of tissues were collected from each fish. These included ovary or testis, liver, digestive tissues (pyloric caecae, anterior stomach, anterior intestine, and middle intestine) skeletal and heart muscle, gill and brain. A further set of tissue samples has also been collected from F1 fish from the cod families generated during year one of the project, with the aim of identifying stress- and immune- relevant genes.

Large-scale sequencing has been carried out on three libraries to date, those generated from brain, heart muscle and ovary tissue. Small-scale sequencing has been conducted on a library generated from gill. In total, 18 477 successful sequencing reactions have been processed during the initial stages of the CGP. All of the sequences generated to date have been deposited in GenBank, and are thus

Library	Tissue	NB fish	NL fish	No. of ESTs	Contigs	Singletons
gmnbbr	brain	10	0	3237	298	2492
gmnbgi	gill	10	0	288	30	227
gmapht	heart	10	10	7867	1371	4083
gmapov	ovary	10	10	7085	1315	3490

Table 1
Libraries and EST sequences generated by the CGP to date. Libraries are generated from multiple individuals from the two populations used in the NB and NL breeding programs. After clustering, some sequences presumed to originate from a single gene are associated together within contigs, with the remaining unique single reads classified as singletons.

freely accessible to cod researchers worldwide. Currently, 87% of cod sequences available in GenBank have been provided by the CGP. A breakdown of sequences generated by this project to date is shown in Table 1. Ultimately the CGP aims to generate 160 000 sequences in total, and with sequences already produced together with those currently in the pipeline, the sequence generation component of the CGP is approximately 20% complete. The next libraries scheduled for sequencing have been produced from material isolated from F1 fish exposed to a variety of stressors, or after stimulating with viral-like or bacterial antigens.

Sets of clusters have been generated for each individual library, but also the entire sequence collection has been clustered (cluster set all_v1). On examination of the all_v1 clusters, the majority of contigs generated contain only two or three sequences, with an average of 3.3 sequences per contig in total. The majority of unique sequences remain represented by a single read. In total, approximately 11 000 unique sequences have been identified, which comprise the "working gene set" for the project at this early stage. Where possible, sequences have been given a preliminary annotation using AutoFACT.[6] This program runs similarity searches against a set of public databases and attempts to identify common themes within the resulting output. If successful, it assigns an AutoFACT annotation to the sequence. Preliminary AutoFACT annotation of a small number of sequences generated from cod gill is shown in Table 2.

Sequences will be used within the CGP to develop a set of gene-linked markers. Three classes of markers are being investigated for their use in downstream studies such as genetic mapping or analysis of QTL. These include microsatellites, exon-primed, intron-crossing (EPIC) markers, and single nucleotide polymorphisms (SNPs). Identification of polymorphism within the sequence clusters has been made possible because each of the EST libraries is generated from tissues isolated from multiple individuals, which are pooled before preparing RNA. A minimum of ten fish are used per library, with our standard procedure being to pool 20 fish per library, ten from each of the two family breeding programs. The use of fish from the same populations as the two breeding programs, or from F1 fish within those programs, ensures that markers developed will be informative in the analysis of quantitative trait loci (QTL) within the family-based breeding component of the CGP.

Microsatellite markers are developed from simple tandem repeats of short DNA sequences, which frequently exhibit copy number changes. A set of published microsatellite markers are being used within the breeding programs to avoid mating related individuals when setting up families, but additional gene-linked microsatellites are also being developed. All sequence generated through the CGP program has been mined for the presence of microsatellite markers using a re-

Sequence	Name of hit	Bits	eValue	AutoFACT Description
gmnbgi_0001a01.pDNRF2	UniRef90_P22232	322	5e-41	Fibrillarin related cluster
gmnbgi_0001a02.pDNRF2				
gmnbgi_0001a02.pDNRm13r				
gmnbgi_0001a03.pDNRF2	UniRef90_Q7ZT04	244	2e-19	CD8 alpha chain related cluster
gmnbgi_0001a03.pDNRm13r	UniRef90_Q2FBJ2	166	1e-10	CD8 alpha chain related cluster
gmnbgi_0001a04.pDNRF2				
gmnbgi_0001a04.pDNRm13r	UniRef90_UPI00005A0C8B	157	5e-10	Cluster related to UPI00005A0C8B; PREDICTED: similar to Plasminogen activator inhibitor 1 RNA-binding protein (PAI1 RNA-binding protein 1) (PAI-RBP1)
gmnbgi_0001a06.pDNRF2				
gmnbgi_0001a06.pDNRm13r				
gmnbgi_0001a08.pDNRF2				

Table 2
Example output from the CGP annotation pipeline. Sequences shown are taken from the gill library, gmnbgi, with sequence name following the format - library; location of clone on a 96/384 well plate; primer. If an AutoFACT annotation has been applied to a sequence, details of the similarity hit, quality metrics (bit score, e-value) for that hit, and the AutoFACT annotation are displayed.

peat-finding program.[7] Approximately 545 potential microsatellite markers have been identified within the set of unique CGP sequences using tandem repeats finder (alignment score threshold set at 40), with approximately 4% of sequences being associated with a microsatellite of this type. A subset of these microsatellites (75 in total) has been selected to assess their degree of polymorphism within the populations used for development of the broodstock programs. Results for microsatellites analysed to date show a high level of polymorphism and degree of heterozygosity within the two cod populations.

The second type of markers being investigated, EPIC markers, rely on the identification of intron length polymorphisms. By comparing the sequence of cod ESTs to the genomic sequence of other species such as the puffer fish (*T. nigroviridis*) or the zebrafish (*D. rerio*), sites where intron sequences have been excised from each cod transcript were predicted by identifying gaps within alignments between the genomic and the EST sequence. Sites of intron excision were confirmed experimentally by designing primers flanking each predicted insertion site, and using cod genomic DNA as a template for PCR. If PCR was successful, the level of intron length polymorphism within the broodstock populations was assessed by analysing length differences between the PCR products generated from several individuals.

The third marker type, SNPs, represent single base changes within sequences which are otherwise identical, and comprise a type of variation observed frequently within DNA. As tissue samples from multiple individuals are pooled in the creation of the EST libraries, SNPs can be identified within individual sequences which are aligned together within the sequence contigs. However, because we are at the early stages in sequence generation, and also because EST libraries have been extremely well normalised, only a few genes have been deeply sampled in the sequence generated to date. Nevertheless, in contigs with >5 component sequences it is possible to identify approximately one SNP every 100bp. Thus, cod exhibits an extremely high level of sequence variation. Currently a medium throughput SNP identification pipeline is being developed to allow identifi-

cation of a set of SNPs that will be informative within the broodstock populations.

Ultimately the CGP aims to identify more than 3000 potential gene-linked markers and use these to create a high-density cod genetic map. This will enable genome-wide comparisons between cod and other sequenced fish species, and may provide the tools required for fast-track identification and analysis of candidate genes for phenotypic traits desirable in aquaculture. The extensive sequence resource, and the large number of marker sequences generated during the course of the CGP, will be invaluable in future molecular genetic studies on cod and other species.

Conclusions

Significant progress has been made in the development of sequence resources for Atlantic cod by the CGP. Sequences have been deposited in GenBank and are available for use by researchers worldwide. Information from this resource has been used to successfully expand collections of several types of sequence-based markers in cod. These markers will be used in genetic mapping, and in providing a better understanding of traits relevant to the successful development of cod aquaculture, such as disease resistance and temperature tolerance. These resources will be important in developing future international collaborations focusing on cod genomics and its application in aquaculture.

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Gamete Collection and Egg Quality Comparison in Atlantic Cod (*Gadus morhua*) and its Importance to a Family-Based Selective Breeding Program

L. Lush, D. Drover, A. Walsh, V. Puvanendran, and J. Symonds

Lynn Lush

Communal spawning tanks allow for minimal stress and handling of broodstock. Atlantic cod (*Gadus morhua*) readily spawn in captivity, producing large volumes of high quality eggs, without practitioner intervention. However, with communal spawning no selection of individual pairs is attempted, leaving the possibility that certain animals can out-compete others for mates and in gamete production. Communal mating may limit the selection of those individuals which could pass on the best traits for aquaculture to the offspring, possibly limiting the production potential of the species for industry. Family-based selective breeding has led to advances in growth for salmon and catfish, and is currently being investigated in Atlantic cod (www.codgene.ca), with the aim to improve production through improved growth, survival, disease and environmental tolerance, and final marketable quality. The collection of high quality gametes is a crucial starting point for a successful breeding program. A comparison between three methods of gamete collection was performed, investigating differences between egg quality and survival in communal, paired mated and stripped spawned Atlantic cod broodstock. Results show no variation in fertilization rate between groups, but other egg parameters are sometimes variable between different collection methods.

Introduction

Communal spawning allows for minimal stress and handling of a broodstock. Atlantic cod (*Gadus morhua*) readily spawn communally in captivity, producing high volumes of high quality eggs both under ambient and photomanipulated conditions.[1] Atlantic cod are batch spawners with females producing up to 19 batches of eggs in a single spawning season.[2] It has been noted that certain individuals in a communally spawning population contribute a higher proportion of egg and sperm production than do others, developing a spawning hierarchy based on size, aggression, and female mate selection.[3] This limits genetic contribution due to limited parents,[4] and potentially limits the production of the species for industry. Selective breeding has led to advances in growth for many fish species, including salmon (*Salmo salar*) and catfish (*Ictalurus punctatus*),[5,6] and is currently being investigated in Atlantic cod, aiming to improve production through improved growth, survival, disease resistance, environmental tolerance, and final marketable quality.

The transition from unselected communal spawning in cod requires the investigation of alternative methods of gamete collection. While cod perform well in communal spawning tanks, there is a concern that stress associated with collecting gametes by other methods may reduce gamete quality and increase post spawn mortality. Thorsen et al.[7] noted that both Atlantic haddock (*Melanogrammus aeglefinus*) and cod do not tolerate manual stripping of eggs as well as some other species, with haddock being the more sensitive. An alternate method, used in other species, has employed paired mating tanks, where a selected male and female are placed in a suitable tank environment and allowed to spawn unassisted and apart from other individuals. Trippel et al.[8] saw success with haddock and cod in mated pairs, with higher fertilization rates noted for cod in this situation.

Another commonly used method of gamete collection in finfish is strip spawning, whereby sperm and unfertilized eggs are collected from animals that are running and subsequently fertilized under laboratory conditions. This method does require an understanding of the ovulatory cycle of the species in question, which is not always easy to ascertain. Thorsen et al.[7] noted that success in strip spawning of marine fish may be difficult to attain as eggs only have a few hours of viability once ovulated, and quality of eggs may be variable or inferior to eggs collected from natural spawnings. Strip spawning is the method of gamete collection used for those species which do not naturally spawn in captivity, such as the Atlantic halibut (*Hippoglossus hippoglossus*) and has been challenging to both practitioner and fish.

A comparison of the quality of eggs, larvae and broodstock between the three methods of gamete collection would be beneficial as then a choice can be made to utilize the method which delivers the highest quality egg, best egg survival, and highest post spawn recovery of broodstock, all with consideration for keeping the spawning season as succinct as possible. Understanding the benefits and limitations of these collection methods would be greatly beneficial to selective breeding studies as the gametes collected would ideally be of the highest quality possible, reducing this influence on family performance. Ultimately, both knowledge of gamete collection and selective breeding will be highly beneficial to industry for production improvements in cod farming (see Symonds et al.[9]).

Materials and Methods

Study One

Atlantic cod caught in NAFO convention area 3Ps, off the south coast of Newfoundland, were brought to the Ocean Sciences Centre in Logy Bay, Newfoundland in the winter of 2002. Fish were held in 35-m^3 flow-through tanks on a four-month advanced photoperiod. Fish were fed three times weekly to satiation on baitfish (herring, mackerel, squid) supplemented with a marine finfish vitamin pellet (Tammy Blair, St. Andrews Biological Station). Communal spawning of the group was monitored annually over the subsequent three years, and egg quality analyzed based on the fertilization rate, average egg diameter, batch volume, and blastomere morphology normality. Fertilization and blastomere normality rates were measured on one hundred eggs per batch, and diameters were measured on ten eggs from each batch. In 2005 fish from this group (n=37) were finclipped and Passive Integrated Transponder (PIT) tagged in the dorsal musculature. Finclips were genotyped by microsatellite analysis to determine relatedness of individuals in the population using a marker panel consisting of Gmo 8,

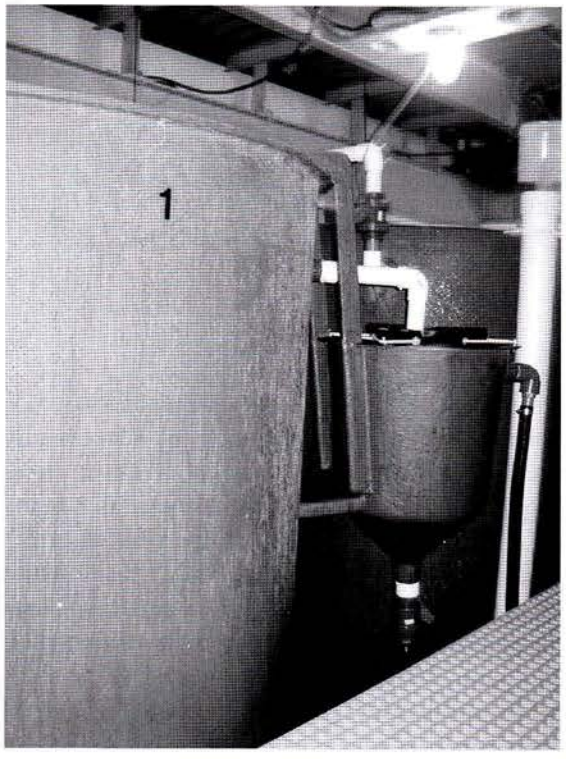


Figure 1
Paired mating tanks at the Ocean Science Centre, Logy Bay, Newfoundland

Gmo19, Gmo37, Tch 5, and Tch11 (Research and Productivity Council, Fredericton, New Brunswick). Fish were sexed using cannulation. Unrelated male and female pairs were then placed in 15-m^3 paired mating tanks (Fig 1) and allowed to acclimate. All tanks were equipped with an external egg collector fitted with a nitex (500 micron) bag. Eggs from spontaneous spawnings of these pairs were collected and egg quality analysis was performed based on the same parameters as collected for the communally spawning fish.

Egg quality and spawning performance of these paired mated individuals was compared to the quality and performance of the same population from the previous year's spawning, based on the same parameters.

Males were removed once a successful spawning event occurred and replaced by a running male unrelated to the female remaining in residence. Males were switched in this fashion throughout the season and females were replaced as they became spent, or unproductive with a newly ripe or ripening female from the communal tank. The purpose of this movement was to generate an adequate number and variety of unique families for a family-based genetic selection study being undertaken in Atlantic Canada, and managed through Genome Atlantic and its collaborators.[9]

Study Two

The experiment preliminarily compared gamete quality from three types of collection methods from a single group of fish in a single spawning season. Communal spawning, paired mating and strip spawning were compared between a group of 2002 year class F1 broodstock undergoing their second year of spawning in captivity. Communal spawning and unrelated paired mated spawning were performed in the same manner as that of the wild broodstock in study one. Approximately two months after the group began communally spawning; the ripe individuals in the population were stripped of eggs or sperm. Following the strip spawning, individuals that were still running were placed in the paired mating tanks. Based on microsatellite analysis (same panel as above) of these fish, unrelated males and females were matched for paired and stripped crossing methods. In fertilizing eggs from the strip spawning collection a dry fertilization method was employed. Eggs were mixed with approximately 3 mL of sperm, which was

pipetted directly onto the eggs after collection. 100 mL of seawater was added and one minute elapsed to allow the sperm to fertilize the eggs. Following this, 500 mL of seawater was added to the gametes and a further five minutes were allowed for any other possible fertilization before moving eggs to incubators. Egg quality analysis, following the same procedure as for the other collection method groups, was performed 24 hours following fertilization. Egg diameters were not collected for the communal spawners, but were for the other two methods.

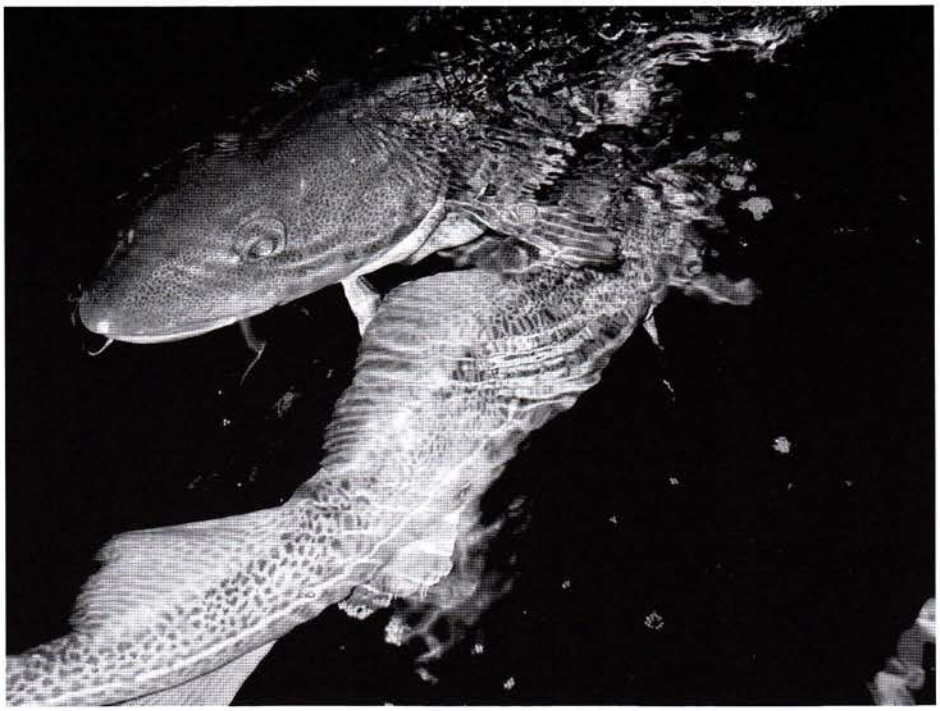
Results

Study One

First paired mated spawning occurred one week after pairs were moved to tanks. Over the period of 104 days, 65 of 93 (70%) ripe pairs attempted successfully spawned. Initial egg quality was poorer, but improvements were observed as females had more time to acclimate to the tanks. Observations indicated that many ripe females showed interest in all males that were placed in the tank. Figure 2 demonstrates activity between a pair of cod in a paired mating tank. Most females remained in residence in the paired mating tank for several weeks, and were introduced to many males over that time span. Some pairs however, were unsuccessful or produced egg batches less than 10 mL. There were a few females in the communal population and those in the paired mating tanks that did not spawn at all during the season.

When comparing the same group of fish from a communal spawning situation to a paired mated spawning in the subsequent year, very few significant differences were observed in egg quality. Egg fertilization was not significantly different between the two groups when compared using a Kruskal-Wallis non parametric test ($p=0.3347$). Fertilization for the communally spawning fish in year one averaged 92% and for the paired mating in year two averaged 87% (Fig. 3). Egg diameters between the two groups also showed no significance difference when compared using a t-test ($p=0.7388$); averaging 1.47 mm for communal spawners and

Figure 2
A cod pair



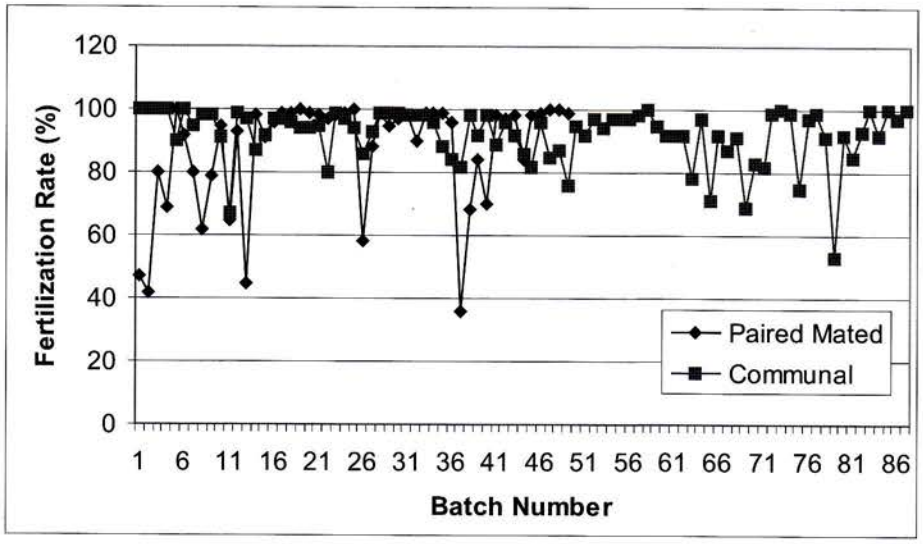


Figure 3
Fertilization rate (%) of communally spawned and paired mated Atlantic cod broodstock in study one.

1.46mm for paired mated fish (Fig 4). When observing the various parameters of egg blastomere normality, only two abnormalities showed any significant difference when compared using a Kruskal-Wallis non-parametric test. Blastomere uniformity ($p=0.0005$) and blastomere/cytoplasm clarity ($p=0.0227$) were significantly different between paired mated and communally spawned cod. Eggs demonstrating proper blastomere uniformity were higher in the communal spawners at 95% versus 91% for paired mated fish. Clarity normality was higher in the paired mated group at 97% compared to 96% in the communal group (Fig. 5).

Study Two

Comparing the three gamete collection methods with the F1 broodstock revealed no significant difference in fertilization between the three groups when compared using a Kruskal Wallis test ($p=0.1885$; Fig. 6). There was a significant difference between the diameters of the paired mated fish and the stripped animals. Diameters of eggs were significantly larger in the stripped spawning fish over the paired mated ($p=0.0019$) when compared using a t-test (Fig. 7).

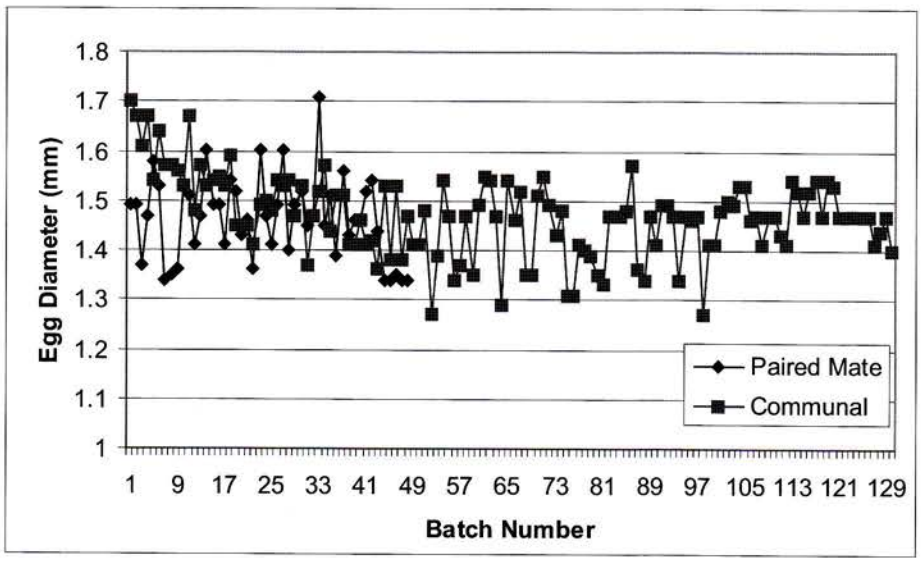


Figure 4
Egg diameter (mm) of communally spawned and paired mated Atlantic cod broodstock in study one.

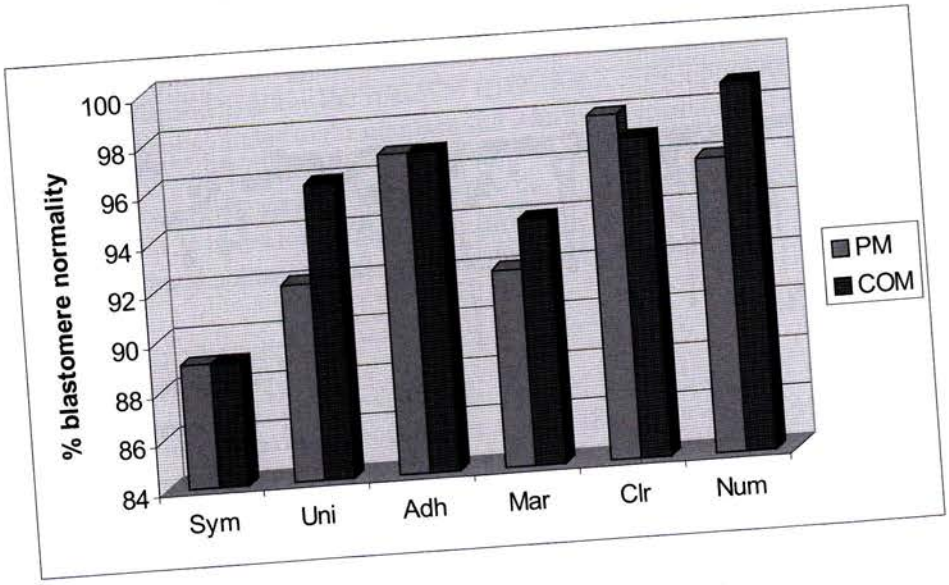


Figure 5
Blastomere morphology normality (%) of communally spawned (COM) and paired mated (PM) Atlantic cod broodstock in study one. (Symmetry-Sym; Uniformity-Uni; Adhesions- Adh; Margins-Mar; Clarity-Clr; Cell Number-Num).

Discussion

Results from study one, indicate that paired mating is a useful method to generate parent identified families for a family-based genetic selection program without compromising egg quality or spawning success. In comparing communal and paired mated spawning, Atlantic cod will readily spawn using both methods, and produce batches of high quality eggs. With paired mating, most females were receptive to most males introduced to the tank either if introduced together or if the male was introduced later, following a period of tank residence by the female. The suggestion by Hutchings et al.[3] that fishing disturbances during the spawning season will disrupt the spawning hierarchy and lead to poorer production and longer periods between ovulations does not seem to translate to this scenario. Spawning may have proceeded quicker without the frequent changing of males, but for running females in the experiment, spawning with a new male often occurred within a reasonable ovulatory period of two to four days. It is possible, that the female given only the option of one male will spawn regardless of mate condition,

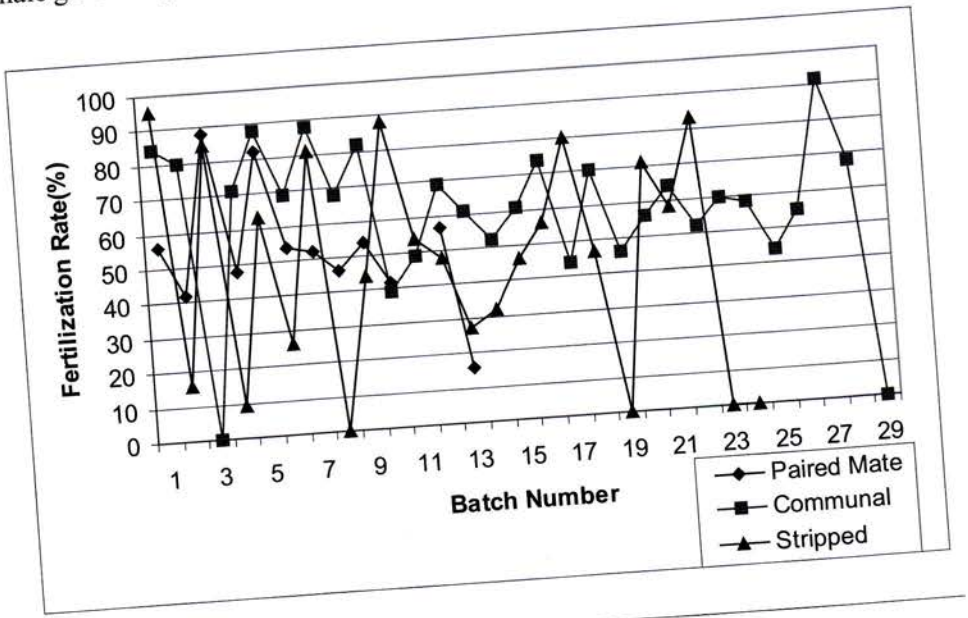


Figure 6
Fertilization rate (%) of communally spawned, paired mated, and stripped F1 broodstock in study two.

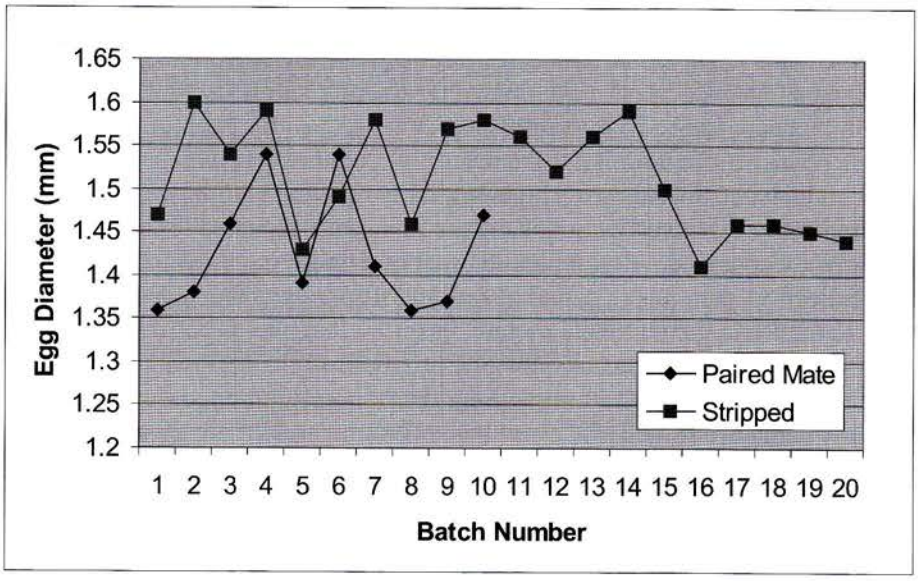


Figure 7
Egg diameter (mm) of paired mated and stripped spawned F1 broodstock in study two.

but there did not seem to be any difficultly with paired mating success using this method. It was also noted that spawning became more successful after a period of acclimation to the paired mating tanks (approximately 15 days) and low fertilized or very small batches of less than ten mL were no longer observed following this period.

Fertilization rates and egg diameters remained statistically similar; as did most blastomere morphology parameters, with the exception of uniformity and clarity between the groups in study one. It is difficult to predict why statistically significant differences would occur in these blastomere parameters only, but it should be noted that for clarity the difference between groups was only one percent, and while statistically significant, may not be biologically significant. Uniformity differed by four percent and again biological significance may be questioned. There are varying viewpoints as to the ability of eggs to hatch successfully when abnormal blastomere morphology occurs at the time of analysis. Certain types of abnormalities seem to be more indicative of poorer hatching, ie symmetry[10] and these predictive measures seem to be variable throughout species[7,10,11] and from year to year within one spawning population.[1]

This study had the limitation that it was unable to compare the performance of individual females and males in the communal spawning tank to the performance of these same individuals in the paired mating scenario. Ideally, using microsatellite DNA markers parentage would be assigned to individual communally spawned eggs at a certain stage and the quality of these eggs could be associated to the parents producing them. However, costs for such a study would be high, but may be of some value for future consideration.

With the gamete collection comparison in study two using hatchery reared F1 broodstock strip spawning was also investigated. No clear conclusion could be drawn as to the best method to collect eggs based on this study. Fertilization rate did not appear to be affected by the method of collection, which indicates all methods are potentially equally viable. The difference in diameters between the paired mated and stripped spawning fish was unexpected, in that it was thought that egg diameters from stripped females would be smaller as proper timing of hydration of oocytes and ovulation may be difficult to predict, and eggs could be stripped before proper final maturation. In the case of this study, it was the paired

mated group eggs that were smaller in size. This could have been a result of the later date that the paired mated data were collected as it has been previously noted that egg diameters decrease as the spawning season progresses.[12] This may help explain this occurrence with this group.

Further data need to be collected to determine conclusively which method is the most conducive to providing the highest quality eggs for a family-based selective breeding study. An investigation using the same pairs repeated for both methods needs to be conducted. Also, a complete set of data for the three methods of gamete collection, communal, paired mated, and stripped spawning need to be compared to ascertain the complete picture, ensuring that data is collected in a tight time frame to reduce effects of spawning progression changes in egg quality. As study two of this paper was only a preliminary investigation, a further study is currently being performed to answer these questions and to help definitively conclude the best method to collect eggs for a family-based genetic selection study.

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Family-Based Atlantic Cod (*Gadus morhua*) Broodstock Development

Amber Garber

J. Symonds, A. Garber, V. Puvanendran, A. Robinson, S. Neil, E. Trippel, S. Walker, D. Boyce, K. Gamperl, L. Lush, G. Nardi, F. Powell, A. Walsh, and S. Bowman

The decline in wild cod populations has resulted in fisheries closures throughout Atlantic Canada. Cod aquaculture is widely recognized for its potential benefits in supplying cod to the marketplace, while providing stability to the established salmon aquaculture industry through species diversification. Broodstock selection is an important aspect of developing a new candidate species for culture. To this end, the Atlantic cod genomics and broodstock development project (CGP, www.codgene.ca) was initiated to create two regional family-based selective breeding programs in Newfoundland & Labrador (NL) and New Brunswick/New Hampshire (NB/NH). In 2006 the CGP produced 103 full- and half-sibling families for communal rearing in sea cages. Initial measurements on all families were collected at 90 days post-hatch (dph) for 36 NL families and 105 dph for 50 NB/NH families, with mean body weight ranging from 0.18 to 0.72 g and 0.75 to 3.11 g in the NL and NB/NH programs, respectively. Data were also collected on juvenile cod at tagging on 49 families from the NB/NH program and 22 families from the NL program. Preliminary data analysis has revealed significant variation between families in observed body weight and the incidence and types of deformities. The initial heritability estimate for body weight at tagging in the NB/NH families is high ($h^2 = 0.498 \pm 0.111$). This estimate is encouraging as it demonstrates the possibility for improvement of important commercial production traits in the developing cod aquaculture industry.

Introduction

Declining wild populations of Atlantic cod worldwide have resulted in renewed interest in farming this species, with cod aquaculture becoming increasingly important in Atlantic Canada. While most of the basic culture protocols are developed, there is continued reliance on unselected wild cod stocks which limits progress of the developing industry. Therefore, the development of captive selective breeding programs is fundamental for future improvements in the effective domestication of this species. Towards this goal an $18.1 million project (CGP, www.codgene.ca) was funded to develop cod family-based selective breeding programs in Newfoundland and Labrador (NL) and New Brunswick/New Hampshire (NB/NH). These separate regional broodstock programs were initiated during the cod spawning season (Dec 2005 to April 2006), and utilized local cod stocks to establish families for the future benefit of the provincial aquaculture industries. The measurement and genetic evaluation of eco-

nomically valuable traits such as growth, sexual maturation, fish health, upper temperature, hypoxia, and stress tolerance, survival and product quality and yield will be conducted on these families (Fig. 1). The benefit of incorporating specific traits into the breeding program design will also be assessed, with the target of producing fast growing, good quality, healthy cod. The CGP also includes a large-scale genomics initiative (based at the Atlantic Genome Centre, Nova Scotia) which will generate substantial molecular tools for Atlantic cod that can be used to improve the accuracy of selection for traits that are difficult or impossible to measure directly on live individuals such as disease resistance or product quality.[1]

Below we provide an overview of the broodstock development component of the CGP and outline the initial results from the families generated by the project.

Materials and Methods

Broodstock

Wild cod broodstock used as parents of the families (December 2005 to April 2006) were obtained from two locations in Canada (Cape Sable (NAFO division 4X) and eastern Newfoundland (NAFO division 3Ps)) and one site in the United States (New Hampshire coast (NAFO division 5Y)). F1 broodstock previously generated from the same NL stocks housed at the Ocean Sciences Centre were also used as parents. Ambient spawning stocks were used in NB and NH, whereas 4-month advanced photoperiod manipulated broodstock were used as parents in NL.[2,3]

Prior to spawning, fin clips obtained from PIT-tagged broodstock were used to obtain genotypes for five microsatellite loci. The microsatellite genotyping was conducted by the Research and Productivity Council, Fredericton, NB, and the data were analysed using the MER program[4] to generate relatedness estimates between all pairwise combinations of broodstock. Only unrelated individuals were crossed during spawning based on this estimate.

Family generation and evaluation

The broodstock were either artificially strip spawned or pair mated to establish full and half sibling families using the Berg and Henryon breeding design, where each sire is mated to two dams and each dam is mated to two sires.[5] Each surviving family was reared separately up to hatch in an incubator (Fig. 2) and then from hatch to tagging in a 470-L family rearing tank (Fig. 3). Families in both programs were incubated at 4.0-6.5°C and reared at 10.0-14.0°C post-hatch up to tagging. A pooled group of 74 families was also generated in the NB/NH program (Group 2 in Fig. 1) to compare individual rearing versus communal rearing as a strategy for family-based selective breeding.

Cod rearing expertise and protocols developed previously by project participants[6] were applied, and adapted where necessary, to successfully rear individual families. Family survival was monitored throughout the hatchery rearing phase. At 90 or 105 days post-hatch (dph) family numbers were standardized to 1500 individuals per tank and initial weight measurements were obtained. In NL, 1782 fish were individually weighed and measured ($n = 50$ each 35 families and $n = 32$ for one additional family). In NB/NH, three to five bulk weights were recorded per family ($n = 50$ families). Juveniles were PIT-tagged at a minimum family mean body weight of ~15 g, size determining the timing of tagging rather than age. During tagging the data recorded on all individuals included body weight, to-

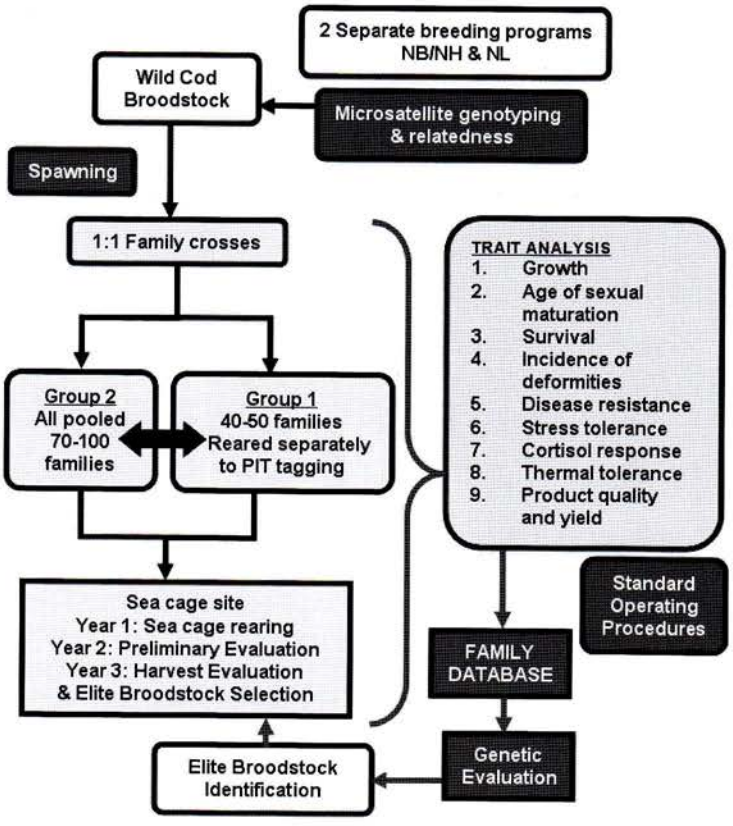


Figure 1
CGP cod family-based selective breeding and trait analysis program design.

tal length and the presence and type of deformities. Only non-deformed healthy individuals were tagged.

Statistical analysis and heritability estimation

Initial family analysis was conducted at 90 and 105 dph. Bar graphs were constructed to visually depict the means and standard errors for each family (Microsoft® Office Excel 2003). The General Linear Model (GLM) was used to analyze variance in weight (g) between families (SAS version 9.1.3, SAS Inc.). Pearson correlation coefficient with probability was calculated between weight and length in the 90 dph NL sample (SAS).

Heritabilities for weight and length at PIT-tagging were estimated using the VCE-5 software[6] with a multiple-trait mixed linear model including the fixed effect of tank and the random effect of fish. All family pedigree information was included but wild-caught parent stock were assumed unrelated. Individual fish weights and lengths were adjusted to an age-constant basis using a repeated measures regression of length or weight on days fed (SAS GLM procedure). All fish were adjusted to 120 days on feed.

Results

In NL, 68 families were generated (48 from paired mating and 20 from hand stripping) and 39 families (Group 1) were transferred post-hatch to rearing tanks for evaluation and trait analysis. Similarly, in NB/NH 122 families were generated (120 from hand stripping and 2 from paired mating) with 50 transferred to individual family tanks (Group 1) and 74 families combined in a pooled group (Group

Figure 2
50-L incubators for rearing individual families up to hatch.

2), 42 of which were shared with Group 1 families to enable performance comparisons at harvest. Numbers were standardized to 1500 individuals maximum per family at either 90 dph (NL) or 105 dph (NB/NH). Sample weights were recorded from each family to compare initial performance (Fig. 4). Significant differences between mean body weights were observed between the families at both sites ($P < 0.0001$), with mean weights ranging from 0.18 to 0.72 g in NL at 90 dph and 0.75 to 3.11 g in NB/NH at 105 dph. Correlation between length and weight in the NL sample was 0.89 ($P < 0.0001$).

PIT-tagging commenced once a family attained a minimum mean body weight of ~15 g. Descriptive statistics for body weight at tagging, as well as age and number of fish at tagging, are given in Table 1. The overall incidence of deformities in the NB/NH program was 16.7% (ranging from 1.1% to 69.7% per family) and 20.4% in the NL program (ranging from 0.0% to 49.5% per family). Family data at tagging from the NB/NH program (n = 11 037 progeny, NB/NH families analyzed together) were used to calculate the heritability of juvenile body weight, $h^2 = 0.498 \pm 0.111$, a heritability of juvenile body length of 0.584 ±0.117 and an additive genetic correlation of 0.968 ±0.013 between juvenile body weight and juvenile body length. The regression coefficients used to adjust weight and length to 120 days on feed were 0.2338 ±0.0067 g/day and

Table 1
Descriptive statistics for body weight, age and number of fish at tagging by broodstock source.

Broodstock Source	Number of families at tagging	Mean number of individuals assessed per family	Total number of individuals assessed	Mean body weight at tagging (g)	Standard deviation weight at tagging	Age range at tagging (dph)
Newfoundland	22	403	8,882	17.84	7.15	172-237
Cape Sable Island	39	327	12,767	22.94	9.40	159-209
New Hampshire	10	329	3,295	20.87	10.08	176-218

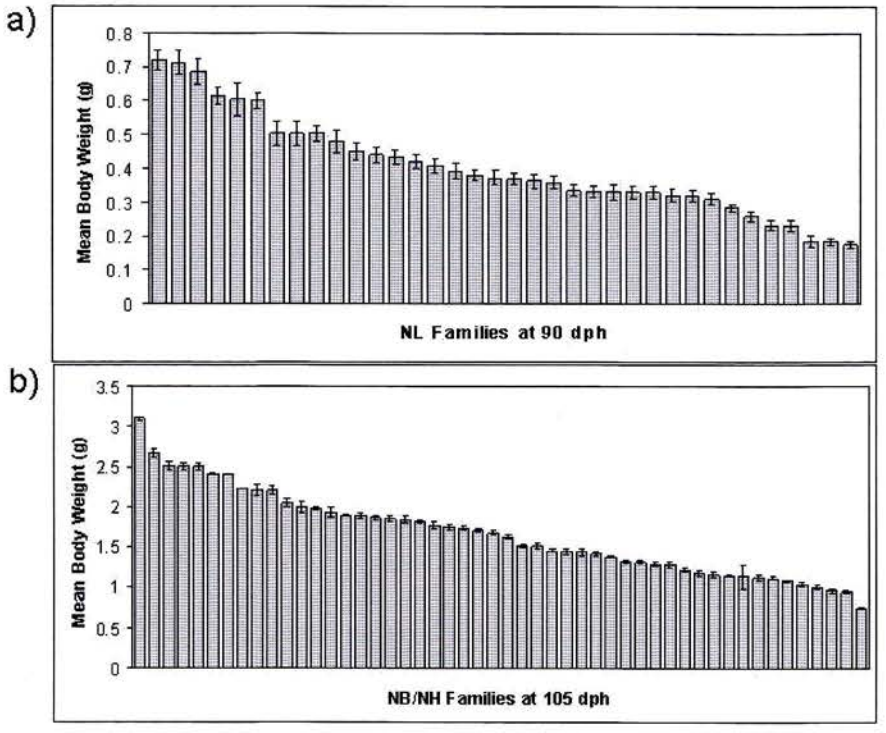
0.0456 ±0.0011 cm/day respectively ($P < 0.0001$ for both). This initial calculation is similar to heritability estimates found for body weight in several other fish species, including Norwegian Atlantic cod (h^2 range for body weight = 0.27-0.64).[7,8]

The Group 2 families were transferred to a single commercial cage in July/August 2006 (~ 41 532 individuals) at a mean weight of ~8 g. In November 2006 the PIT-tagged Group 1 families were transferred to two commercial sea cage sites in New Brunswick (~5000 individuals per cage) and one site off the south coast of Newfoundland (~3000 individuals) for further rearing and evaluation. The Group 1 families will be reared for an additional year in the cages before a preliminary assessment is conducted (body weight, deformities, sex assessed by ultrasound, and sexual maturation status). The final harvest assessment of the Group 1 and Group 2 families, and subsequent genetic evaluation and elite broodstock selection, will take place after approximately two years in the cages (Fig. 1), assuming a three-year generation cycle. Family identification of the Group 2 families will be determined post-harvest by microsatellite DNA analysis. In addition to sea cage performance trials, families will also undergo stress response, thermal tolerance, and disease challenge tests to determine the heritability of these traits and to assess their potential for future incorporation into a marker assisted selection program.

Ultimately the data collected on all families at all sites will be combined to develop a database for analysis and genetic evaluation of individuals and families. An overall profile will be created for each family, summarising performance throughout the three year evaluation process. The trait data collected will be used for estimating genetic parameters (heritabilities, genetic and phenotypic correlations) and developing genetic evaluation procedures using a multi-trait animal model and Best Linear Unbiased Prediction (BLUP), incorporating all available pedigree information. The family broodstock will be ranked according to the results of the genetic evaluation and the elite individuals will be selected as parents

Figure 3
470 L individual family rearing tank (post-hatch to PIT-tagging) with automatic feeder.

Figure 4
Mean (± standard error) body weights of full and half sibling Atlantic cod families at a) 90 dph in Newfoundland and b) 105 dph in New Brunswick/New Hampshire.



of the next generation of families and for the production of juveniles for commercial culture by the CGP's industry partners in 2009.

Discussion and Conclusions

A major aim of the CGP is to develop phenotypic assays for the assessment of economically valuable traits identified by industry partners (Fig. 1), and to collect accurate and repeatable measures of the traits under evaluation. The goal is also to collect sufficient data on all traits for genetic parameter estimation (heritabilities, genetic and phenotypic correlations) and to evaluate their potential incorporation into the breeding program through traditional and/or marker assisted selection.

Initial results from the first year class of families have demonstrated that individual cod families can be successfully reared and evaluated, and that significant phenotypic differences in traits such as body weight are observed between families. At tagging the preliminary heritability estimate for body weight in the NB/NH program was high and this provides an indication that additive genetic variation for body weight exists in Canadian cod broodstock populations and that this trait should respond well to selection. Medium to high estimates for genetic and phenotypic correlations between Norwegian Atlantic cod body weights recorded three times during growth from juvenile to harvest, including weight at tagging have been reported.[8] The correlations between weight at tagging with the two subsequent recordings indicated that tagging information can be utilized within a cod breeding program. If similar correlations are obtained within the two Canadian programs, the prediction is that additive genetic variation for body weight will be detected at harvest and will be used as part of the analysis to select elite broodstock for the industry. In the published literature there are several estimates of response to selection for increased growth rate in fish.[9,10] An average figure of response estimates for efficient breeding programs is around 12-15 % genetic gain per generation for growth rate. This

means that it should be possible to double growth rate in less than seven generations, and thus provide significant benefits to the developing cod aquaculture industry through the development and continued operation of family-based selective breeding programs for cod.

Acknowledgements

We thank the technical teams at the Aquaculture Research and Development Facility, Ocean Sciences Centre, NL, Fisheries and Oceans Canada, Biological Station and Huntsman Marine Science Centre, St. Andrews, NB, and GreatBay Aquaculture, NH, for their expertise and dedication which made the production and evaluation of cod families possible. Funding was provided by Genome Canada/Genome Atlantic, provincial funding bodies in the Atlantic Provinces, industrial partners, and in-kind from participating institutions. More extensive details of project participants can be found at www.codgene.ca.

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Recent Developments at the University of New Hampshire Open Ocean Aquaculture Site

Michael Chambers

Michael Chambers, Richard Langan, Hunt Howell, Barbaros Celikkol, Win Watson, Rollie Barnaby, Jud DeCew, and Chris Rillahan

The University of New Hampshire (UNH) established an open ocean aquaculture R&D facility 10 km off the New Hampshire coast in the Gulf of Maine in 1999. The offshore facility consists of a submerged mooring system that accommodates up to four experimental fish containment systems, submerged longlines for molluscan shellfish culture, and surface structures that include automatic feed buoys, fish tracking instrumentation, and environmental monitoring equipment. Over the last year, the AquaPod™ and JPS net pens were evaluated at the offshore site, Atlantic cod were harvested and a second, higher density cohort was re-stocked. Finally, blue mussel culture technologies were successfully transferred to a local fisherman who deployed 12 submerged longlines in open ocean State waters.

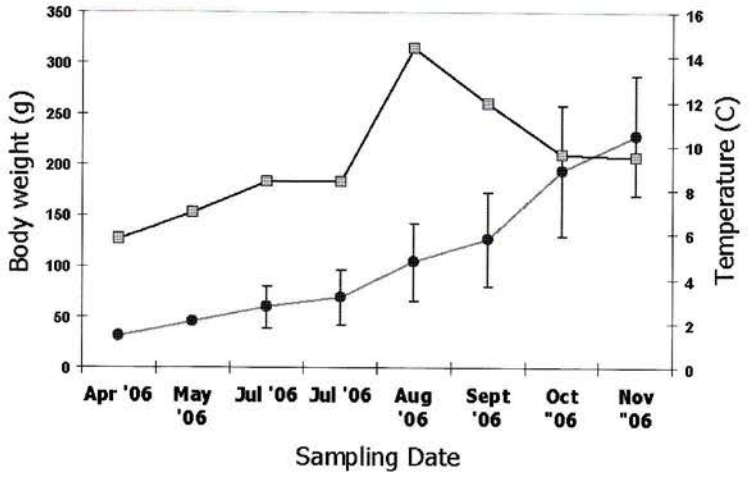
Introduction

Encouraged by promising results from a number of small-scale engineering projects and hatchery research on the production of coldwater species in the mid 1990s, the University of New Hampshire (UNH) initiated the Open Ocean Aquaculture Project in 1998. The goal of the project is to stimulate the development of an environmentally sustainable offshore aquaculture industry, thereby increasing seafood production, creating new employment opportunities, and contributing to regional and national economic and community development. With funding from the national Oceanic and Atmospheric Administration (NOAA) and in partnership with local fishing cooperatives and a commercial marine fish hatchery, UNH established an offshore aquaculture R&D facility in the Gulf of Maine in 1999. The project team, that has included several regional institutions, has focused on coordinated research efforts in the development of engineering design and assessment tools; deployment and evaluation of engineered systems including moorings, cages, feeders, and remote observation and operations systems; development of rearing techniques for a number of native marine species, and implementation of management practices to mitigate potential environmental impacts of fish farming.

Cod Aquaculture

Following the harvest of an initial experimental production run of Atlantic cod (*Gadus morhua*) as reported by Chambers and Howell,[1,2] a second growout trial was initiated in April 2006. Fifty thousand Atlantic cod were stocked into a

Figure 1
Cod growth (circles) compared to temperature (squares) over time.



3000-m^3 Sea Station™, submersible cage located at the offshore facility. Great Bay Aquaculture, from Portsmouth, NH produced the juveniles that were stocked at 35 g mean weight. They have been fed between 0.85-1.5% body weight/day of a Burris™ Marine Grower, semi-sinking pellet, consisting of 50% protein and 14% lipid. Feed was delivered to the cage twice per day using a solar and wind powered automated feed buoy. Food conversion ratio to date has been good (0.98), as has the specific growth rate (3.2%/d). Growth in weight has been steady (Fig. 1), and existing biomass in the cage is estimated at 12 900 kg.

Survival as of January 2007 was at 88%. Most of the mortality (9%) has been attributed to stress and overcrowding during their 5.5-month stay in a submerged, 200-m^3 nursery net within the cage. Once the fish were released into the main net (at 150 g mean weight), mortality has been insignificant.

Harvesting will commence in the summer 2007 when a mean weight of 800 g has been achieved. Groups of cod will be captured at a depth of 25 m, isolated, decompressed and transported live to inshore pens. From there, the cod will be delivered to premium live markets in Boston, MA.

Cod Tracking in the Net Pen

A US Coast Guard navigational buoy was retrofitted to hold a Hydroacoustic Technologies Inc. (HTI) receiver, CPU, digital video recorder (DVR), and battery banks. The buoy was moored to the center of the offshore grid and cables were attached to the cage for placement of hydrophones, video cameras and a CTD. Cod were implanted with an ultrasonic transmitter that allowed them to be tracked every two seconds, in three dimensions by the four hydrophones, receiver and HTI software (Fig. 2). The three cameras inside the cage (top, middle, and bottom) monitored the overall position of the cod population, as well as their movements in the vicinity of the feed delivery tube. Finally, oceanographic parameters at the site were collected from the project's environmental monitoring buoy[3] and used to correlate fish behavior with environmental conditions.

To date we have tracked a total 48 juvenile cod for a total of 1718 hours, yielding an estimated ~45 billion data points. Additionally, the DVR has captured large-scale movements of the population for hundreds of hours. We are currently analyzing those data and we plan to submit a paper for publication in the spring, and present our results at the World Aquaculture Conference in February 2007.

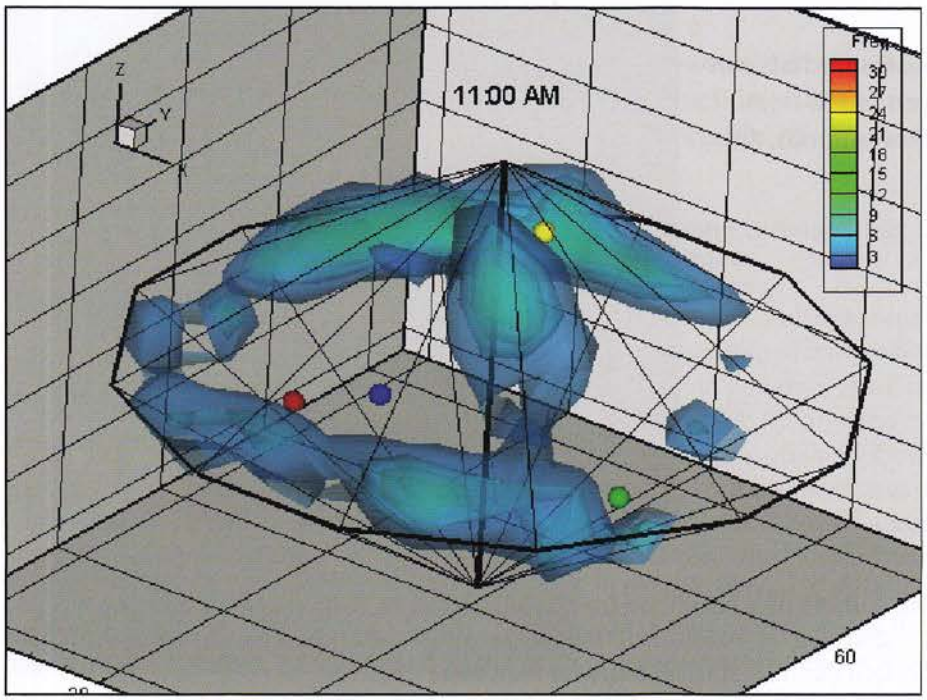


Figure 2
Density plot of tagged cod inside a 3000-m^3 Sea Station™ fish cage.

Preliminary acoustic and video observations indicate that cod behave independently, swim at an average speed of 0.15-1.0 body length per second and do not feed every day that food is available.

Mussel Production

UNH has developed the technology and production methods to farm blue mussels (*Mytilis edulis*) in a submerged, open ocean environment as reported by Langan and Horton.[4] Removed from the stress of near-shore waters, they grow thinner shells and larger meats.

A commercial fisherman in NH is using this technology to launch the first commercial offshore mussel farm in the US. At its current size of 12 lines, this farm can produce up to 150 000 pounds of mussels annually.

Each longline spans 600 feet and is anchored at each end to the seafloor by two-ton granite blocks. Two clusters of submersible floats raise the line to form

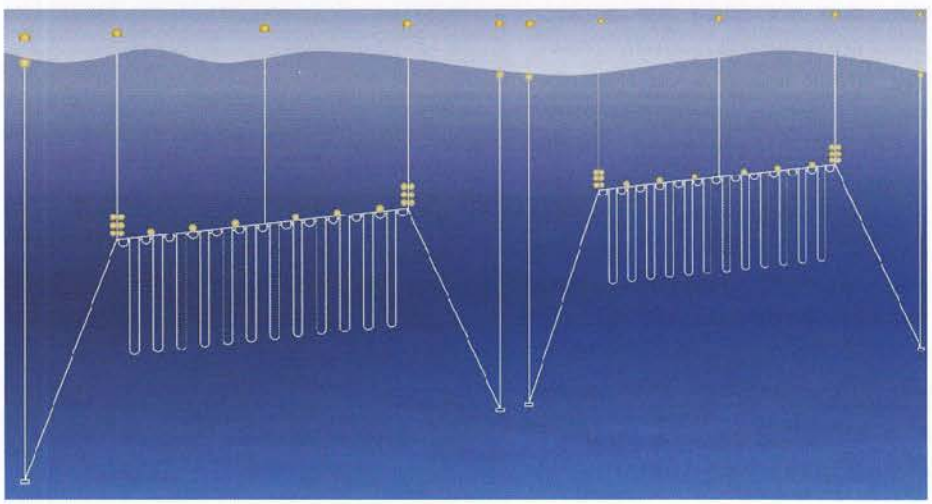


Figure 3
Two submerged mussel longlines.

Figure 4
A 500-m^3 AquaPod™ fish pen being deployed at the Port Authority in Portsmouth, NH.

the corners of a backbone from which loops of mussel grow-out ropes are suspended (Fig. 3). Additional floats along the backbone help maintain the line at the desired depth as mussel biomass increases.

Seed used for the farm came from the project's offshore, finfish cages and local nurseries. Research has demonstrated that with the proper timing, location, and collection apparatus, wild seed collection is a reliable source of juvenile mussels. To seed the growout ropes, the juvenile mussels (20-25 mm) are fed into a custom-designed machine based on New Zealand technology that funnels the mussels onto a growing line. The seeded lines are then covered by a biodegradable cotton mesh, sock to keep the seed in place until the mussels have a chance to attach to the line. The strong rope core allows farmers to extend the length of the growout lines and take advantage of vertical depth. Once seeded, the lines are lowered into the water for growout. The longlines are tended using hydraulic-powered starwheels, especially designed for this application. Growout takes up to nine months, during which the farmer must periodically inspect the crop and add additional floatation to maintain the depth most appropriate for growth.

When the mussels grow to 55 mm, they are ready for market. They are removed from the growing ropes using a custom-designed stripping machine and then washed, declumped, and de-bearded (byssal threads removed) for marketing.

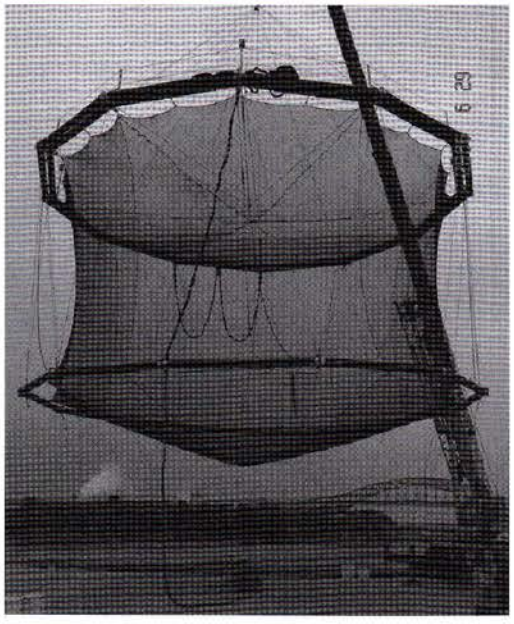


Figure 5
The JPS/SBIR cage being deployed at the Port Authority.

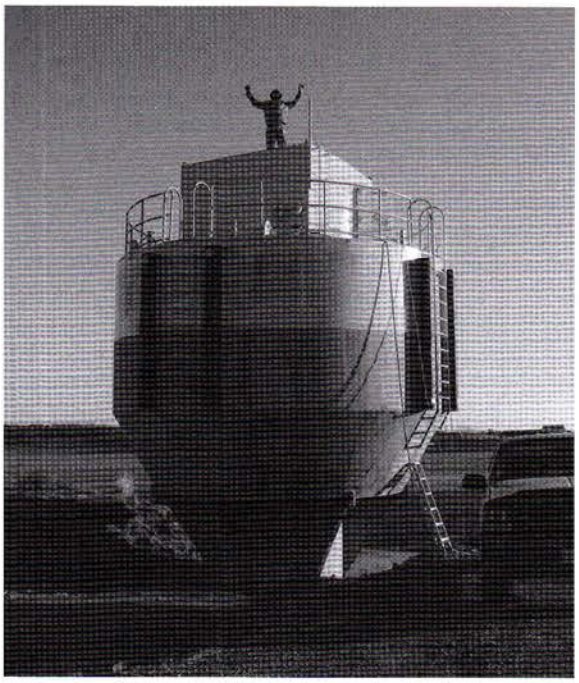


Figure 6
The 20-ton feed buoy under construction at AEG, Hillsborough, NB, Canada.

Evaluating Submersible Fish Cages

Innovative, sub-sea cages have been numerically modeled, tank-tested, and in some cases field-tested at the project's offshore facility. One such system was a 500-m^3 AquaPod™ cage developed by Ocean Farm Technologies, LLC (Fig. 4). This rigid, spherical net pen was also measured for drag force and fluid velocity to investigate flow reduction as it was towed offshore.[5] During the seven-month deployment, the cage maintained its structural integrity in the energetic sea conditions of the North Atlantic.

Another system analyzed at UNH was a low cost, submersible, plastic net pen. A NOAA Small Business Innovation Research (SBIR) grant was awarded to JPS Industries and UNH to develop and evaluate an 1100-m^3 net pen. Buoyancy was controlled by flooding or inflating the upper and lower collars and an airlift chamber attached to the cage and counterweight (Fig. 5). The airlift would allow the cage to be raised in stages thus allowing cod to decompress before being harvested for live marketing.

Hydrostatic tests demonstrated that the system could be submerged and re-surfaced in steps.[6] In December 2006, the cage was recovered from the offshore site and is undergoing a thorough engineering analysis of components and wear points. These results will be taken into consideration before the full-scale version is constructed in the summer of 2007.

The American Soybean Association has been funding UNH to support the development of a high-density, low-volume net pen. This 100-m^3 pen utilizes a single point mooring and changes depth depending on sea and current conditions. Results of the engineering assessment of the cage are reported by DeCew et al.[7] and sea trials are expected in NH in the spring of 2007.

Feeding Fish Offshore

Based upon earlier experiences developing automated feeders for submerged, open ocean feed farms, an SBIR grant was awarded to UNH and Nets Systems for the design and construction of a 20-ton feed buoy. The buoy is needed to increase feeding capacity at the farm and to serve as a communication and control platform for remote operation and observation at the main campus in Durham, NH. The buoy is unique in that it feeds submerged cages hydraulically and has been designed to withstand storm waves over 10 m in height. To aid the design process, computer and physical scale modeling was conducted at the Jere Chase Ocean

Engineering Lab. In February of 2006, a construction award was granted to Aquaculture Engineering Group Inc. (AEG). Construction is ongoing in Hillsborough, NB Canada with a delivery date to the OOA site in the spring of 2007 (Fig. 6).

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