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Hemoglobin type, egg buoyancy, and antifreeze glycoprotein production as mechanisms for adaptive variation in Gulf of Maine Atlantic cod (Gadus morhua)

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HEMOGLOBIN TYPE, EGG BUOYANCY, AND ANTIFREEZE GLYCOPROTEIN PRODUCTION AS MECHANISMS FOR ADAPTIVE VARIATION IN GULF OF MAINE ATLANTIC COD (*GADUS MORHUA)*

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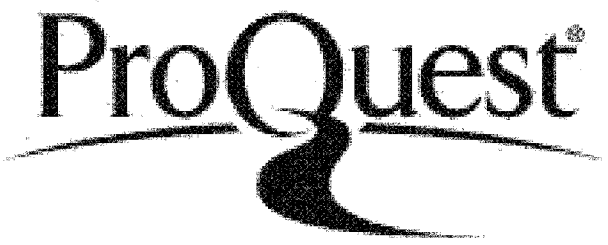

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ABSTRACT

HEMOGLOBIN TYPE, EGG BUOYANCY, AND ANTIFREEZE GLYCOPROTEIN PRODUCTION AS MECHANISMS FOR ADAPTIVE VARIATION IN GULF OF MAINE ATLANTIC COD (*GADUS MORHUA*)

by

Amanda Clapp

University of New Hampshire, September, 2012

Three physiological traits were examined to compare captive populations of winter- and spring-spawning cod from the Gulf of Maine, as differences in these traits have been observed in other locally-adapted subpopulations. Daily mean neutral buoyancy of hatchery-spawned eggs was observed at three temperatures (5, 10, and 12°C) and three photoperiods (15:9, 9:15, and 24:0 L: D) at 12°C. Body fluids of larvae held at 0°C for five days were tested for antifreeze glycoproteins (AFGPs) to determine the onset of AFGP production. Juveniles held at 0°C were bled between 20 and 35 days to determine induction time for AFGP production. Hemoglobin type was determined by isoelectric focusing of blood from hatchery-reared adults. No significant differences were found in mean neutral buoyancy (~1.024 g/mL) or hemoglobin type (mostly HbI-1/2). No AFGPs were detected in larvae. AFGPs were first produced by juveniles on day 30, although no AFGP production differences were found between populations.

CHAPTER I

INTRODUCTION

Locally-adapted subpopulations of Atlantic cod, *Gadus morhua*, are found on both the northwest and northeast Atlantic coasts and exhibit differences in morphology, behavior and physiological characteristics (Moen et al., 2008, Nielsen et al., 2009, Bradbury et al., 2010). As cod adapt to local environmental conditions, they form metapopulations with distinct spawning aggregations and varying stock dynamics (Heath et al., 2008, Holmes et al., 2008, Hutchinson, 2008). The intrastock diversity has a genetic basis and has been linked to environmental gradients such as temperature and salinity (Hutchings et al., 2007, Hutchings and Fraser, 2008, Nielsen et al., 2009). In addition to genetic differences (Beacham et al., 2002, Knutsen et al., 2003, 2011, Pampoulie et al., 2006, O'Leary et al., 2007), locally-adapted populations differ in life history characteristics (Olsen et al., 2004), timing of spawning (Kovach et al., 2010), migration patterns (Robichaud and Rose, 2004, Wright et al., 2006), phenotypes (Marcil et al., 2006), growth and survival (Case et al., 2006, Hutchings et al., 2007, Jónsdóttir et al., 2008), reproduction (Westin and Nissling, 1991), antifreeze glycoprotein production (Goddard et al., 1999), and hemoglobin polymorphisms (Sick, 1965a, b, Andersen et al., 2009). Recognizing biologically meaningful management units based on this intrastock

diversity is needed to preserve diversity and manage subpopulations effectively (Secor et al., 2009, Schindler et al., 2010).

Two cod stocks are recognized in US waters by current fishery regulations, however, more genetic subgroups have been recently identified (Wirgin et al., 2007, Kovach et al., 2010). Three genetically distinct stocks were identified in US waters (Kovach et al., 2010). One group, the Northern Spring Complex (NSC), spawns in the spring in the coastal waters of the Gulf of Maine at Bigelow Bight, Ipswich Bay, and Massachusetts Bay. The Southern Complex (SC) spawns in the spring at Stellwagon Bank, and in the winter at Jeffrey's Ledge and Massachusetts Bay, as well as at Nantucket Shoals and Cox Ledge. The third group spawns in the winter at the northeast peak of Georges Bank. The NSC and SC were found to differ at *Pan*I and Gmo132, two genetic loci that exhibit natural selection. The Georges Bank group was found to be intermediate between the other two. The difference in spawning season at overlapping locations and in *Pan*I and Gmo132 suggest that these two stocks have adapted to the same environment in different ways.

Adaptive variation has also been found at other loci and specifically associated with local environmental conditions. For instance, Canadian and European cod stocks show differences in hemoglobin type, egg buoyancy, and antifreeze glycoprotein production that have been associated with temperature and salinity gradients (Nissling and Westin, 1991, Goddard et al., 1999, Petersen and Steffensen, 2003). To date, no studies have investigated the link between environmental conditions and adaptive variation in US cod stocks.

Atlantic cod has been the focus of a major commercial fishery for hundreds of years. Over the past hundred years, the number and size of cod landed has decreased dramatically due to increased fishing pressure (Lilly et al., 2008), and perhaps environmental changes. Although supply has diminished, the demand for cod remains high, and cod aquaculture has been viewed as a possible method to bridge this gap. Currently, cod aquaculture is underway in Europe and Canada, as well as the United States. To be successful, maximum growth must be attained at the lowest possible cost and this may be realized by matching locally-adapted stocks with their most favorable environmental conditions. Previous studies on European and Canadian cod stocks have found that genetic differences in antifreeze glycoprotein production and hemoglobin type may be associated with phenotypes that demonstrate improved growth under specific environmental conditions. This information may be useful to culturists tailoring stocks to site-specific conditions.

Hemoglobin

Hemoglobin is an oxygen-binding tetrameric protein in the red blood cells of vertebrates. The molecule typically consists of two heterologous alpha and beta polypeptide subunits, each surrounding an iron-centered porphyrin ring (heme). The ferrous iron binds oxygen for transport and the interactions with the polypeptide subunits specifies the oxygen affinity.

Cod have multiple hemoglobin isoforms which are controlled by a gene with two codominant alleles (Andersen et al., 2009, Borza et al., 2009, Halldórsdóttir and Arnason, 2009). Cod can be homozygous for HbI-1 or HbI-2, or heterozygous (HbI-1/2). Types HbI-1 and HbI-2 differ by two amino acid substitutions in the beta chain (Andersen et al.,

2009). Other, less common, isoforms have also been found in some geographic locations (Jamieson and Birley, 1989, Husebo et al., 2004). In Europe, hemoglobin genotype has been associated with geographic origin (Sick, 1965a, b, Jamieson and Birley, 1989, Fyhn et al., 1994, Nordeide and Pettersen, 1998) such that cod in higher (e.g. Greenland) and lower (e.g. North Sea) latitudes are more likely to have HbI-2, and HbI-1 genotypes, respectively (Sick, 1965b, Petersen and Steffensen, 2003).

The Hb subtype associated with a particular cod population may have important implications for aquaculture, as the amino acids comprising the polypeptide chains can influence oxygen binding, and possibly growth, at differential water temperatures and oxygen concentrations. For instance, HbI-2 has been shown to have higher oxygen binding affinity at low temperatures, and cod with this Hb isoform have higher growth rates in cooler waters compared to those with the HbI-1 genotype (Peterson and Steffensen, 2003, Imsland et al., 2007). Gamperl et al. (2009) suggested that cod with HbI-1 may have lower initial survival under some conditions based on results from heterozygous crosses. HbI-2 cod also have greater tolerance and less of a stress response to hypoxia (Brix et al., 1998, Methling et al., 2010). Collectively, these studies suggest HbI-2 cod may have better growth at cool temperatures, even in crowded aquaculture conditions.

Egg Buoyancy and Development

Egg and larvae retention in spawning areas is necessary for maintaining demographically distinct subpopulations of cod (Stenevik et al., 2008). As cod eggs are pelagic, their vertical distribution in the water column is one factor that affects their transport and the degree of larval retention (Sundby, 1983, Huret et al., 2007). Pelagic, marine teleost eggs

that lack oil globules obtain buoyancy by hydration during maturation (post-vitellogenesis) in the ovary. This occurs by the proteolysis of yolk proteins into free amino acids, which creates an osmotic gradient and an influx of water (Craik and Harvey, 1987). Consequently, pelagic eggs are typically 90-94% water (Craik and Harvey, 1987, Thorsen et al., 1996).

The degree of buoyancy obtained by cod eggs is not uniform throughout their range and is dependent on genetic and environmental factors. Anderson and de Young (1994) demonstrated that the density of Newfoundland cod eggs was influenced by the ambient salinity in which the fish spawned. In the brackish waters of the Baltic Sea, cod spawning is restricted to deeper basins with higher salinities (10-18 ppt; Nissling and Westin, 1991), and physical and biochemical changes in egg structure are necessary to attain buoyancy (Kjesbu et al., 1992, Thorsen et al., 1996). Relative to marine stocks, eggs produced by Baltic cod are larger, have lower specific gravity, higher water content and thinner chorions (Kjesbu et al., 1992, Thorsen et al., 1996). While it is not clear in cod if these differences are due to physiological adjustments to environmental conditions or genetic adaptation, in other Baltic species (e.g. flounder; *Platichthys flesus* and plaice; *Platessa platessa*), studies have shown that these changes are due to genetic selection (Solemdal, 1967, 1973). It has been hypothesized that differential egg buoyancy among subpopulations contributes to local retention in Norwegian fjords (Myksoll et al., 2011). In support of this contention, genetic differences were found in fish from locally adapted populations that differed in egg buoyancy in controlled spawning experiments conducted in full strength seawater (Stenevik et al., 2008).

Antifreeze Proteins

Antifreeze proteins (AFPs) and glycoproteins (AFGPs) are used by many species of plants and animals to cope with freezing temperatures (Harding et al., 2003). Proteins conveying freeze resistance have been found in many unrelated species such as spiders, mites, insects (including *Coleoptera, Collembola, Plecoptera, Orthoptera, Hemiptera, Mecoptera, Lepidoptera, Diptera, Neuropteran*), and plants (including winter rye, kale, carrots, winter wheat, and perennial ryegrass) (Li et al., 2005). This appearance of AFPs and AFGPs in many unrelated species makes it likely that each group developed the proteins independently (i.e. convergent evolution; Logsdon and Doolittle, 1997). Understanding antifreeze proteins and glycoproteins has several commercial applications as they can be used to make cells cold tolerant, leading to applications in food preservation, genetic engineering of freeze resistant crops, and cryosurgery (Goddard and Fletcher, 1994).

These proteins are made by the organisms in response to environmental cues, which vary by species (Hew et al., 1981, Fletcher et al., 1982b, 1987). Most fishes living in waters that reach temperatures below freezing for any portion of the year produce AFPs that can usually be found in their blood. Without these proteins, tissues, particularly those in contact with the water (e.g. gill filaments), would incur damage from freezing. AFPs and AFGPs depress the plasma freezing point by inhibiting growth of ice crystals (Harding et al., 2003, Zepeda et al., 2008).

The freeze resistance mechanisms of fishes living in seawater below freezing temperatures have been studied since the late 1950's. Blood components (urea, glucose, glycerol, Na+, K+, Cl-, and non-protein nitrogen) of supercooled Arctic fishes were

investigated by Gordon et al. (1962), but none of the components measured were present in high enough concentrations to give the degree of freezing point depression observed. Analysis of blood components from three fishes from the genus *Trematomus* in the Antarctic revealed that glycoproteins were responsible for at least 30% of the observed freezing point depression (DeVries and Wohlschlag, 1969). These glycoproteins had a composition of approximately 50% protein and 34% carbohydrate, with alanine, threonine, and galactosamine comprising the major components. Further investigation of these glycoproteins in Antarctic fishes (*Trematomus* and *Dissostichus*) indicated additive effects with other blood components contributing to freezing point depression (DeVries et al., 1970).

Arctic cod, *Boreogadus saida,* was also found to have antifreeze glycoproteins very similar to those of *Trematomus* (Osuga and Feeney, 1978). The general sequence of these glycoproteins was determined to be an alanine-alanine-threonine repeat with galactosamine attached to each threonine, with few amino acid substitutions. Of the eight differently sized AFGPs (numbered 1-8), the smaller ones (6-8) demonstrated sequence differences in number and position of proline substitutions between *Trematomus* and *Boreogadus*. Hew et al. (1981) identified AFGPs in captive Atlantic cod held in captivity under ambient winter conditions. AFGPs from Atlantic cod were similar to those of other northern cods, with molecular weights from 2.5 to 33kDa and synthesized primarily in the liver (Hew et al., 1981, Cheng et al., 2006).

In diverse fishes, four types of antifreeze proteins, without glycoprotein moieties, have been identified (numbered I-IV). Type I AFP, found in sculpins (*Myoxocephalus scorpius, M. verrucosus*), right-eyed flounders (e.g. *Pleuronectes americanus*), and

cunner (*Tautogolabrus adspersus*) is a 3.3-4.5 kDa alanine-rich α-helix (Fletcher et al., 1982a, 2001, Kao et al., 1986, Harding et al., 2003). Sea ravens (*Hemitripterus americanus*), smelt (*Osmerus mordax*), and herring (*Clupea harengus*) have type II AFPs, that are 11-24 kDa proteins with 14% alanine, many disulfide bonds, all 20 common amino acids, and about 7.6% half cystine (Kao et al., 1986, Ewart et al., 2000, Fletcher et al., 2001, Harding et al., 2003). Haddock (*Melanogrammus aeglefinus*) may also have type II AFP, but only very low levels have been detected (Ewart et al., 2000). Type III AFP is a 6.5 kDa β-sandwich with little alanine that is found in ocean pout (*Macrozoarces americanus*), wolfish (*Anarhichas lupus*), and eel pout (*Lycodes reticulatus*) (Fletcher et al., 2001, Harding et al., 2003). Type IV AFP, found in longhorn sculpin (*Myoxocephalus octodecimspinosus*) is a 12 kDa alanine rich helical bundle (Fletcher et al., 2001, Harding et al., 2003). Of the various types of AF(G)Ps found in fishes, freeze-resistance is positively correlated with size and the presence of carbohydrate moieties, such that greatest protection is conveyed with AFGPs over 10 kDa (Kao et al. 1986).

Photoperiod and temperature are the primary cues for seasonal cycles of AF(G)P production, and the specific cue(s) are species-specific. Ocean pout have been shown to have cycles triggered by water temperature, whereas Atlantic and spotted wolfish have cycles triggered by photoperiod and require low water temperatures for AFP synthesis (Desjardins et al., 2006). Several fishes that produce antifreeze proteins have been found to show differences in production levels or patterns based on their geographic distribution or AFP gene copy number. For instance, greater AFP production is seen in Arctic fishes, where production is constant, year-round, compared to cyclical (seasonal) production

seen in fishes from warmer areas (e.g. shorthorn sculpin; Fletcher et al., 1982a, Enevoldsen et al., 2003). Production may also be greater within species along a latitudinal range, as in the case of ocean pout and winter flounder where individuals from more northern areas produce greater AFP, due to AFP gene amplification, than individuals from more southerly (and milder) regions (Fletcher et al., 1985, Scott et al., 1985, Hayes et al., 1991). Population differences in AFGP production have also been found in Atlantic cod. Goddard et al. (1999) collected juvenile cod from four locations around Newfoundland, held them under ambient conditions, and analyzed monthly blood samples for levels of AFGP. They found that the northern most group had the highest AFGP concentration and also the fastest clearance rate. They theorized that because high levels of AFGPs in the blood increase its viscosity, rapid clearance was advantageous to reduce metabolic stress on the circulatory system.

The most common technique used to analyze AF(G)Ps in blood is with a nanolitre osmometer. The osmometer can be used to raise and lower the temperature of a very small sample (3 nanoliters) to determine its freezing and melting points. The difference between those temperatures is the thermal hysteresis (TH), which is a direct indicator of the amount of AF(G)P present in the sample (Kao et al., 1986). Baseline levels of TH vary between studies, but are usually between 0.04 and 0.08°C in Newfoundland cod (Goddard et al., 1992, 1997) and therefore, 0.10°C is generally indicative of AF(G)P's presence. When present, AF(G)Ps also alter the shape of the ice crystals, resulting in pyramidal or hexagonal growth (i.e. crystals with defined planes) (Ewart et al., 2000, Fletcher et al., 2001, Enevoldsen et al., 2003, Zepeda et al., 2008). It has further been

shown that AFGPs bind ice crystals to halt growth, which is resumed only after AFGP detachment (Zepeda et al. 2008).

The antifreeze production cycle of winter flounder has been studied in depth. Fletcher et al. (1984) found that in this species AFP production is triggered by deceasing photoperiod, and that the clearance by increasing temperature. AFP production is induced when the pituitary stops releasing a synthesis inhibitor that works at the transcription level in the liver (Fletcher et al., 1984, Fourney et al., 1984). The synthesis inhibitor was identified as growth hormone (GH) by Idler et al. (1989), who administered the hormone to control and hypophysectomized fish. GH and AFP levels are inversely correlated during the winter months in this species.

While less is known about antifreeze glycoprotein synthesis in cod, the cues for initiation of production have been studied. Fletcher (1982b) determined that cod in Newfoundland have a seasonal antifreeze production cycle, with AFGPs first appearing in January, peaking in February and March, and disappearing by July. Investigations with adult cod sampled monthly while held under combinations of ambient or controlled light cycles (18:6 or 24:0 L:D), and ambient or controlled temperature (0-5°C), demonstrated that low temperature (<2°C), not light, initiated antifreeze production (Fletcher et al., 1987). This study also examined plasma clearance rates at different temperatures, and determined that AFGPs clearance was faster at higher temperatures, but eventually cleared even at 0°C. This suggests that day length and temperature both likely play a role in regulating AFGP clearance (Fletcher et al., 1987).

Studies of Antarctic notothenioids demonstrated that eggs and larvae of these species do not have enough AFGPs to convey freezing-resistance, but that the chorion of

the egg, and later the integument of the larvae, must act as barriers to freezing until sufficient antifreeze levels can be attained (Cziko et al., 2006). This is similar to freezing-resistance in cod embryos and larvae. Valerio et al. (1992) did not detect antifreeze glycoproteins in cod embryos or larvae, but also found a high degree of freeze resistance, especially in embryos. Similar to Antarctic notothenioids, the chorion and larval integument must act as a barrier to ice propagation.

Juvenile and adult cod exhibit different levels of antifreeze production and thermal preference ranges. A study of wild, adult and juvenile cod held under ambient conditions demonstrated that juveniles produce greater levels of AFGPs than adults (Kao and Fletcher, 1988). Juvenile cod with lengths of 15 to 40 cm were shown to have the highest AFGP production of any size juveniles when held under ambient temperature and light conditions, with induction occurring at approximately 2°C (Goddard et al., 1992). By frequently sampling adult cod held in captivity under ambient conditions, Goddard et al. (1994) determined that it required about 10 days after exposure to 0°C water for AFGPs to appear in the blood. Using this information, they were able to determine the thermal history of cod in the bays of Newfoundland. They found that cod that stay inshore tend to inhabit the pockets of warmer water for as long as possible, and then return to them as they reform in the spring, in order to avoid the subfreezing water temperatures (Goddard et al., 1994). Laboratory investigations of thermal preference in adult and juvenile cod with and without AFGPs found that adults prefer colder waters than juveniles, and that juveniles with AFGPs were more likely to enter cold water than those without AFGPs (Goddard et al., 1997). They hypothesized that brief encounters with colder water may be a behavioral mechanism to increase AFGP production.

Cod aquaculture was initiated in the US in 1999 with the first commercial harvest occurring in 2009. Little is known about the stock-specific characteristics of the cod inhabiting US waters and the following experiments were undertaken to gain information about the mechanisms that maintain stock diversity and whether unique traits may benefit breeding efforts for the nascent aquaculture industry.

CHAPTER II

INVESTIGATIONS OF ADAPTIVE VARIATION IN GULF OF MAINE ATLANTIC COD (*GADUS MORHUA*)

Introduction

Biological diversity, in the form of genetic, phenotypic, behavioral or other adaptive variation, contributes to the stability and long-term sustainability of exploited species (Schindler et al., 2010). This population or intrastock diversity promotes adaption to local environmental conditions and may confer resilience to environmental change, and should therefore be considered explicitly in fisheries management strategies (Hilborn et al., 2003, Hutchinson et al., 2008, Secor et al., 2009). Instrastock diversity has long been recognized as an important characteristic of anadromous species, notably salmonids (Taylor, 1991); there is now increasing recognition of its importance in marine systems (Conover et al., 2006, Hauser and Carvalho, 2008, Selkoe et al., 2008).

Atlantic cod (*Gadus morhua*) is one of the most studied examples of a marine species known to exhibit intrastock diversity throughout its range. Atlantic cod exist as metapopulations with distinct spawning aggregations and population genetic structuring on various spatial scales (e.g., Knutsen et al., 2003, O'Leary et al., 2007, Heath et al., 2008). Intraspecific diversity is manifested in variation in life histories (Olsen et al., 2004), timing of spawning (Kovach et al., 2010), migration patterns (Robichaud and Rose,

2004, Wright et al., 2006), phenotypes (Marcil et al., 2006), growth and survival (Case et al., 2006, Hutchings et al., 2007, Jónsdóttir et al., 2008) and reproduction (egg buoyancy and sperm motility; Westin and Nissling, 1991). Observed variation in life history traits has been shown to have a genetic basis and be linked to environmental gradients (Hutchings et al., 2007, Hutchings and Fraser, 2008, Nielsen et al., 2009). Genome scans have identified adaptive divergence correlated with gradients in temperature and salinity on broad and local scales in both the western and eastern Atlantic (Moen et al., 2008, Nielsen et al., 2009, Bradbury et al., 2010). In the northeast Atlantic, polymorphisms have been identified in several markers under natural selection, including variation in the pantophysin I (*PanI)* locus (e.g. Petersen and Steffenson, 2003, Pogson and Fevolden, 2003) according to selection pressures related to temperature, salinity, and depth (Karlsson and Mork, 2003, Case et al., 2005, Arnason et al., 2009), temperature-associated hemoglobin polymorphisms (Sick 1965a, b, Andersen et al., 2009), and geographic variation in antifreeze protein production (Goddard et al., 1999). These findings suggest a potentially strong role of local adaptation in structuring populations of northeast Atlantic cod.

Comparatively less is known about intrastock diversity in northwest Atlantic cod. In the Gulf of Maine cod stock in U.S. waters, a potentially important component of intrastock diversity occurs in the form of spatially overlapping, but seasonally separated, and genetically distinct spawning aggregates in Ipswich and Massachusetts Bays (Wirgin et al., 2007, Kovach et al., 2010). Genetic differentiation between these population complexes is primarily a result of differentiation at non-neutral loci (either directly or indirectly influenced by selection), suggesting a role for adaptive divergence (Kovach et

al., 2010). Potentially limited ecological exchange between the two population groups is also evidenced by divergent patterns of adult movement (Howell et al., 2008, Tallack, 2009) and larval recruitment (Churchill et al., 2011). The purpose of our study was to investigate the potential role of adaptation in maintaining differentiation of these populations. We hypothesized that the two populations developed differences in their physiological responses as a result of the different thermal regimes experienced by their early life history stages in the wild. We tested this hypothesis using controlled experiments with captive broodstock from the two divergent Gulf of Maine spawning populations. Specifically, we investigated differences in two factors, egg buoyancy and antifreeze protein production, which have been previously shown to vary among populations of Atlantic cod (Nissling and Westin, 1991, Goddard et al., 1999).

Methods

Broodstock

Wild-caught, adult cod from the coastal spring and winter-spawning populations were maintained in captivity for > 5 years in separate circular tanks (4.6 m diameter x 1.8 m depth) that were incorporated into recirculating systems (Great Bay Aquaculture LLC; Portsmouth, NH). All fish were caught between Cape Ann and Portsmouth, NH and were in spawning condition at the time of capture. The broodstock were maintained on a simulated natural photo-thermal regime with temperatures fluctuating from 5-10°C and lighting (20 lux, crepuscular; 70 lux, daylight) provided by incandescent lamps. The broodstock were fed a custom-made diet comprised of fishmeal and squid hydrosylate and commercial diets (Breed M, Inve, Salt Lake City, UT, USA; Vitalis-Cal, Skretting, Cojobar, Spain), 2-3 times weekly. The winter and spring-spawning fish maintained

separate spawning periods (December- January; May-June) and volitionally spawned annually. Fertilized eggs were collected in 1.0 mm nylon mesh egg collectors that were emptied each morning during the spawning seasons. For juvenile studies, embryos were incubated at 10°C until hatch and larvae were initially fed enriched rotifers (Ori-Green, Skretting) and weaned to Skretting Gemma Micro. Juveniles were maintained at 10-12°C and fed appropriate size commercial diets (Skretting Gemma Diamond) until the experiments were conducted.

Effects of Temperature on Egg Buoyancy

Fertilized eggs (< 24 hrs post-spawning) were collected from winter and spring-spawning fish on 3 dates and transferred to 3 controlled temperature rooms (5, 10, and 12°C) at the University of New Hampshire (UNH). On each collection date, 75 mL of eggs were incubated in two McDonald jars containing 3 L of filtered seawater (35 ppt) at each temperature. All rooms were maintained on 24-hr light, and dead eggs were removed and 50% water changes were performed daily.

Mean neutral buoyancy, the salinity at which eggs and water were of equal density, was determined daily from two days post-spawn until hatching using modified methods of Liu et al. (1993). Triplicate, borosilicate glass tubes (15 x 2 cm), delineated in 1.0 cm increments, were filled to the 10 cm mark with filtered (5 um) seawater ranging from 28 to 35 ppt in 0.5 ppt increments. Polypropylene transfer pipettes were used to gently position 10 eggs/tube 1cm below the water surface and the relative position of all eggs was recorded after 20 minutes. Eggs suspended in the water column 2-8 cm from the surface were considered neutrally buoyant and the mean buoyancy was calculated for the eggs in each tube.

Three to six eggs were also observed with a dissecting microscope (Leica S8APO, Leica Microsystems, Richmond, IL, USA) and photographed with a digital camera (Canon S40 Powershot, Canon, Lake Success, NY, USA) daily for determination of developmental stage. Developmental stages between fertilization and hatch were identified based on the descriptions of Hardy (1978), Thompson and Riley (1981) and Geffen and Nash (2011); IB (blastula – embryonic axis), II (embryonic axis – blastopore closure), III (embryo 180-270° around yolk), IV (embryo 270-360° around yolk), V (embryo 360° around yolk – beginning of hatching).

As water density is influenced by temperature, for comparisons, densities were normalized using guidelines established by the Intergovernmental Oceanographic Commission (IOC, 2010), and embryonic development in the different temperature treatments was normalized by degree days (days post spawning x temperature °C).

Effects of Photoperiod on Egg Buoyancy

As light has been shown to affect egg buoyancy in halibut (Mangor-Jensen and Waiwood, 1995), an experiment was conducted to determine photoperiodic effects on developing cod embryos. Fertilized eggs from the spring-spawning stock were collected on 3 dates and transported to a controlled-temperature room (12°C) at the University of New Hampshire (UNH). On each collection date, the eggs were divided into 9 -13.5 mL aliquots and incubated in triplicate 1 L glass beakers containing 500 mL of filtered seawater (35 ppt) in simulated winter (9 hr light: 15 hr dark), simulated summer (15 hr light: 9 hr dark), and constant light. Photoperiod treatments were separated by heavy black polyethylene to block extraneous light. Incandescent bulbs (~75 lux) provided illumination in all treatments. Dead eggs were siphoned from the bottom of beakers and

50% water changes were conducted daily. All maintenance and sampling was performed during the daylight period of all treatments. Buoyancy was measured as indicated above.

Antifreeze Glycoprotein Production in Juveniles

Juvenile cod from both spawning stocks were transported to the Aquaculture Research Center (UNH) and held in 1600-L tanks that were incorporated into separate recirculating systems. The fish were held for approximately 120 days at 10-12°C, and fed appropriate size feed until the start of the experiments. Preliminary experiments using winter-spawned juveniles (17-25 cm, 0+ yr) determined that AFGP was not induced in fish held at held at 0 or 2°C for 14 days. Therefore, time-course experiments were conducted using juvenile fish held at 0°C for both spawning populations.

Seven winter and spring-spawned juveniles (13-24 cm, 0+ yr) were held in a controlled- temperature chamber comprised of a 74-L insulated aquarium with a plexiglass lid, attached to a benchtop chiller (Neslab RTE-740, Thermo Scientific, Portsmouth, NH, USA). The water temperature was lowered from 12°C to 0°C by 0.5°C per day and was thereafter held constant. Temperature, dissolved oxygen, and water quality were monitored daily and fish were not fed throughout the trial. On days 20, 25, 30, and 35 the fish were bled from the caudal vasculature using 1ml tuberculin syringes fitted with 26-gauge ½" needles. One fish was bled on day 20, and two fish were bled on each of the other days. The blood was transferred to heparinized microfuge tubes with 40 µL aprotinin maintained on ice until centrifuged (4°C and 10,000 rpm for 7 min; Beckman Coulter Microfuge 22R Centrifuge, Beckman Coulter, Brea, CA, USA). The plasma was recovered and stored at -70°C for later analysis, and blood samples from fish held at 10°C were used as controls.

Thermal hysteresis of plasma samples was determined with a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand) following the method of Kao et al. (1986). Drops of plasma ($\leq$ 1 μL) were placed in Cargill's B immersion oil in the wells of the sample disc on the cooling stage of the osmometer. Observations of ice crystal formation (freezing point) and melting (melting point) were made using a compound microscope at 100x (Olympus compound microscope; Olympus, Center Valley, PA, USA) and corresponding temperatures were recorded. Antifreeze (glyco) proteins decrease plasma freezing points without affecting melting points (Kao et al., 1986). The differences in freezing and melting points (thermal hysteresis) were calculated. The shape of the frozen plasma crystal was also noted, as pyramidal crystal growth is indicative of AFGP production (Zepeda et al., 2008). In addition, antifreeze glycoprotein presence was confirmed using 2-dimensional (2-D) electrophoresis, as described below (Chen et al., 1997).

Plasma was added to 4 volumes of isoelectric focusing (IEF) buffer (8M urea, 2% Triton X-100, 40mM DTT, 0.002% bromophenol blue, 0.5% IPG buffer; GE Healthcare, Piscataway, NJ, USA) and protein content was determined (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). Plasma samples corresponding to 200 μg protein were brought to 250 μL total volumes with DeStreak Rehydration Solution (GE Healthcare) and were subjected to IEF on nonlinear pH 3-10 Immobiline Drystrips (GE Healthcare) on an Ettan IPGphor II (Amersham Biosciences, Piscataway, NJ, USA) with the following running conditions: rehydrate 12 hours, step & hold 500 V for 1 hour, gradient 1000 V for 1 hour, gradient 8000 V for 2 hours 30 minutes, and gradient 8000 V for 25 minutes.

To prepare for second dimension electrophoresis, IEF gels were rinsed in dH_2O for 10 minutes, and then placed in equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 6% β-mercaptoethanol) for 15 minutes. Excess equilibration buffer was removed and gels were rinsed with SDS running buffer (2.5 mM Tris base, 19.2 mM glycine, 0.01% SDS).

First dimension IEF gels were embedded in 1% (w/v) agarose in SDS running buffer at the top of the 15% SDS-PAGE gels (15% acrylamide-bisacrylamide solution, 0.1% SDS, 0.36 M Tris-HCl, 0.002% TEMED, 0.002% APS) along with one well of Precision Plus Protein All Blue standards (Bio-Rad). SDS-PAGE gels were run in a Bio-Rad Protein II xi cell (Bio-Rad) with SDS running buffer at 20 mA for 15 minutes and 50 mA for 3 hours. After electrophoresis, SDS-PAGE gels were rinsed in dH_2O for 20 minutes then stained with Coomassie Brilliant Blue R-250 (Fisher Scientific, Pittsburg, PA, USA) for at least one hour. Gels were de-stained overnight (5% acetic, 25% methanol) then rinsed in dH_2O for 1 hour. Images of each gel were captured with a digital camera (DSC-H55, Sony, New York, NY, USA) and examined for the presence of AFGP.

Antifreeze Glycoprotein Production in Larvae

Larval cod from both-spawning stocks were transported from Great Bay Aquaculture (Portsmouth, NH) to the Ritzman Aquaculture Laboratory (UNH) at 5, 15, 30, 45, and 60 days post hatch (dph) and placed into a 74L insulated aquarium attached to a benchtop chiller (Neslab RTE 140, Thermo Scientific, Newington, NH, USA). The water temperature was lowered from 9°C to 0°C by 1°C per 12 hours and then held constant. Larvae were fed age-appropriate diets as follows: <45 dph fed rotifers, 45-60 dph fed Artemia, ≥60 dph fed Skretting Gemma Wean (Skretting, Cojobar, Spain).

Temperature and dissolved oxygen were monitored and debris removed from the bottom of the tank daily. On the day the water reached 1°C and each following day, 0.5 mL of larvae were collected. This volume corresponds to approximately 55, 40, 25, 15, and 5 larvae respectively for the 5, 15, 30, 45, and 60 dph trials.

The collected larvae were rinsed in distilled water, homogenized (sonifier, Branson Ultrasonics Corporation, Danbury, CT, USA), and centrifuged (10000 rpm 5 minutes; Beckman Coulter Microfuge 22R Centrifuge, Beckman Coulter, Brea, CA, USA) and the supernatant was collected and lyophilized (freeze dryer, Labconco Corporation, Kansas City, MO, USA). Samples were stored at -70°C and rehydrated with just enough distilled water to re-dissolve the sample immediately prior to analysis. Sample preparation is based on Valerio et al. (1992). Thermal hysteresis of larval homogenates was determined with a nanolitre osmometer as described above and the shape of ice crystals was also noted.

Statistical Analyses

Mean neutral buoyancy of eggs one day post-fertilization and one day prior to hatch were compared with a paired t-test to determine if buoyancy changed during the incubation period. Comparisons between mean neutral buoyancy of spawning groups and temperature treatments, as well as time to hatch in degree-days, were compared using ANOVA (JMP 8; SAS Institute Inc., Cary, NC, USA). Differences between means with a probability (p) less than 0.05 were considered significant. Thermal hysteresis of samples from common collection dates were averaged for each spawning group.

Results

Effects of Temperature on Egg Buoyancy

No significant differences in mean neutral buoyancy were found between stocks ($p = 0.792$) or treatment temperatures ($p = 0.594$) (Figure 1). Mean neutral buoyancy was approximately 1.024 g/mL and no differences were found in embryonic development rates when normalized by degree days (paired t-test, $p = 0.122$; Figure 2, Table 1). Developmental stages are shown in Figure 3. Mean egg hatch occurred at 88.44 ± 11.21 degree days (Figure 4).

Effects of Photoperiod on Egg Buoyancy

Mean neutral buoyancy data were not found to be normally distributed (Shapiro-Wilk, $p < 0.001$), and non-parametric tests were therefore used for analysis. No significant difference in mean neutral buoyancy was found between photoperiodic treatments (Kruskal-Wallis, $p = 0.795$) and mean neutral buoyancy did not significantly increase or decrease between fertilization and hatching (Wilcoxon Sign-Rank, $p = 0.303$; Figure 5). Mean neutral buoyancy for each photoperiodic treatment was approximately 1.026 g/mL and no differences were found in embryonic development rates (Figure 5, Table 2). Hatch occurred on day 6 after fertilization in all treatments.

Antifreeze Glycoprotein Production in Juveniles

Juveniles produced detectable levels of AFGPs after 30 and 35 days exposure to 0°C, but did not do so after 20 and 25 days (Table 3). Only the spring-spawned juveniles at 35 days had a thermal hysteresis value over 0.10°C (Figure 6), but all samples at 30 and 35 days exhibited pyramidal ice crystal growth (Table 3, Figure 7). Thermal hysteresis of samples at 30 and 35 days were over 0.05°C and demonstrated an increasing

trend from 30 to 35 days (Figure 6). The presence of AFGPs was confirmed in 30 and 35-day samples with 2-D electrophoresis (Table 3, Figure 8).

Antifreeze Glycoprotein Production in Larvae

None of the larval samples had thermal hysteresis values over 0.05°C (Figure 9) or produced ice crystals that exhibited pyramidal growth. Thermal hysteresis levels were comparable for both spawning groups and all age groups examined. These results indicate that the larvae did not produce antifreeze glycoproteins during this experiment.

Discussion

Atlantic cod populations exhibit extensive population diversity, including temperature-associated adaptations on both sides of the Atlantic Ocean (Bradbury et al., 2010). In the present study, we were interested in determining if the genetic differentiation previously observed between winter- and spring-spawning populations in the Gulf of Maine (Kovach et al., 2010) reflect divergent adaptations to differing thermal regimes. We tested for population differences in two physiological factors, egg buoyancy and antifreeze production, known to differ among northwest Atlantic cod populations (Nissling and Westin, 1991, Goddard et al., 1999).

Cod, like many other polar and subpolar marine fishes, produce antifreeze proteins or glycoproteins (AFGPs) to permit survival in water of potentially subfreezing temperatures (Fletcher et al., 1987). In cod, AFGPs produced in response to cold water temperatures bind ice crystals and prevent their growth, thereby improving blood and extracellular freeze resistance and protecting cell membrane integrity (Rubinsky et al., 1990, 1991). In response to AFGP production, experimental plasma freezing points in Newfoundland cod were lowered from summer values of -0.6 - 0.8°C (juveniles and

adults) to approximately -1.5°C and -1.2°C in juveniles and adults, respectively (Hew et al., 1981, Fletcher et al., 1987, Kao and Fletcher, 1988, Goddard et al., 1992). Extended exposure to subfreezing temperatures results in proportionally greater AFGP production throughout the winter months (Goddard et al., 1994). Furthermore, population differences in AFGP production capacity were found in juvenile Newfoundland cod reared in common garden experiments. Those originating from more northerly latitudes produced significantly higher levels (~50%) than those from more southern climes (Goddard et al., 1999).

In the present experiment, juvenile cod from both the spring and winter-spawning complexes produced AFGP after 30 days exposure to cold water. These results are in agreement with those of Fletcher et al. (1987) who observed AFGP production, in levels similar to those observed in this study, after 35 days of cold water (0 °C) exposure. The degree of thermal hysteresis observed in the present study suggests that AFGPs were only produced in low levels, as would be expected by the duration of exposure to cold water. The similar response time for AFGP production may be due to the common environment that both populations inhabit or reflect the fact that juveniles of both populations were approximately the same age and size when subjected to cold-water treatments. AFGP expression in cod is initiated during the juvenile, but not embryonic or larval phases, and therefore in the wild, population-specific production may differ during the juvenile's first winter because of size or age-related differential exposure.

The age and developmental stage at which cod first begin producing AFGPs is unknown, but they were not detected during the embryonic and pre-feeding larval stages in previous studies (Valerio et al., 1992). Valerio et al. (1992) suggested several

mechanisms that prevent freezing during these early stages in the absence of AFGPs. For embryos, ocean currents may transport them out of icy waters and/or extra-embryonic membranes (chorions) may prevent ice formation (Valerio et al., 1992, Cziko et al., 2006). As pre-feeding larvae do not ingest seawater for osmoregulation, and lack external gill filaments for respiration, delicate tissues are therefore not exposed to icy water during this stage. The absence of AFGP in larval samples in the present experiment may indicate that either larval cod can not produce AFGPs, or require longer periods of time at cold temperatures to do so. While problematic because of high larval mortality at cold temperatures, additional studies are necessary to determine the ontogeny of AFGP production.

Egg and larvae retention in spawning areas is necessary for maintaining demographically distinct subpopulations of cod (Stenevik et al., 2008). As cod eggs are pelagic, their vertical distribution in the water column is one factor that affects their transport and the degree of larval retention (Sundby, 1983, Huret et al., 2007). Pelagic, marine teleost eggs that lack oil globules obtain buoyancy by hydration during maturation (post-vitellogenesis) in the ovary. This occurs by the proteolysis of yolk proteins into free amino acids, which creates an osmotic gradient and an influx of water (Craik and Harvey, 1987). Consequently, pelagic eggs are typically 90-94% water (Craik and Harvey, 1987, Thorsen et al., 1996). The degree of buoyancy obtained by cod eggs is not uniform throughout their range and is dependent on genetic and environmental factors. It has been hypothesized that differential egg buoyancy among subpopulations contributes to local retention in Norwegian fjords (Myksoll et al., 2011). In support of this contention, genetic differences were found in fish from locally adapted populations that

differed in egg buoyancy in controlled spawning experiments conducted in full strength seawater (Stenevik et al., 2008).

In the present study, no differences in egg buoyancy were found between the winter and spring-spawning stocks, nor was buoyancy influenced by incubation temperature or photoperiod. Egg buoyancy also did not change during development, which contrasts with the results of Mangor-Jensen (1987) who found decreased density in the days prior to hatch. Inherent variation in buoyancy exists within batches of spawned eggs, and the discrepancy between these studies may reflect the selection of a fraction of uniformly buoyant eggs, during early development, in the study by Mangor-Jensen (1987). While no differences in egg buoyancy between populations were found in the present study, this does not preclude the possibility of egg buoyancy differing seasonally from fish spawning in the Gulf of Maine. In this study, fish from both populations received the same, high-quality diet. In the wild, however, it is possible that prey availability and quality differs during the population-specific periods of vitellogenesis. Kjesbu et al. (1992) demonstrated that cod egg buoyancy is dependent upon broodstock condition (nutrition) prior to the spawning period. In well-fed (high condition) fish, specific gravity and chorion thickness decreased with successive spawning but remained constant in low condition fish. It is also possible that environmental salinity levels, and commensurate buoyancy, may differ between the respective spawning seasons of the spring and winter-spawning stocks. Inshore, spring-spawning cod are known to form spawning aggregates in bays adjacent to major rivers (e.g. Ipswich Bay, Merrimack River) that may be influenced by high influxes of fresh water from spring runoff. Salinity-related differences in buoyancy for spring- and winter-spawned fish would be compatible

with differences in larval retention due to seasonal differences in coastal circulation patterns (Churchill et al., 2011).

In conclusion, no differences in AFGP production or egg buoyancy were found between captive populations of winter and spring-spawning cod from the Gulf of Maine. The similarity in these physiological parameters may not reflect the physiology of fish in the wild and may be due to the common diets fed both groups, and similar fish size during cold-water treatment. The time-course for AFGP production was established for juveniles of both spawning stocks and a genome-wide screening may be useful in discerning genetic differences between these populations.

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Tables and Figures

Table 1. Developmental stages in degree days of spring- and winter-spawned embryos incubated at 5, 10, and 12°C (based on Hardy (1978); Thompson and Riley (1981); Geffen and Nash (2011)).

Stage	Degree days (mean ± standard deviation)					
	Spring 5°C	Spring 10°C	Spring 12°C	Winter 5°C	Winter 10°C	Winter 12°C
IB	11.3 ± 2.5	13.3 ± 5.8	12.0 ± 0.0	12.0 ± 6.7	12.0 ± 4.5	20.0 ± 13.9
II/III	22.5 ± 2.9	27.5 ± 5.0	30.0 ± 6.9	27.0 ± 6.7	24.0 ± 5.5	26.4 ± 19.7
III	40.0 ± 0.0	40.0 ± 0.0	40.0 ± 6.9	42.0 ± 5.7	36.7 ± 5.8	36.0 ± 0.0
III/IV	43.8 ± 6.3	50.0 ± 0.0	54.0 ± 8.5	48.8 ± 4.8	47.5 ± 12.6	56.0 ± 13.9
IV	50.0 ± 7.1	60.0 ± 0.0	60.0 ± 0.0	57.5 ± 2.9	60.0 ± 8.2	64.8 ± 13.7
IV/V	56.3 ± 7.5	70.0 ± 0.0	72.0 ± 0.0	65.0 ± 5.0	70.0 ± 0.0	66.0 ± 8.5
V	75.0 ± 0.0	80.0 ± 0.0	84.0 ± 0.0	84.0 ± 8.2	82.5 ± 5.0	92.0 ± 6.9
Hatch	75.0 ± 7.1	82.5 ± 5.0	90.0 ± 6.9	96.0 ± 7.4	90.0 ± 6.3	103.2 ± 18.2

Table 2. Developmental stages in degree days of spring-spawned embryos incubated at 12°C under photoperiod treatments (based on Hardy (1978); Thompson and Riley (1981); Geffen and Nash (2011)). All photoperiod treatments developed at the same rate.

Stage	Days post-spawn	Degree days
IB	1	12
II/III	2	24
III	3	36
III/IV	4	48
IV/V	5	60
Hatch	6	84

Table 3. Indicators of plasma antifreeze glycoprotein (AFGP) induction in juvenile Atlantic cod held at 0°C. Mean thermal hysteresis (TH), pyramidal ice crystal growth, and (AFGP) presence in 2D gels.

Spawning Group	Days at 0°C	TH (°C)	Pyramidal growth	AFGP in 2D gels
Spring	19	0.03	no	no
Spring	25	0.03	no	no
Spring	30	0.06	yes	yes
Spring	35	0.11	yes	yes
Winter	20	0.04	no	no
Winter	25	0.02	no	no
Winter	30	0.08	yes	yes
Winter	35	0.08	yes	yes

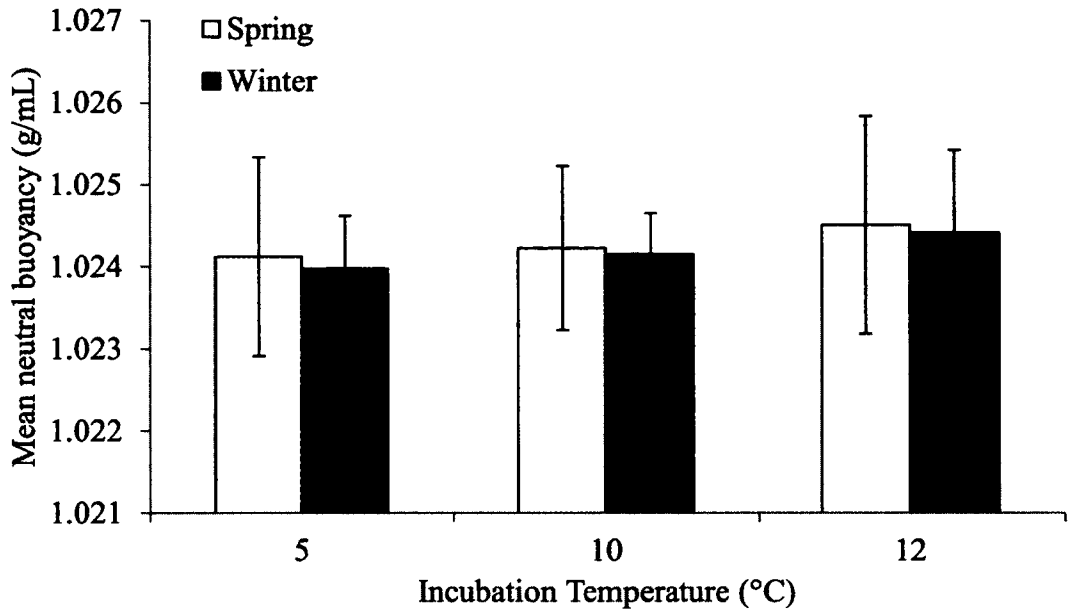


Figure 1. Mean neutral buoyancy through development of spring- (open bars) and winter-spawned (filled bars) embryos held at 5, 10, and 12°C.

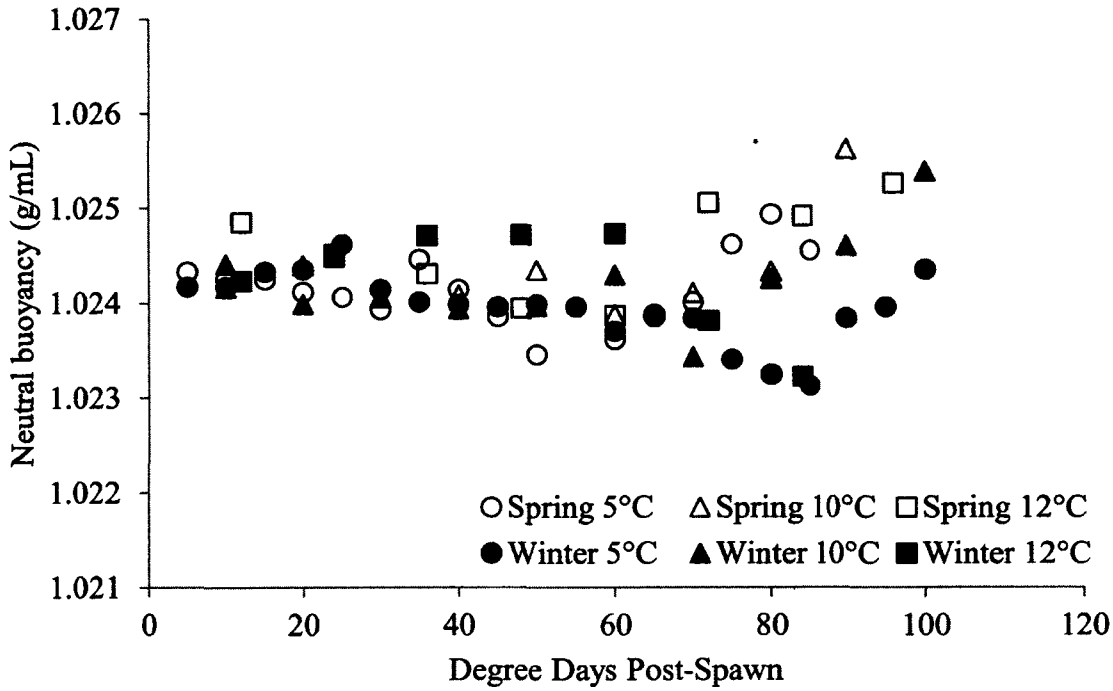


Figure 2. Neutral buoyancy through development of spring- and winter-spawned embryos held at 5, 10, and 12°C. Age is shown as degree days (days * temperature °C).

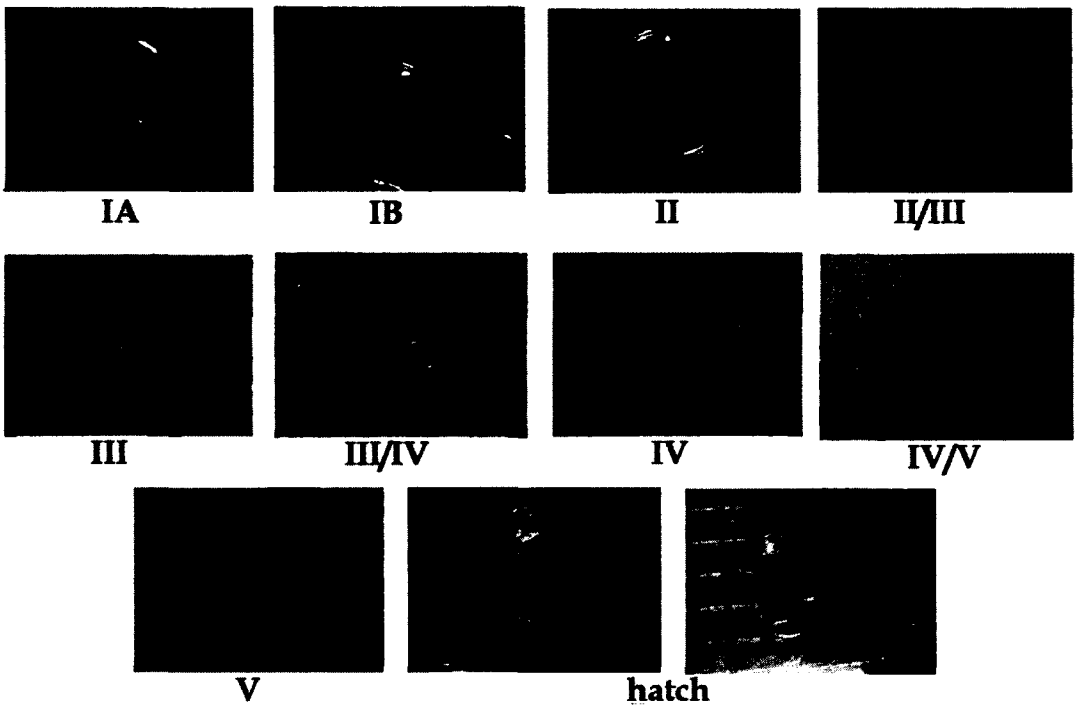


Figure 3. Developmental stages of cod eggs from post-fertilization to hatch (based on Hardy (1978); Thompson and Riley (1981); Geffen and Nash (2011)).

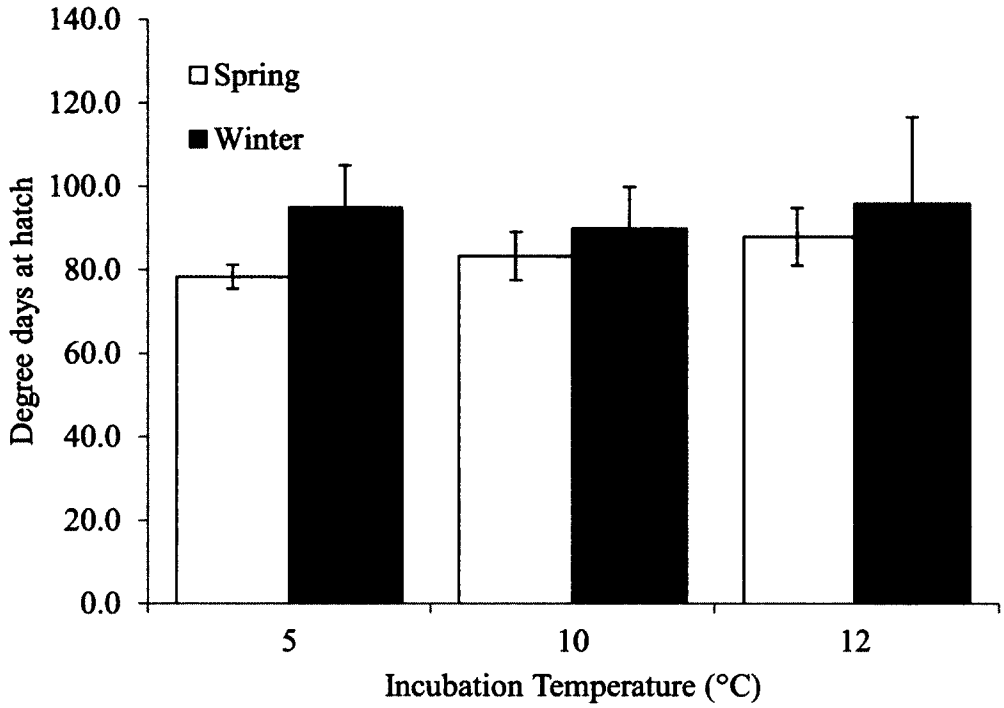


Figure 4. Age in degree days at hatching of spring- (Spring, open bars) and winter-spawned (Winter, filled bars) eggs held at 5, 10, or 12°C.

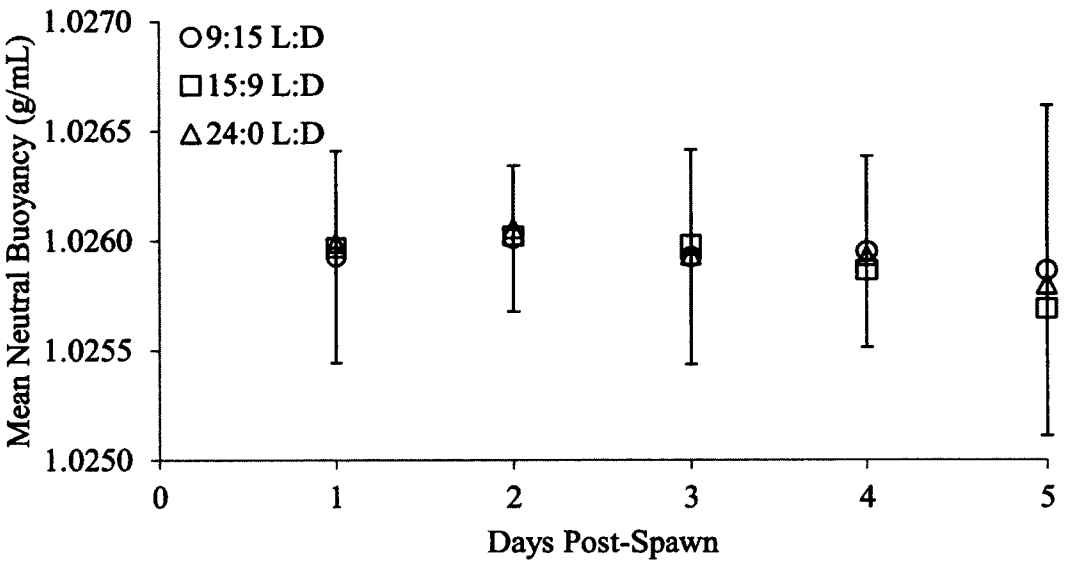


Figure 5. Daily mean neutral buoyancy (± standard deviation) of spring-spawned eggs held under three photoperiods at 12°C. Circles, squares, and triangles represent 9 hr: 15 hr, 15hr: 9hr, and 24 hr: 0 hr light: dark (L: D) cycles respectively.

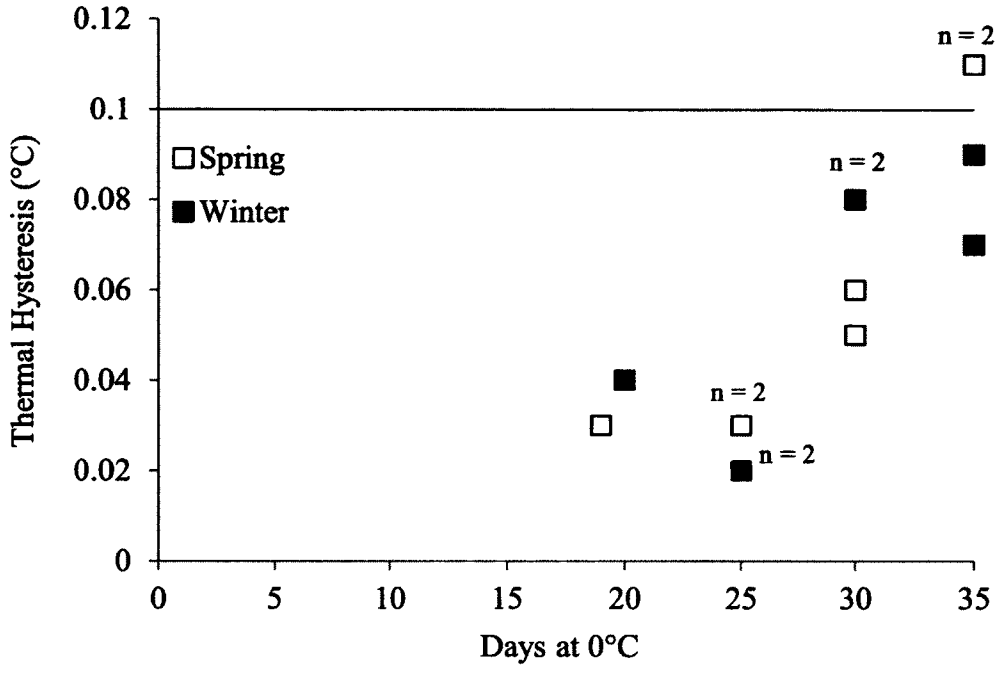


Figure 6. Plasma thermal hysteresis of spring- (open squares) and winter-spawned (filled squares) juvenile cod (13-24 cm) held at 0°C until sampling. Symbols represent 1 sample except where noted.

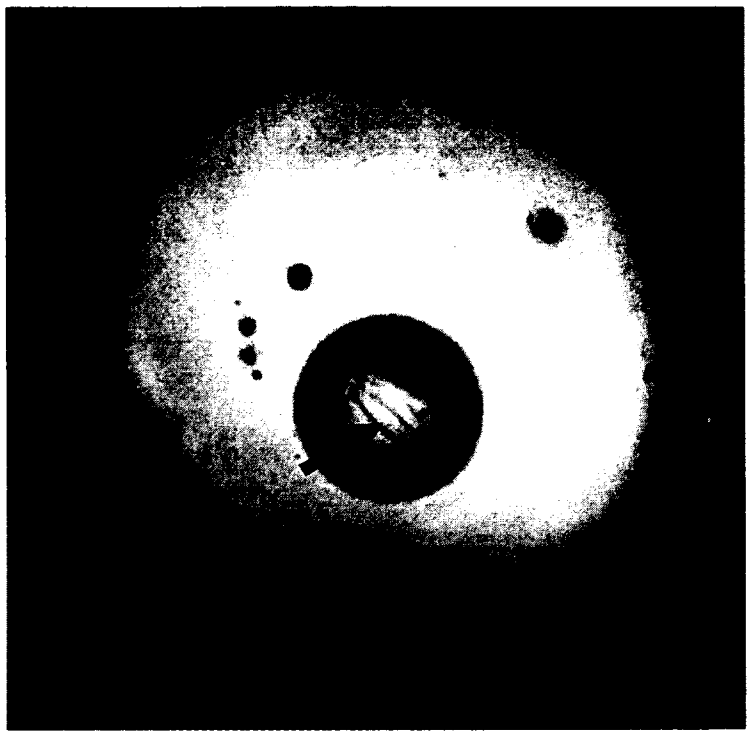
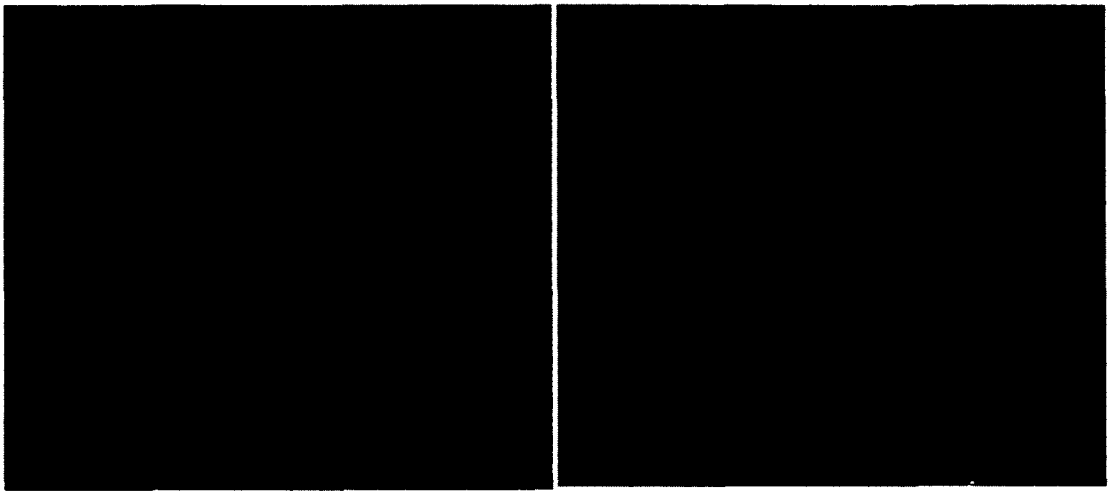


Figure 7. Black arrow indicates an ice crystal exhibiting pyramidal growth in a drop of cod plasma suspended in immersion oil.



No antifreeze glycoprotein

Antifreeze glycoprotein

Figure 8. 2D electrophoresis gels without and with antifreeze glycoprotein. The protein is indicated by the circle on the right gel. 10 and 25 kD are indicated in the ladder on the left side of each gel.

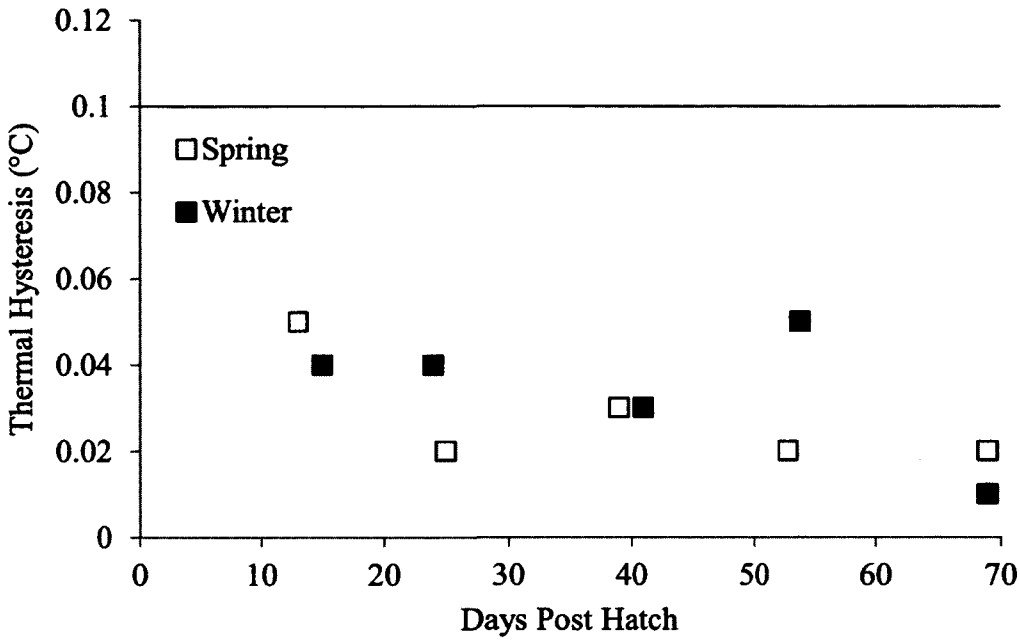


Figure 9. Thermal hysteresis of larval homogenates from spring- (open squares) and winter-spawned (open squares) larval cod held at 0°C (± 1°C) for 5 days. A thermal hysteresis of 0.10°C (solid line) indicates antifreeze glycoprotein presence. Days post hatch is the age of larvae at the time of sampling.

CHAPTER III

INVESTIGATION OF HEMOGLOBIN POLYMORPHISM IN GULF OF MAINE ATLANTIC COD

Introduction

Hemoglobin polymorphisms were first identified in European cod stocks in the 1960s (Sick, 1965a, b). Two codominant alleles of the hemoglobin β subunit, designated HbI-1 and HbI-2, that differ by two amino acid substitutions (Andersen et al., 2009), are the most common alleles, resulting in homozygote (HbI-1/1; HbI-2/2) and heterozygote (HbI-1/2) genotypes. Other, less common, isoforms have also been found in some locations (Jamieson and Birley, 1989, Husebø et al., 2004). Hemoglobin types have been used to differentiate stocks from different geographic areas as well as onshore and offshore populations (Fyhn et al., 1994, Nordeide and Petersen, 1998). While the north-south cline in hemoglobin types is more pronounced in Europe than in the western Atlantic (Jamieson and Birley, 1989), it is possible that cod stocks in the Gulf of Maine may also exhibit hemoglobin polymorphism.

The hemoglobin polymorphism in cod has been associated with traits that are of interest to the aquaculture industry. Cod with HbI-2 tend to prefer cooler waters, have a higher binding affinity for oxygen at low temperatures and higher initial growth rates at lower temperatures (Petersen and Steffensen, 2003, Imsland et al., 2007). Based on the results from heterozygote crosses, Gamperl et al. (2009) suggested that cod with HbI-1

may have lower initial survival under some conditions. HbI-2 cod may also have greater tolerance to hypoxia and a less severe stress response in hypoxic conditions (Brix et al., 1998, Methling et al., 2010). These data suggest that genotypic HbI-2/2 cod may demonstrate greater growth in aquaculture facilities (e.g. high densities) even under low temperature conditions. We hypothesized that the two divergent spawning populations differ in hemoglobin polymorphism.

Methods

Juvenile cod (1+ yr, > 20 cm) from spring- and winter-spawning stocks were transported from Great Bay Aquaculture (Portsmouth, NH) to the Aquaculture Research Center (UNH) and held in 1600-L tanks that were incorporated into separate recirculating systems. Fish were held at 8-12°C under a simulated natural photoperiod and fed appropriate size feed (Skretting, Cojobar, Spain). Blood was taken from the caudal vasculature of 16 winter- and 14 spring-spawned cod (2 yrs) using tuberculin syringes fitted with 20-gauge needles. The blood was transferred to heparinized microfuge tubes containing 40 µL aprotinin maintained on ice until centrifuged (4°C and 10,000 rpm for 7 min; Beckman Coulter Microfuge 22R Centrifuge, Beckman Coulter, Brea, CA, USA). The packed cells were stored at -70°C for later analysis.

To extract hemoglobin, blood cells were thawed, lysed with distilled water, centrifuged at 7000 rpm for 10 minutes, and the supernatant collected. Lysate was added to 4 volumes of isoelectric focusing (IEF) buffer (8M urea, 2% Triton X-100, 40mM DTT, 0.002% bromophenol blue, 0.5% IPG buffer; GE Healthcare, Piscataway, NJ, USA) and protein content was determined (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). Samples corresponding to 200 µg protein were brought to 250 µL total volumes

with DeStreak Rehydration Solution (GE Healthcare) and were subjected to IEF on linear pH 5-8 IEF gels (Bio-Rad) on an Ettan IPGphor II (Amersham Biosciences, Piscataway, NJ, USA) with the following run conditions: rehydrate 12 hours, step & hold 500V for 1 hour, gradient 1000V for 1 hour, gradient 8000V for 2 hours 30 minutes, and gradient 8000V for 25 minutes.

After electrophoresis, gels were placed in fixative (5% acetic, 25% methanol) for 5 minutes. Gels were then rinsed with distilled water for 5 minutes. Gels were stained with GelCode Blue stain (Fisher Scientific, Pittsburg, PA, USA) for 2 hours. After staining, gels were again rinsed in distilled water for 1 hour. Gels were photographed with a digital camera (DSC-H55, Sony, New York, NY, USA), and the images were evaluated using ImageJ (US National Institute of Health, Bethesda, MD, USA) to determine hemoglobin type based banding patterns described by Fyhn et al. (1994) and Brix et al. (2005). Fisher's exact test was used to compare the ratios of hemoglobin types in the two spawning stocks.

Results

Hemoglobin type was determined as HbI-1, HbI-1/2, or HbI-2 for all gels. Most samples from both spawning groups were identified as HbI-1/2 with the remainder identified as HbI-2. Spring-spawned cod had 11 HbI-1/2 and 3 HbI-2 and winter-spawned cod had 14 HbI-1/2 and 2 HbI-2 (Figure 10). No significant differences between stocks were detected ($n = 30$, $p = 0.6424$).

Discussion

Only two previous studies reported hemoglobin types from fish sampled in the Gulf of Maine (Sick 1965b; Jamieson and Birley 1989) and in both of them the majority

of fish had HbI-2. Several difficulties arise in trying to draw comparisons from results obtained in those previous studies and the present one. Jamieson and Birley (1989) sampled cod from Georges Bank, and it is likely that those fish comprised a separate genetic group from the fish of the Northern and Southern complexes sampled here (Kovach et al., 2010). Cod in the Sick (1965b) study were sampled near Woods Hole, MA, and of 75 fish genotyped 10 of had the HbI-1/2 genotype while 64 had HbI-2. Currently, cod sampled from near Woods Hole would be comprised of fish from the winter-spawning stock, but because of possible changes in stock composition and movement patterns, it is difficult to speculate about the stock of origin in previous studies. In the current study, the HbI-1/2 isoform was found to be the predominant form in fish from both captive spawning groups. The sampled fish originated from volitionally-spawning fish (> 20 individuals/population) obtained from the wild. It is not known, however, how many broodstock participated in spawning or whether the sampled offspring were related. Therefore, the results of this study must be viewed with caution, until a larger sample size, from wild fish is analyzed.

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Figures

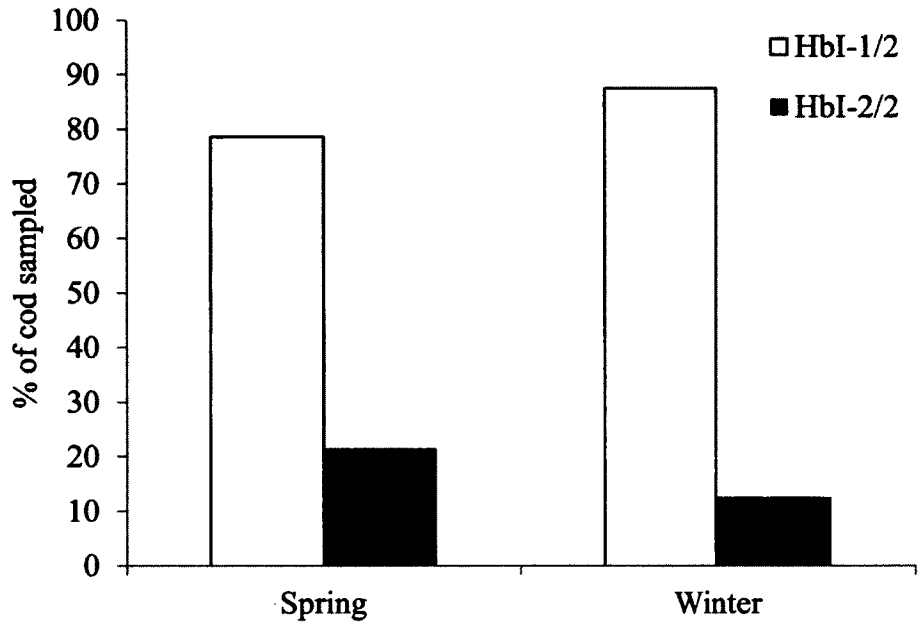

Figure 10. Frequency of observed hemoglobin types for spring-spawned and winter-spawned juvenile cod.

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APPENDIX

University *of* New Hampshire

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Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

23-Sep-2010

Berlinsky, David L
Biological Sciences, Rudman Hall
Durham, NH 03824

IACUC #: 080903
Project: The effects of temperature on growth, reproduction and antifreeze production in Atlantic cod
Category: C
Next Review Date: 22-Oct-2011

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your study is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,
Jessica Bolker

Jessica A. Bolker, Ph.D.
Chair

cc: File