TEMPERATURE AND DIETARY EFFECTS ON THE PHYSIOLOGY OF TWO GADIDS, THE ATLANTIC COD (*Gadus morhua*) AND HADDOCK (*Melanogrammus aeglefinus*)

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Temperature and Dietary Effects on the Physiology of Two Gadids, the Atlantic Cod (*Gadus morhua*) and Haddock (*Melanogrammus aeglefinus*)

by

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Abstract

Atlantic cod and haddock grown in sea cages are exposed to changes in water temperatures that can have a significant impact on their physiology and/or biology. During winter months these species are exposed to low temperatures that have a negative impact on their food consumption and growth rates. On the other hand, during summer months, these fish can be exposed to temperatures above their upper critical temperature that can affect their stress physiology, immunology and ultimately lead to high mortality. To investigate the effects of low temperature on several aspects of the biology of these two species, I used tank respirometry to explore whether feeding low protein diets at cold temperatures (2 °C) would decrease the metabolic cost of digestion and the time required for digestion/gastric evacuation, thus leaving more available energy for growth. In addition, using biochemical and molecular biological tools, I studied the effects of high water temperatures (acute and chronic challenges) on the stress response and immune function of juvenile Atlantic cod and determined their upper critical temperature. My results showed that: 1) low water temperature (2 °C) significantly reduces food consumption, growth rate, gross conversion efficiency and absolute specific dynamic action (SDA; mg O_2) and slows gastric evacuation; 2) reducing dietary protein levels during the winter is unlikely to be of benefit to Atlantic cod and haddock culture operations. With regards to studies at elevated water temperature, I identified oxygen consumption and changes in plasma total cortisol levels as reliable indicators of high temperature stress in Atlantic cod, and showed that juveniles of this species can tolerate short-term exposure to temperatures above 20 °C, although their stress response differs when exposed to acute vs. chronic thermal challenges. Moreover, my results indicated

that their immune function during chronic temperature stress is influenced by interactions between thermal effects and temperature-induced stress, and that not all immune-related parameters are affected by exposure to high temperatures.

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List of Abbreviations and Symbols

ANOVA	Analysis of Variance
Approx. or ~	Approximately
ARDF	Aquaculture Research and Development Facility
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BM	Body Mass
CART	Cocaine and Amphetamine Regulated Transcript
cDNA	Complementary DNA
CM	Cumulative Mortality
C_T	Threshold Cycle
CTM	Critical Thermal Maximum
dl	Deciliter
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EF-1α	Elongation Factor 1 alpha
FC	Food Consumption
Fig	Figure
g	Grams
GCE	Gross Conversion Efficiency
GE	Gastric Evacuation
h	Hour (s)
HP	High Protein Diet
HPA	Hypothalamus-Pituitary-Adrenocortical
HPI	Hypothalamus-Pituitary-Interrenal
HSI	Hepatosomatic Index
hsp	Heat Shock Protein
IgM	Immunoglobulin M
IgM-H	Immunoglobulin M Heavy Chain
IgM-L	Immunoglobulin M Light Chain
IL-1β	Interleukin-1 beta
IU	International Units
kDa	Kilo Dalton
kg	Kilogram
kPa	Kilopascal
ln	Natural Logarithm
LP	Low Protein Diet
MHC	Major Histocompatibility Complex
min	Minute (s)
ml	Milliliter (s)
M-MLV	Moloney Murine Leukemia Virus
mRNA	Messenger Ribonucleic Acid
MO_2	Oxygen Consumption

ng	Nanogram
nm	Nanometer
O_2	Oxygen
O_{2f}	Final Oxygen Concentration
O_{2i}	Initial Oxygen Concentration
PBS	Phosphate Buffered Saline
Q_{10}	The factor by which the rate (R) of a biological process increases for every 10 °C in temperature
QPCR	Quantitative Polymerase Chain Reaction
QRTPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RB	Respiratory Burst
rcf	Relative Centrifugal Force
RM-ANOVA	Repeated Measures Analysis of Variance
RMO_2	Routine Oxygen Consumption
RMR	Routine Metabolic Rate
RNA	Ribonucleic Acid
RQ	Relative Quantity
SDA	Specific Dynamic Action
SDA_{DUR}	Duration of Specific Dynamic Action
$SDA_{DUR\ ENER}$	Duration of Specific Dynamic Action per Gram of Food
SDA_{ENER}	Specific Dynamic Action per Gram of Food
SDA_{MAX}	Peak Oxygen Consumption
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
S.E.	Standard Error
SGR	Specific Growth Rate
SGR_A	Adjusted Specific Growth Rate
TMS	Tricaine Methanesulfonate
TS	Temperature Stressed
TTBS	Tween-20 Tris Buffered Saline
U	Units
V	Volts
Vol	Volume
β2-M	Beta-2- microglobulin
°C	Degree Celsius
µg	Microgram
µl	Microliters

Co-Authorship Statement

In this thesis, I was responsible for the intellectual design and carrying out of the experiments, the analysis of the data and the preparation of the manuscripts. Exceptions are: a) Dr. Santosh Lall designed both fish feeds used in the experiments in Chapters 2 and 3; b) Dr. Kurt Gamperl designed the respirometry system used in chapters 2 and 4; c) the design of the gene expression analyses by qPCR in Chapter 5 was done in collaboration with Dr. Matt Rise; and d) Dr. David Schneider assisted with statistical design in Chapter 2 and 3.

Chapter 1. Introduction

Aquaculture is the farming or husbandry of aquatic resources (animals and plants) that involves some degree of human intervention in the rearing process to enhance production and the individual or corporate ownership of the stock being cultivated (Boghen, 1995; FAO, 2009). Species produced in aquaculture can be used in numerous ways including the enhancement of natural populations in different bodies of water (e.g. rivers and lakes) to support sport fisheries, for fee-fishing ponds or for hobby activities. However, aquaculture is most commonly recognized as a means of producing food for human consumption and as a source of jobs and profit (Boghen, 1995).

The exact origin of aquaculture is unsure, due in part to the fact that there appear to be several starting points, depending on the species and geographical location. However, it is known that fish culture formed part of Chinese society around 4000 years ago, and that around the same time, the Japanese cultured oysters and the Egyptians reared tilapia (Boghen, 1995). In Europe, aquaculture started with the ancient Romans and Greeks in the form of the capture and fattening of wild fish in ponds, but it only developed during the middle ages. On the American continent, the ancient Hawaiians and Mayans also practiced aquaculture (Ackefors *et al.*, 1994).

The aquaculture of numerous species has become a significant part of the world`s fisheries production, and in the last five decades it has increased greatly. For example, in the mid-sixties, aquaculture production was approximately 1 million tonnes, by the start of the 1980's it had increased to around 9 million tonnes, and by 1990 it had nearly doubled again (Boghen, 1995). Further, while aquaculture production in 1992

accounted for 18.6% of the world's fisheries production, at present, it accounts for close to 50% of the global fish production for human consumption, a 30% jump in 15 years (FAO, 2009).

In Canada, aquaculture has expanded greatly over the last decade, and in 2005 this industry's production was close to 154 million tonnes, with an approximate value of $715 million dollars (DFO, 2005). Every province, and the Yukon Territory, have aquaculture operations, and furthermore, fish farms have become a stronghold in many communities across the country and provide around 14,000 full-time, year-round, jobs (DFO, 2005). The Canadian aquaculture industry receives most of its production value from the culture of salmonid species, which in 2005 had an estimated value of $545,741,000 dollars. However, the production of shellfish species has also increased greatly, and during the same year, the production of mussels, oysters and clams was valued at $57,641,000 dollars (DFO, 2006). Nevertheless, future growth and stabilization, and avoidance of price fluctuations that could cause problems for the finfish culture industry, may be largely dependent on species diversification (Harmon, 2003).

1.1 Farming of Atlantic Cod and Haddock

The Atlantic cod (*Gadus morhua*) is a member of the family Gadidae, which includes 59 species. It is a cold-water marine fish that inhabits mainly northern seas, and in the Northwest Atlantic is found in shallow waters of about 5 m up to depths of 600 m. This species inhabits the coasts of east and west Greenland and in Canada, it is distributed as far north as Frobisher Bay and extends into Ungava Bay. Although it is

more abundant along the Labrador coast and off Newfoundland, its distribution extends as far south as Cape Hatteras, NC (USA). The haddock (*Melangrammus aeglefinus*) belongs to the same family as the Atlantic cod, and ranges from the Strait of Belle Isle (between Labrador and Newfoundland) to Cape Cod (USA). In Canadian waters, they occur mainly on the continental shelf from the Bay of Fundy to the east coast of Cape Breton, and eastward to the Grand Banks, most commonly at depths of 45 to 240 m.

Atlantic cod stocks have been in decline or fished to their limit since the 1970's. This is especially true in the Northwest Atlantic where a collapse in the Canadian stocks resulted in a complete closure of the fishery in 1992. However, stocks closer to Europe, although decreased, are still open for commercial fishing (Brown *et al.*, 2003; Bailey *et al.*, 2005). In addition to the decline in Atlantic cod, there have been considerable decreases in the harvest of other white fish species such as Alaskan Pollack (*Theragra chalcogramma*) and hake (*Merluccius bilinearis*), and this has strained the world market for these species. In recent years there has been significant interest in the culture of Atlantic cod and haddock in Atlantic Canada, as: 1) both of these species are prime white flesh fish that have established markets in North America and Europe; and 2) the increased price for both species, in conjunction with a fall in prices for salmonid species, have attracted the attention of the aquaculture industry (Le François *et al.*, 2002; Chambers and Howell, 2006; Rosenlund and Skretting, 2006).

Atlantic cod aquaculture is not a new industry, as farming of this species for stock enhancement (i.e. the production of larvae that are then released into the marine environment) has been ongoing for over a century in countries such as Canada, the

USA, Denmark, Sweden, Norway and the Faroe Islands (Brown *et al.*, 1995; Svåsand *et al.*, 2000; Chambers and Howell, 2006). However, the impact of this practice on the recruitment of wild populations has been considered minimal (Svåsand *et al.*, 2000; Bailey *et al.*, 2005). The culture of Atlantic cod for human consumption had little success until the mid 1980's, due to problems with survival during the larval stage, larval feeding and mass rearing (Brown *et al.*, 1995), and it was only in 1986 that the current concept of Atlantic cod aquaculture was established in Newfoundland; this new industry established, in part, by adapting technologies used in the salmon industry and through new investment (Brown *et al.*, 1995). Currently, Canada, the United States, Scotland and Norway all have programmes aimed at enhancing and developing the Atlantic cod aquaculture industry (Brown *et al.*, 2003), and Norway (the world leader in Atlantic cod production) estimates that its production of farmed Atlantic cod will approach 400,000 tonnes by the year 2010 (Bailey *et al.*, 2005).

At present, Atlantic cod are grown in net pens, and juveniles have the potential to double their weight every 3 to 4 months, once they have been weaned onto commercial pelleted diets and are reared at optimal temperatures of 8 – 12 °C (Pedersen and Jobling, 1989; Brown *et al.*, 1995; Bjornsson *et al.*, 2001b; Peck *et al.*, 2003b). Furthermore, the data available from commercial hatcheries indicate that cultured Atlantic cod grow faster than wild individuals, and that fish grown in net pens can reach 2 kg in around 1.5 years and 3 kg in 3 years (Chambers and Howell, 2006).

Interestingly, haddock were first identified as a candidate for cold-water aquaculture in the United Kingdom, and its high consumer demand, as well as declines in the wild fishery in the 1990's, led to the start of a demonstration project on its farming potential. In North America, research on its potential for aquaculture has been

underway at various universities in the United States (e.g. the University of Maine, the Massachusetts Institute of Technology and the University of New Hampshire), and in Scotland at the Ardtoe Marine Laboratory (Harmon, 2003; Moran and Goudey, 2003; Chambers and Howell, 2006). However, development of haddock aquaculture has occurred mainly in Atlantic Canada (Aiken, 2003), due in part to the partnership between the private sector (Heritage Salmon Ltd) and Canadian government laboratories. As a result of this partnership, production of cultured haddock reached 180 tonnes in 2002 and was expected to increase three fold in 2005 (Harmon, 2003; Chambers and Howell, 2006). Unfortunately, the commercial partner terminated its research programme. Nonetheless, valuable information was gained; data collected indicate that cultured individuals grow faster than their wild counterparts and that they can reach 2.5 kg in 3 years.

Most of the research on Atlantic cod and haddock culture has been focused on parental effects and environmental conditions during egg incubation, on the larval period (Downing and Litvak, 1999; Buckley *et al.*, 2000; Downing and Litvak, 2000; Downing and Litvak, 2002; Trippel and Neil, 2003; Monk *et al.*, 2006; 2008), on the control of sexual maturation (Martin-Robichaud and Berlinsky, 2004; Norberg *et al.*, 2004; Taranger *et al.*, 2006), and on feeding and dietary requirements (Kim and Lall, 2001; Nanton *et al.*, 2001; Lall *et al.*, 2003; Hamre, 2006; Hamre and Mangor-Jensen, 2006; Grisdale-Helland *et al.*, 2008). However, most of these studies do not directly address issues concerning the environmental conditions that these fish face in cage culture, as well as their impact on growth, mortality and disease resistance. Furthermore, there is a lack of information on the time of year and size at which the cultured fish should be transferred from land-based hatcheries to sea-cages; the latter is

important as growth in the sea-cages depends on seawater temperatures and the exposure of the cage site to the elements. In addition, small juveniles are not considered strong enough for transfer to sea-cages during winter months, and are more susceptible to strong water currents and wave action (Kjesbu *et al.*, 2006).

1.2 Some Challenges Facing Atlantic Cod and Haddock Culture

Due to the fact that they are limited in their movements in the water column, cage-cultured finfish are exposed to seasonal fluctuations in several environmental factors that have significant effects on their physiology. Of these, temperature is probably the most important as it affects practically all physiological systems, as well as cellular processes, and consequently metabolism, food intake, nutritional efficiency and growth (Burel *et al.*, 1996; Crockett and Londraville, 2006). Temperature may affect fish in different ways depending on the season. In the case of farmed fish, several studies have shown that during the winter months, when water temperature falls below 5 °C, fish significantly decreased their food consumption and showed changes in feeding behaviour that lead to diminished growth rates (Brown *et al.*, 1989; Clark *et al.*, 1995; Claireaux *et al.*, 2000; Purchase and Brown, 2001). This represents a significant challenge for the aquaculture industry due to the increased time until the fish reaches market size, and these problems have been associated with the relationships that exist between ration, metabolic scope [the difference between the active (or maximum) and the standard (or maintenance) metabolic rates] and specific dynamic action (SDA; the cost of ingestion, digestion, and nutrient absorption/assimilation) (Jobling, 1981a; Soofiani and Hawkins, 1982; Soofiani and

Priede, 1985; Claireaux *et al.*, 2000; Claireaux and Lefrançois, 2007). For example, the metabolic scope of fish is limited at cold temperature, and this has been hypothesized to restrict the amount of metabolic energy that a particular fish can devote to SDA. Based on the above, and the results of Gotceitas *et al.* (1999), it is clear that food ration and composition must be optimized to achieve significant growth in Atlantic cod during winter. However, at present, Atlantic cod and haddock are generally fed commercial diets that are formulated for optimal growth at water temperatures between 10 and 12 °C, and are energetically expensive to digest due to their high protein content (~50 – 58%). Further, there are only a few studies on the metabolic physiology, husbandry and effect of diet composition on haddock and Atlantic cod (Kim and Lall, 2001; Morais *et al.*, 2001; Nanton *et al.*, 2001; Peck *et al.*, 2003a; Peck *et al.*, 2004; Tibbetts *et al.*, 2005), and these have rarely been conducted at temperatures below 8 °C. By reducing the protein content of these diets, it may be possible to reduce the metabolic cost of digestion (LeGrow and Beamish, 1986), and thus allow Atlantic cod and haddock to increase their food consumption/feeding frequency, and/or to devote more energy to growth during the winter season. However, experiments to test these assumptions have not been performed.

At the other end side of the spectrum, during summer, fish held in sea-cages in Newfoundland and Labrador can be exposed to temperatures that can reach 20 °C at the water's surface and even at depths of 6 m. Furthermore, rapid temperature changes (e.g. daily changes) of around 10 °C are also typical (e.g. due to inversion of the thermocline; Fig. 1.1) (Gollock *et al.*, 2006). These changes in water temperature can thermally stress fish causing physiological alterations including elevations in circulating cortisol levels, the suppression of immune functions and feeding, these

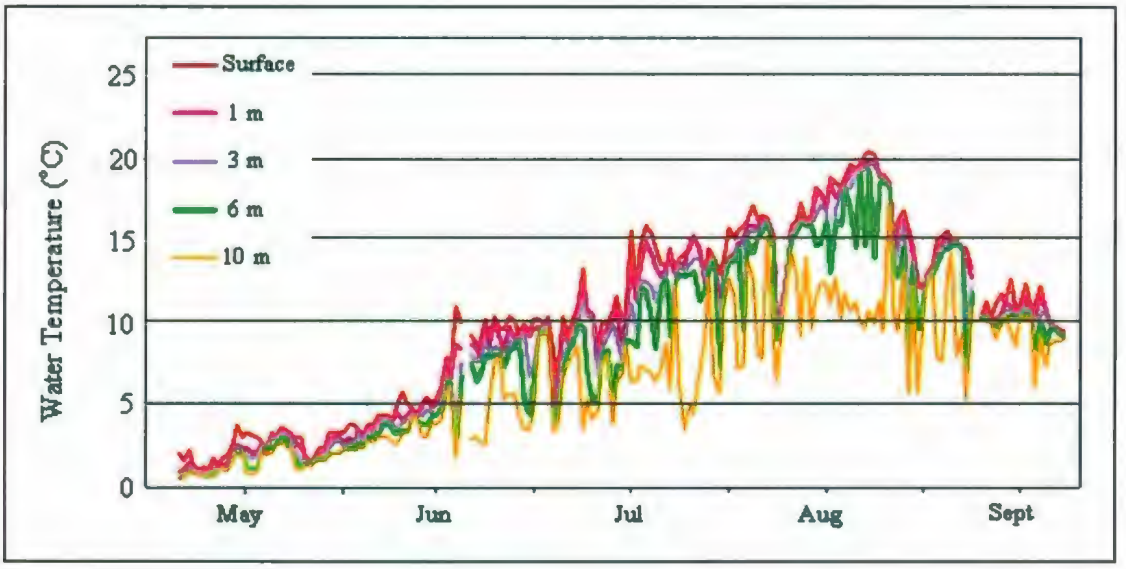

Fig. 1.1. Water temperature profile for an Atlantic cod sea-cage site located in Turnip Cove, Newfoundland for the year 2002.

effects ultimately leading to reduced growth and or increased mortality (Bly and Clem, 1992; Zou *et al.*, 2000; Gollock *et al.*, 2006). There have been several studies performed on the thermal biology of Atlantic cod. However, the goal of most of these studies was to determine the thermal preferendum for the species, or their optimal temperature for growth. At present, there is limited data on the temperature tolerance of various life history stages of cod and haddock, and studies are lacking on the effect that high temperatures may have on the immune system and overall health of these species. Fish health is a fundamental issue in aquaculture, given the variety of diseases that have already been identified in gadoids and that more are expected to develop with increased culturing. Thus, it is important to learn how environment factors impact the immune responses of cultured fish (Kjesbu *et al.*, 2006). Furthermore, understanding how temperature affects the biology of cultured fish is essential for selecting suitable areas for the establishment of cage-sites.

1.3 Overall Objectives

The main goal of my doctoral research was to improve the growth, survival and overall welfare of farmed Atlantic cod and haddock, particularly during the sea-cage stages of rearing. To accomplish this, I utilized several methodologies to better understand how seasonal temperature changes (approximately 2 to 20 °C; summer and winter-like temperatures, respectively) influenced routine and post-feeding metabolism, stress and immunological parameters, and finally digestive physiology, food consumption, growth and conversion efficiency.

Studies conducted in chapters 2 and 3 were carried out at fall and winter-like temperatures (2 – 11 °C), and investigated whether reducing the protein content of diets could reduce the cost of digestion and improve gastric evacuation in cod and haddock, and thus improve the growth of both species through enhanced feed conversion and appetite/food consumption. In chapters 4 and 5, experiments were carried out at summer-like temperatures (10 to > 20 °C) and were designed to elucidate the effects of high water temperature on the survival, metabolic and stress physiology, and immunology of juvenile Atlantic cod.

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Chapter 2. Effects of Dietary Protein and Lipid Level, and Water Temperature, on the Post-Feeding Oxygen Consumption of Two Gadoids, the Atlantic Cod (*Gadus morhua* L.) and Haddock (*Melanogrammus aeglefinus* L.).

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2.1 Abstract

Tank respirometry was used to study whether changing protein:lipid level (55.1% protein with 10.7% lipid vs. 42.2% protein and 16.4 % lipid; both diets isoenergetic) would affect the food consumption (FC), routine metabolic rate (RMR) and cost and duration of digestion (SDA) of juvenile Atlantic cod and haddock (40 - 50 g) acclimated to 11, 6 and 2 °C. Protein:lipid level did not affect any of the measured parameters. However, numerous temperature and species effects were observed. The RMR of cod was 25-35% higher than for haddock. The FC of both species decreased from ~2.0 to 0.6% of body mass between 11 and 2 °C. Although maximum post-feeding oxygen consumption (30–50% above RMR) and SDA duration (55 to 85 h) were not significantly affected by temperature, SDA duration per g of food increased greatly from 11 to 2 °C (e.g. from 3 to 10 h g food $^{-1}$). Finally, while SDA comprised between 3.3–5.2% of dietary energy content, absolute SDA (mg O_2) decreased by ~60-65% in cod and ~75% in haddock from 11 to 2 °C. These results show that protein:lipid has little influence on SDA, and thus, suggest that feeding low protein diets at cold temperatures is unlikely to improve growth.

2.2 Introduction

In recent years, there has been significant interest in Atlantic cod (*Gadus morhua*) and haddock (*Melangrammus aeglefinus*) culture in Atlantic Canada due to the precipitous decline in wild populations (Rosenlund and Halldorsson, 2007); http://www.fao.org/fishery/species/2218; http://www.fao.org/fishery/statistics/global-capture-production/en), and that both of these species are prime white flesh fish that command a high market price and have high consumer demand in North America and Europe (Kjesbu *et al.*, 2006; Rosenlund and Skretting, 2006). However, the aquaculture of Atlantic cod and haddock in North America is still at a pre-commercial stage, in large part, because seasonal fluctuations in several environmental factors make the cage-culture of these fish challenging. Of these environmental factors, temperature is probably the most important as it can range from < 0 to ~ 20 °C, and changes in temperature affect physiological processes such as metabolism, food intake, growth and survival (Burel *et al.*, 1996; Imsland and Jonassen, 2001; Luo and Xie, 2008). Although there are many studies that have evaluated the effects of temperature on fishes (Person-Le Ruyet *et al.*, 2004; Tort *et al.*, 2004b; Pérez-Casanova *et al.*, 2008), only a few have directly examined the impact of environmental conditions that haddock and Atlantic cod face in cage-culture have on their growth, mortality and disease resistance. For example, research shows that Atlantic cod decrease their food consumption and experience changes in feeding behaviour and decreases in growth rate during months where water temperatures are low (Brown *et al.*, 1989; Clark *et al.*, 1995; Purchase and Brown, 2001). In the case of haddock, although early growth and survival co-vary with temperature (Ottersen and Loeng, 2000), few authors have

examined the effect of temperature on the growth physiology of this species (Laurence, 1978; Tytler, 1978; Peck *et al.*, 2003a). Furthermore, it appears that the biggest challenge with regards to the aquaculture of this species in North Atlantic is overcoming slow growth rates associated with cold water temperatures (Frantsi *et al.*, 2002).

Decreases in fish appetite (Mallekh and Lagardere, 2002), growth (Clark *et al.*, 1995; Claireaux *et al.*, 2000) and activity (Clark *et al.*, 1995) at low temperatures (< 5 °C) are likely related to the inter-relationships between metabolic scope (the difference between maximum and standard metabolism), specific dynamic action (SDA, the energy used for ingestion, digestion, and nutrient absorption/assimilation) and food ration (Muir and Nimmi, 1972; Jobling, 1981a; Soofiani and Hawkins, 1982; Soofiani and Priede, 1985; Claireaux *et al.*, 2000). For example, it appears that Atlantic cod in cold water can only direct a limited amount of metabolic energy to SDA due to a reduction in their metabolic scope (Claireaux *et al.*, 2000), and that food ration and composition must be optimized to achieve significant growth under these conditions (Gotceitas *et al.*, 1999). However, there is insufficient data on which to base feeding protocols or diet formulations for use at cold temperatures, because studies that provide relevant information on SDA in Atlantic cod, haddock and other fishes (Jobling and Davies, 1980; Soofiani and Hawkins, 1982; LeGrow and Beamish, 1986; Lyndon *et al.*, 1992; Blaikie and Kerr, 1996; Peck *et al.*, 2003a; 2005), on the relationship between Atlantic cod husbandry and growth (Lambert and Dutil, 2001), or that provide detailed analyses on the optimization of diets for these species (Lie *et al.*, 1988; Kim and Lall, 2001; Morais *et al.*, 2001; Nanton *et al.*, 2001; Tibbetts *et al.*, 2005) have rarely been conducted at temperatures below 8 °C.

During sea-cage rearing, Atlantic cod and haddock juveniles are generally fed commercial diets that are formulated for optimum growth at temperatures of 10 – 12 °C, and that are energetically expensive to digest due to their high protein content (~50 – 58%). However, it may be possible to improve the growth of these species at cold temperatures, and reduce production costs, by feeding diets containing lower protein levels. This is because, with the exception of a recent study by Eliason *et al.* (2007) on rainbow trout, *Oncorhynchus mykiss* (Walbaum), most studies on fish have shown that SDA can be reduced signicantly by reducing the dietary protein content (LeGrow and Beamish, 1986). Based on the above findings and that dietary protein is the most expensive component of fish diets (De Silva and Anderson, 1995; Médale and Guillaume, 2001; Morais *et al.*, 2001), we investigated whether reducing the protein:lipid content of Atlantic cod/haddock diets would decrease the magnitude and duration of SDA at cold temperatures. Although the potential advantage of decreasing the magnitude of SDA (the energetic cost of digestion) is obvious, reductions in the duration of SDA could potentially hasten the return of appetite, and thus result in increased feeding frequency and growth.

2.3 Materials and Methods

These studies were conducted in accordance with the guidelines published by the Canadian Council on Animal Care, and approved by the Animal Care Committee at Memorial University (protocol 04-01-KG).

2.3.1 Rearing Conditions

Juvenile Atlantic cod used in these experiments were communally reared in production tanks following standard rearing protocols in place at the Aquaculture Research and Development Facility (ARDF) of the Ocean Sciences Centre, Memorial University of Newfoundland. Juveniles were held in 6000 L tanks supplied with filtered and oxygenated seawater (temperature 11 ± 1 °C; air saturation > 90%) and a photoperiod of 12h light: 12h dark, and were fed twice daily (9:00 and 16:00 h) at a rate of 1.5% of average body mass (BM) per day with a commercial diet (EWOS Canada Ltd, Surrey, BC, Canada; 55% protein, 15% lipid). Haddock were reared from eggs at the Institute for Marine Biosciences, National Research Council (IMB-NRC) Marine Research Station (Ketch Harbour, NS, Canada) following the techniques described in Frantsi *et al.* (2002). Juveniles were initially held in tanks supplied with seawater (temperature 10 ± 1 °C; air saturation > 90%), and fed twice daily (8:00 and 15:00 h) at a rate of 1.5% of BM per day with a commercial diet (Zeigler Bros., Inc., Gardners, PA, USA; 50% protein, 15% lipid). However, the juvenile haddock were transported to the ARDF prior to experimentation. At the ARDF they were initially held in two 4000 L holding tanks supplied with seawater (temperature 11 ± 1 °C; air saturation > 90%) and a 12h light: 12h dark photoperiod, and fed twice daily (9:00 and 16:00 h) at a rate of 1.5% of body day^{-1} with the EWOS commercial diet.

2.3.2 Acclimation

Two months prior to the respirometry experiments (see below), the fish were acclimated to one of three temperatures: 11 °C which is within the optimum

temperature range for juvenile Atlantic cod growth (Bjornsson *et al.*, 2001a; Peck *et al.*, 2003b), 2 °C which poses significant challenges for these species in cage-culture (Brown *et al.*, 1989; Clark *et al.*, 1995), and 6 °C which is intermediate between the two temperatures. However, no 6 °C experiments were performed on haddock, as insufficient numbers of fish were available to conduct experiments at all three temperatures. During this period both species were maintained at 12h light:12h dark, and fed the commercial diet at a rate of 1.5% BM every second day. This change in protocol was used to ensure that the fish achieved an optimum size for the respirometer study, and to minimize size differences between fish held at the different temperatures. At the end of the acclimation period, there was no significant difference in the mass of any of the 5 groups (3 Atlantic cod, 2 haddock) with the mean mass for the smallest and largest groups being 37.2 ± 1.0 and 42.6 ± 2.5 g, respectively.

2.3.3 Respirometry

One week prior to the beginning of the oxygen consumption measurements, groups of temperature acclimated (2, 6 or 11 °C) Atlantic cod or haddock (approx. 30-40 fish; ~ 1000 g total wet mass) were placed into one of 4 custom built 280 L, computer-controlled, tank respirometers (Chapter 4, see page 95; Pérez-Casanova *et al.*, 2008) to allow the fish to acclimate to their new conditions and to recover from handling/netting stress. Two tank respirometers were randomly assigned to the control group (high protein diet, HP) and two to the experimental group (low protein diet, LP). For the first 3 days, the fish were fed to satiation once a day and were maintained on a 12 h light: 12 h dark photoperiod. However, the fish were food deprived for 4 days

prior to the measurement of routine oxygen consumption and SDA. Both diets fed to the cod and haddock in the respirometers were prepared as 4 mm extruded pellets. Two isoenergetic diets were formulated, the first containing 42.2 % protein with 16.4 % lipid (LP) and the second with 55.1 % protein and 10.7% lipid (HP) (see Tables 2.1 and 2.2 for further details on diet composition and proximate analysis). The HP diet was similar in protein level to that currently used at Atlantic cod aquaculture cage-sites (~55% protein). These diets, however, were isocaloric (actual protein:lipid levels of 55:11% and 42:15%, respectively), and contained krill hydrosylate (30 g kg^{-1}) to increase the palatability and enhance food consumption at low water temperature. These diets were produced at the IMB-NRC according to the procedure described by Kim and Lall (2001).

Oxygen concentration in the respirometers was measured 5 times prior to feeding to determine routine oxygen consumption (RMO_2; mg O_2 kg^{-1} h^{-1}) (see below). After the 5^{th} measurement, the fish were fed one of the two diets until apparent satiation was reached (satiation determined as the point when fish were not actively feeding from the water column and pellets remained at the bottom of the respirometers for more than 2 min). After removing the uneaten pellets, the respirometers were sealed and water flow was restored for 1 h. Thereafter, oxygen consumption measurements were automatically performed by the computer-controlled respirometry system. This system turned off the water flow into each tank for 30 min. every 2 h, and measured the drop in water oxygen content. These measurements continued until oxygen consumption reached levels similar to those seen prior to feeding (i.e. routine levels). The total time elapsed was recorded as the duration of SDA (SDA_{DUR}, in h).

This experiment was conducted 4 times at each water temperature for both Atlantic cod and haddock, and thus 8 measurements of oxygen consumption were made for each temperature/diet combination for both species. Oxygen consumption was calculated according to Crocker and Cech (1997) and Perez-Casanova *et al.* (2008) as:

$$MO_2 = (O_{2i} - O_{2f})\,(Vol)\,g^{-1}\,t^{-1}$$

where O_{2i} = initial water oxygen concentration (mg l^{-1}), O_{2f} = final water oxygen concentration (mg l^{-1}), Vol = volume of tank respirometer (l), g = fish mass (g) and t = measurement time (h).

SDA (mg O_2) was calculated by subtracting (RMO_2 x SDA_{DUR}) from the total O_2 consumed during the period of SDA, and peak oxygen consumption (SDA_{MAX}, in mg O_2 h^{-1}) was recorded as the maximum increase in oxygen consumption above baseline for each tank. The food consumed by each tank was reported as % body mass (taking into account the food not eaten). Further, since all the fish were fed to satiation and the amount of food ingested was different between groups and temperatures, we also calculated SDA_{DUR} and SDA as a function of the amount of food or energy ingested: $SDA_{DUR\ ENER}$, (h g $food^{-1}$) and SDA_{ENER} (as % of ingested energy). To transform SDA (mg O_2) to SDA_{ENER}, an oxycalorific coefficient was calculated for each diet according to Brafield (1985): 13.80 J mg^{-1} O_2 for the HP diet and 13.98 J mg^{-1} O_2 for the LP diet.

Table 2.1. Composition of experimental diets fed to the juvenile Atlantic cod and haddock.

Ingredient	Diet	
	Low Protein	High Protein
	(%)	(%)
Herring meal[a]	28.40	52.70
Crab meal[b]	6.00	6.00
Krill hydrolysate[c]	3.00	3.00
Corn gluten meal[d]	10	10
Wheat middling[e]	38.8	21.8
Herring oil[f]	11.20	3.9
Vitamin mixture[g]	1..6	1.6
Mineral mixture[h]	1.00	1.00

[a]Scotia Garden Seafood Incorporated (Yarmouth, NS, Canada)
[b]St. Laurent Gulf Products Limited (Caraquet, NB, Canada)
[c]Aqion (Colorado Springs, CO, USA)
[d]Corey Feed Mills Ltd. (Fredericton, NB, Canada)
[e]Dover Mills Ltd. (Halifax, NS, Canada)
[f]Herring oil was stabilized with 0.06% ethoxyquin (Comeau seafood, Saulnierville, NS, Canada), POV 0.46 meq kg^{-1} oil
[g]Vitamin added to supply the following (per kg): vitamin A (retinol acetate), 6000 IU; vitamin D_3 (cholecalciferol), 4000 IU; vitamin E (dl-α-tocopheryl acetate), 400 IU; vitamin K_3 (menadione sodium bisulfite), 40 mg; thiamin (thiamin HCl), 50 mg; riboflavin, 50 mg; d-calcium pantothenate, 150 mg; biotin, 1 mg; folic acid 15 mg; vitamin B_{12}, 0.15 mg; niacin, 200 mg; pyridoxine HCl, 20 mg; ascorbic acid (ascorbyl monophosphate, 200 mg; inositol, 400 mg; choline chloride, 300g; butylated hydroxytoluene (BHT), 15 mg; butylated hydroxyanisole (BHA), 15mg.
[h] Minerals added to supply the following (per kg diet): manganous sulfate ($MnSO_4 \cdot H_2O$, 32.5 % Mn), 40 mg; ferrous sulfate ($FeSO_4 \cdot H_2O \cdot 7H_2O$, 20.1% Fe), 30 mg; copper sulphate ($CuSO_4 \cdot 7H_2O$, 25.4% Cu), 5mg; zinc sulfate ($ZnSO_4 \cdot 7H_2O$, 22.7% Zn), 75 mg; cobalt chloride ($CoCl_2 \cdot 6H_2O$, 24.8% Co), 2.5 mg; sodium selenite (Na_2SeO_3, 45.6% Se), 1 mg; sodium fluoride (NaF, 42.5% F), 4 mg.

Table 2.2. Results of proximate analyses and bomb calorimetry performed on the experimental diets fed to the juvenile Atlantic cod and haddock.

Parameter	Diet	
	Low Protein	High Protein
Protein (%)	42.3	55.0
Lipid (%)	16.4	10.6
Ash (%)	6.5	9.1
Carbohydrate (%)	26.3	17.3
Moisture (%)	10.7	12.2
Energy (cal g^{-1})	5282.6	5346.5

2.3.4 Statistical Analyses

A general linear model (Method 1) was used to analyze the effects of diet, temperature and species on SDA, SDA_{MAX}, SDA_{DUR} and food consumption of cod at 11, 6 and 2 °C and haddock at 11 and 2 °C. However, when significant interactions were encountered using this model, the data were then analyzed, for each species, using a 2-way ANOVA (Method 2, main effects temperature and diet; see Table 4).When main effects were identified as significant ($P < 0.05$) for species, temperature or diet, Student Newman-Keuls post-hoc tests were performed to identify where significant differences were within each of these experimental parameters. Throughout the text, and in figures and tables, values are reported as means $\pm$ S.E. Statistical analyses were performed using STATISTICA for Windows 98 (StatSoft Inc., Tulsa, OK, USA).

2.4 Results

Changes in the protein and lipid content of the two experimental diets had no significant ($P > 0.05$) effect on any of the measured parameters. In contrast, numerous species and temperature effects were observed (see Tables 2.3 and 2.4; Fig. 2.1- 2.3). Routine oxygen consumption was strongly temperature dependent and averaged ~ 140, 95 and 60 mg O_2 kg^{-1} hr^{-1} for Atlantic cod at 11, 6 and 2 °C, respectively. The significant decrease in routine MO_2 with temperature resulted in Q_{10} [the factor by which the rate of a biological process increases for every 10 °C in temperature] values for Atlantic cod of 2.0 between 11 and 6 °C, 3 between 6 and 2 °C, and 2.8 between 11 and 2 °C. Although the Q_{10} value (3.07) for haddock oxygen consumption between 11

and 2 °C was similar to that measured in the Atlantic cod, values for routine MO_2 were approximately 25% and 35% lower at 11 at 2 °C, respectively (Table 2.3).

Water temperature also had a significant negative effect on the food consumption of both species, with food consumption decreasing from ~ 2.0% body mass at 11 and 6 °C to approx. 0.6 % body mass at 2 °C (Fig. 2.2C); a decrease of 60-70%. Surprisingly, however, this decrease in food consumption had no major effects on the profile of post-feeding MO_2 and only relatively minor effects on SDA_{DUR}. For example, oxygen consumption peaked at 30 – 50% above routine MO_2 shortly (3-5 h) after the fish reach satiety and decreased slowly thereafter (Fig. 2.1). Further, SDA_{DUR} in Atlantic cod ranged from ~ 65 to 85 h at the three temperatures, and only decreased significantly (by ~ 22%) in haddock between 11 and 2 °C (Fig. 2.2A). The disparity between the effects of temperature on food consumption and SDA_{DUR} was apparently because it took longer to digest/assimilate each gram of food consumed as temperature decreased; $SDA_{DUR\ ENER}$ approx. 3 h g $food^{-1}$ at 11 °C but increasing to ~ 8 – 12 h g $food^{-1}$ at 2 °C (Fig. 2B). Overall, species had a significant effect on SDA_{DUR}, with SDA_{DUR} in Atlantic cod approx. 25% greater than in haddock at 2 °C (Table 2.4, Fig. 2.2A). This difference in SDA_{DUR} was associated with a trend of increased $SDA_{DUR\ ENER}$ in Atlantic cod as compared to haddock, however, no overall species effect was detected for this parameter ($P = 0.15$; Table 2.4).

In contrast to SDA_{DUR}, the magnitude of SDA decreased considerably with temperature, and reflected the diminished food consumption as water temperature declined. For example, SDA decreased substantially in both species between 6 and 2 °C, and the overall decrease in SDA between 11 and 2 °C was ~ 60-65% in Atlantic cod and ~ 75-80% in haddock (Fig. 2.3A). This close association between food

consumption and SDA was because, in both species, temperature had no significant effect on SDA as a percentage of the ingested energy (SDA_{ENER}); SDA_{ENER} ranging from 2.3 – 3.7% (Fig. 2.3B). The general linear model for SDA_{MAX} indicated that there were no significant effects at any diet/temperature combination for either species.

2.5 Discussion

2.5.1 Routine Oxygen Consumption

Routine MO_2 values for the Atlantic cod in our study [140 and 95 mg O_2 kg^{-1} h^{-1} at 11 and 6 °C, respectively; 77 and 52 mg O_2 $kg^{-0.8}$ h^{-1} when corrected for allometric scaling (Saunders, 1963; Reidy *et al.*, 1995)] are within the range of values reported by other authors. For example, in studies using swimming-tunnel respirometry: Tang *et al.* (1994) and Reidy *et al.* (1995) reported a RMR of 96 mg O_2 $kg^{-0.8}$ h^{-1} and 88 mg O_2 $kg^{-0.8}$ h^{-1}, respectively, for adult cod at 5 °C; Bushnell *et al.* (1994) reported a value of 76.1 mg O_2 kg^{-1} h^{-1} for juvenile cod at 5.5 °C; Jordan and Steffensen (2007) measured RMR values of 60 and 71 mg O_2 kg^{-1} h^{-1} for juvenile fish at 10 °C; whereas Soofiani and Hawkins (1982) reported RMR values similar to those in the present study using individual flow through respirometers (133 and 92 mg O_2 kg^{-1} h^{-1} at 10 and 7 °C, respectively). From the above data it is clear that there is a high degree of variability in RMR values for Atlantic cod amongst studies. This is likely to be largely due to the diverse methodologies used by the authors. For example, the use of groups of fish (30 – 40 fish) in tank respirometers (present study) allowed the juvenile Atlantic cod and haddock to swim freely and to interact with conspecifics, thus increasing the level of

Table 2.3. Routine oxygen consumption (in mg O_2 kg^{-1} h^{-1}) of juvenile Atlantic cod and haddock at temperatures ranging from 2 to 11 °C. Values are means ± S.E.

		11 °C	6 °C	2 °C
Cod				
	HP Diet	$136.6 \pm 6.0^{a*}$	96.1 ± 1.4^{b}	$60.1 \pm 4.2^{c*}$
	LP Diet	$143.9 \pm 7.1^{a*}$	93.7 ± 2.2^{b}	$57.0 \pm 2.6^{c*}$
Haddock				
	HP Diet	107.0 ± 4.2^{a}	ND	37.7 ± 1.5^{b}
	LP Diet	103.6 ± 3.8^{a}	ND	37.7 ± 1.5^{b}

* Indicates a significant difference between species.
Dissimilar letters indicate differences across temperatures.
ND = Not Determined

Parameter	Method	Species	Source	*F*	d.f.	*P*
SDA_{DUR}	1	Cod and Haddock	Sp	7.25	1	0.009
			Diet	0.78	1	0.381
			Temp	7.25	1	0.009
			Sp*Diet	0.17	1	0.678
			Sp*Temp	15.94	1	<0.001
			Diet*Temp	0.95	1	0.334
			Sp*Diet*Temp	0.48	1	0.489
	2	Cod	Diet	0.71	1	0.406
			Temp	6.46	2	0.004
			Diet*Temp	0.52	2	0.598
		Haddock	Diet	0.12	1	0.729
			Temp	25.30	1	<0.001
			Diet*Temp	0.04	1	0.839
$SDA_{DURENER}$	1	Cod and Haddock	Sp	2.16	1	0.147
			Diet	0.35	1	0.555
			Temp	87.74	1	<0.001
			Sp*Diet	0.28	1	0.601
			Sp*Temp	3.82	1	0.056
			Diet*Temp	1.74	1	0.192
			Sp*Diet*Temp	1.43	1	0.237
	2	Cod	Diet	0.69	1	0.412
			Temp	38.29	2	<0.001
			Diet*Temp	1.71	2	0.194
		Haddock	Diet	0.00	1	0.955
			Temp	37.99	1	<0.001
			Diet*Temp	0.01	1	0.918
Food Consumption	1	Cod and Haddock	Sp	3.23	1	0.078
			Diet	2.05	1	0.157
			Temp	212.14	1	<0.001
			Sp*Diet	2.66	1	0.109
			Sp*Temp	1.29	1	0.262
			Diet*Temp	3.34	1	0.073
			Sp*Diet*Temp	2.49	1	0.129

Cont...

Parameter	Method	Species	Source	*F*	d.f.	*P*
	2	Cod	Diet	1.44	1	0.237
			Temp	44.47	2	<0.001
			Diet*Temp	2.88	2	0.067
		Haddock	Diet	0.06	1	0.803
			Temp	294.19	1	<0.001
			Diet*Temp	0.10	1	0.753
SDA	1	Cod and Haddock	Sp	0.76	1	0.386
			Diet	0.66	1	0.420
			Temp	112.46	1	<0.001
			Sp*Diet	0.02	1	0.891
			Sp*Temp	1.96	1	0.167
			Diet*Temp	0.58	1	0.449
			Sp*Diet*Temp	0.00	1	0.966
	2	Cod	Diet	0.98	1	0.328
			Temp	28.86	2	<0.001
			Diet*Temp	0.32	2	0.729
		Haddock	Diet	0.42	1	0.524
			Temp	66.61	1	<0.001
			Diet*Temp	0.30	1	0.589
SDA_{ENER}	1	Cod and Haddock	Sp	2.52	1	0.118
			Diet	0.17	1	0.678
			Temp	0.96	1	0.332
			Sp*Diet	0.29	1	0.593
			Sp*Temp	4.44	1	0.040
			Diet*Temp	0.01	1	0.929
			Sp*Diet*Temp	0.89	1	0.348
	2	Cod	Diet	0.16	1	0.691
			Temp	2.69	2	0.080
			Diet*Temp	0.50	2	0.608
		Haddock	Diet	0.63	1	0.433
			Temp	0.88	1	0.356
			Diet*Temp	0.51	1	0.482

Cont...

Parameter	Method	Species	Source	*F*	d.f.	*P*
SDA_{MAX}	1	Cod and Haddock	Sp	3.93	1	0.052
			Diet	0.35	1	0.553
			Temp	0.29	1	0.588
			Sp*Diet	0.00	1	0.945
			Sp*Temp	0.41	1	0.525
			Diet*Temp	0.43	1	0.514
			Sp*Diet*Temp	0.02	1	0.876
	2	Cod	Diet	7.77	2	0.001
			Temp	0.10	1	0.748
			Diet*Temp	0.33	2	0.719
		Haddock	Diet	0.10	1	0.756
			Temp	0.49	1	0.488
			Diet*Temp	0.09	1	0.769

Sp = Species; Temp = Temperature; Method 1 = 2 sp, 2 temperatures, 2 diets; Method 2 = Species analyzed indicidually: Atlantic cod, 3 temperatures, 2 diets and haddock, 2 temperatures, 2 diets.

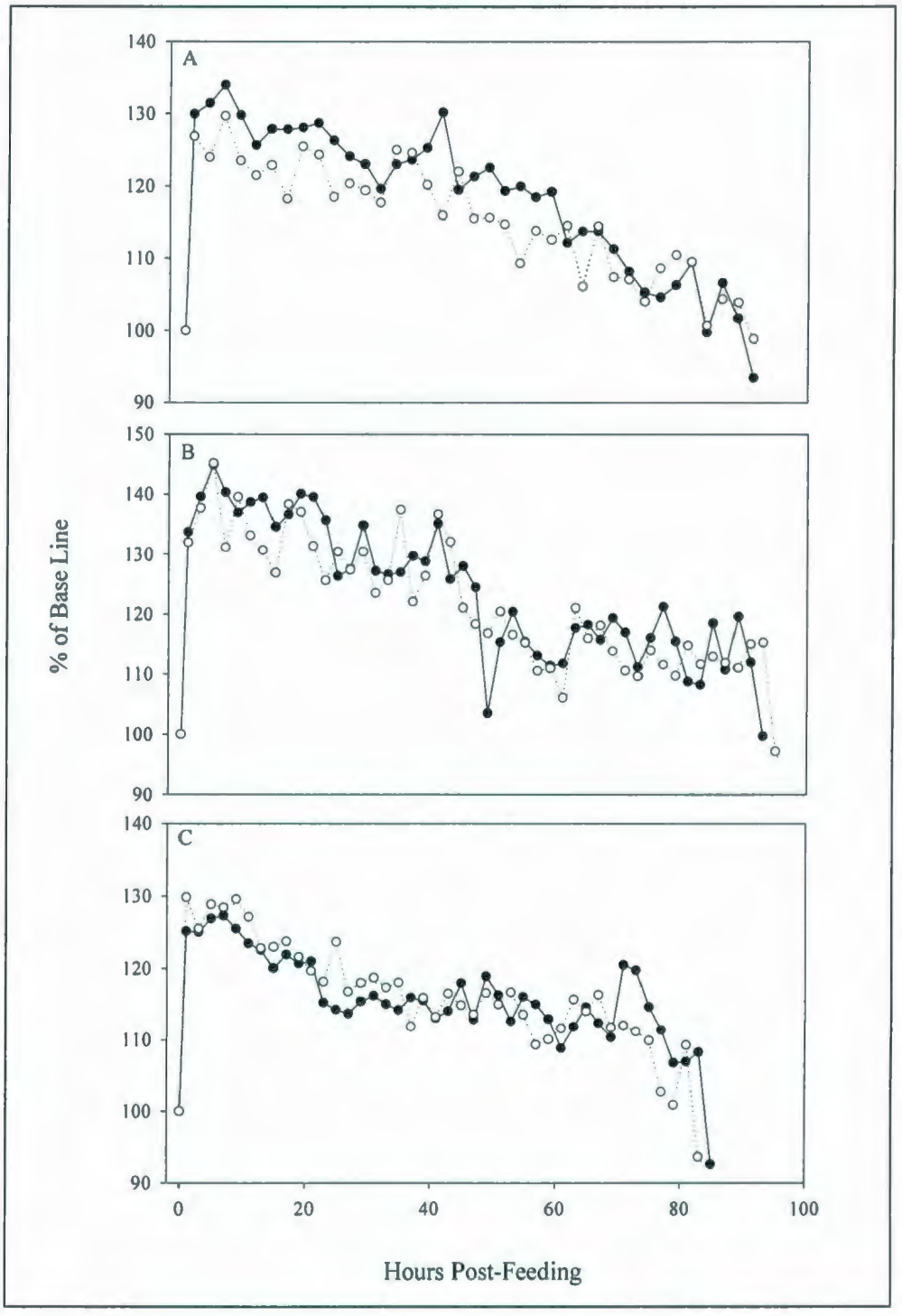

Fig. 2.1. Changes in oxygen consumption in juvenile Atlantic cod after feeding with either a high (55%; filled symbols) or low (42%; open symbols) protein meal at 11 (A), 6 (B) or 2 °C (C). Values are reported as a percentage relative to the fish's routine oxygen consumption (RMO_2) prior to feeding (i.e. baseline is set to 100%). Each plot represents the mean of 1 experimental run (2 tanks each for low and high protein diets).

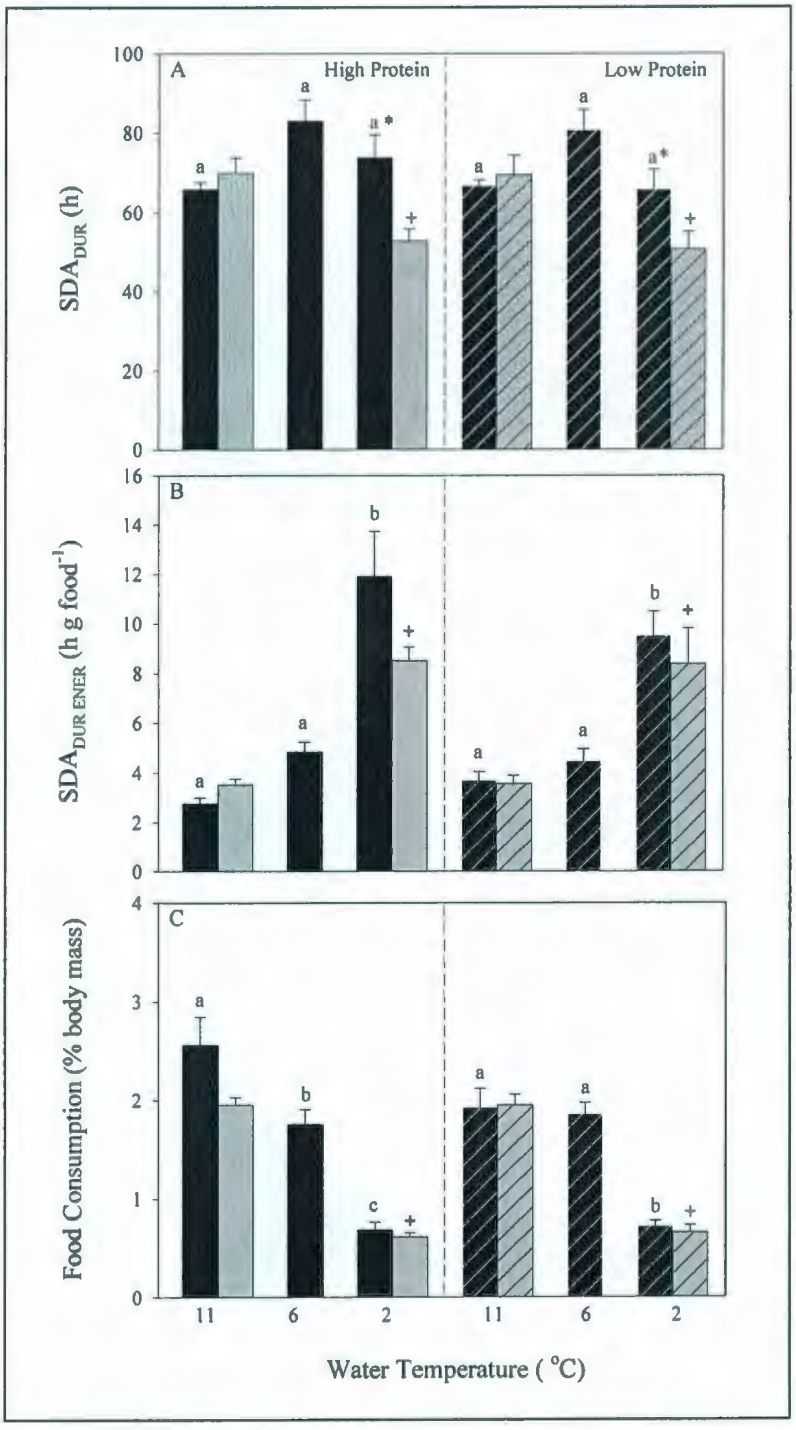

Fig. 2.2. Duration of SDA (A), duration of SDA per g of food (B) and food consumption (C) of juvenile Atlantic cod (black columns) and haddock (grey columns) fed either a high protein or low protein meal at 11, 6 and 2 °C. See Table 2.3 for statistical analysis. Values are means ± S.E. (N = 8). Within each diet, Atlantic cod values without a letter in common are statistically different ($P < 0.05$), a + indicates a significant difference between haddock at 11 and 2 °C, and an * indicates a significant difference between the two species at a particular temperature.

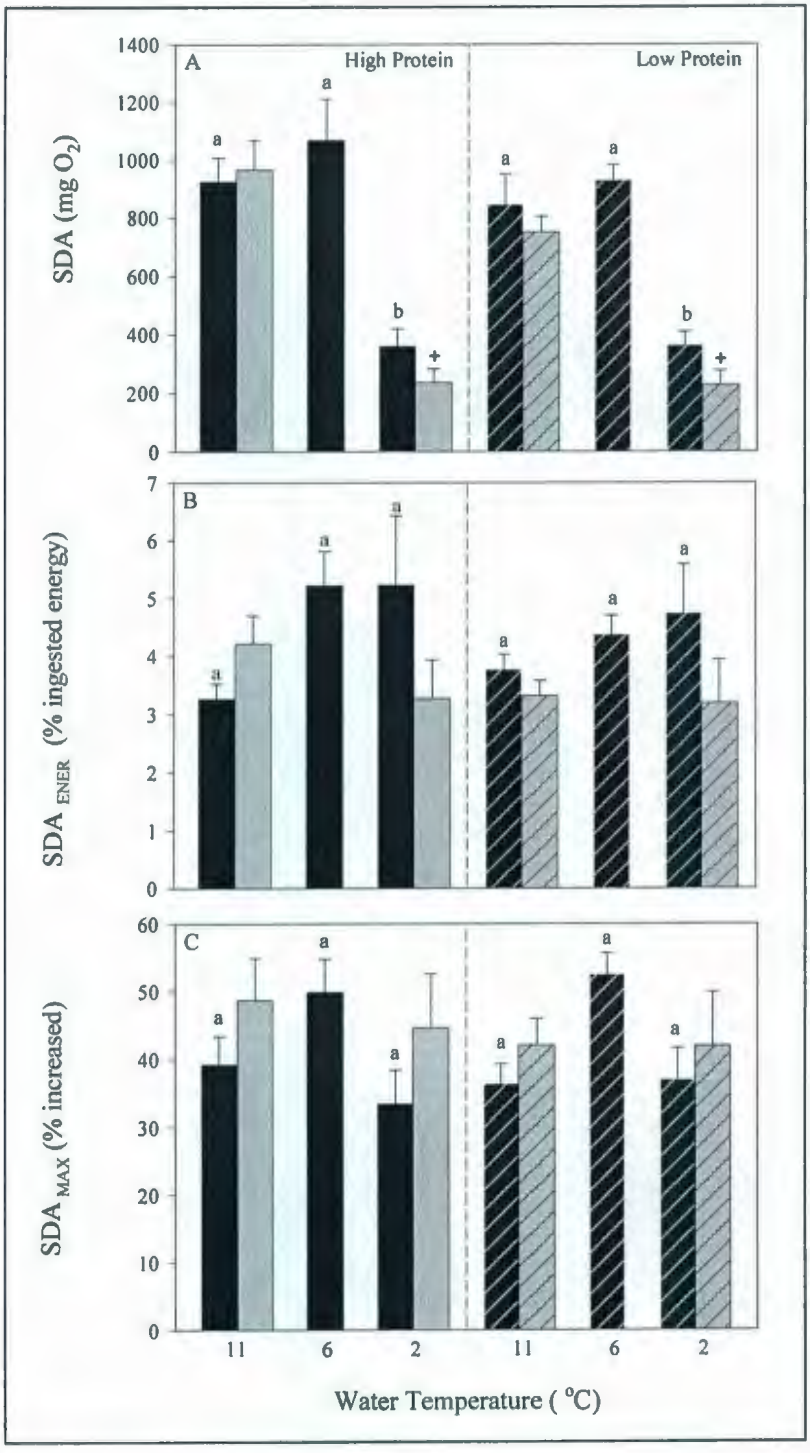

Fig. 2.3. Total cost of digestion (SDA) (A), SDA as a percentage of ingested energy (B) and peak oxygen consumption (C) of juvenile Atlantic cod (black columns) and haddock (grey columns) fed either a high protein (left panel) or a low protein (right panel) meal at 11, 6 and 2 °C. See Table 2.3 for statistical analysis. Values are means $\pm$ S.E. (N = 8). Within each diet, Atlantic cod values without a letter in common are statistically different ($P < 0.05$), a + indicates a significant difference between haddock at 11 and 2 °C, and an * indicates a significant difference between the two species at a particular temperature.

activity (in accordance with our occasional observations). In contrast, fish in swim-tunnels are usually tested alone and do not have the liberty of extensive movement, but on the other hand, may be stressed by the confinement and lack of social interaction. However, population-based differences in standard/routine metabolic rates exist (Nelson *et al.*, 1994; Sylvestre *et al.*, 2007), and resting metabolism may be affected by several factors such as prolonged food deprivation (Fu *et al.*, 2005), sex, and seasonality (Karamushko and Christiansen, 2002). Thus, metabolic rate may vary between studies, even if measured at similar temperatures.

Our experiments showed that RMR was reduced significantly in both Atlantic cod and haddock as water temperature was decreased from 11 to 6 °C, and finally to 2 °C, and that the RMO_2 of haddock was significantly lower than that of Atlantic cod when tested at equivalent temperatures. This difference was approximately 25% at 11 °C and 35% at 2 °C, similar to the difference reported by Tytler (1978) for these two species at 10 °C. The reasons for this difference in RMR between species are unknown. However, these results suggest that the maintenance ration of juvenile haddock is less than that of juvenile Atlantic cod, and thus that haddock could potentially grow better than Atlantic cod at ration levels below satiation. The better growth of haddock, as compared with Atlantic cod, has been previously demonstrated by Peck *et al.* (2003b). Further, these authors reported that the maximal gross growth efficiency for age-0 haddock was greater than that of Atlantic cod when individuals of both species where reared at 8 °C, and that at 12 °C it was ~3 times greater.

Despite the variability in RMR between species, and as compared with other studies, the Q_{10} values obtained in the present study (2.8 and 3.1 for Atlantic cod and

haddock between 11 and 2 °C) were similar to those obtained by other authors. For example, Peck *et al.* (2005) reported average Q_{10} values of 2.3 between 5 and 15 °C for juvenile haddock, Peck *et al.* (2003a) obtained Q_{10} values of 3.2 for juvenile Atlantic cod of 3.0 g between 4.5 and 8 °C, and Soofiani and Hawkins (1982) obtained average values of 3.8 between 7 and 10 °C for Atlantic cod (calculated using their Table 2; range = 2.4 - 4.5).

2.5.2 Temperature Effects on Food Consumption

In the present study, exposing the fish to low water temperatures resulted in decreased feeding levels, e.g. food consumption decreased from ~ 2.0% body mass at 11 and 6 °C to ~ 0.6 % body mass at 2 °C. Similar decreases in food consumption at cold water temperatures have been reported for a number of fish species. For example, Atlantic salmon parr (*Salmo salar* L.) held at 2 °C, and fed either a low or high lipid diet, had a significantly lower (~75%) food intake than at 8 °C (Bendiksen *et al.*, 2002). Gilthead sea bream (*Sparus aurata*) held at 18 °C and subjected to a gradual decrease in water temperature to 11 °C showed a marked decrease (~70%) in food consumption (Tort *et al.*, 2004b). Further, a substantial decrease in food consumption at lower water temperatures has been previously described for Atlantic cod and haddock. Brown *et al.* (1989) reported a lower food intake in Atlantic cod held at 0.6 °C as compared with fish held at 4.5 (~37% reduction) and 8.3 °C (~58% decrease). Peck *et al.* (2003b) reported that maximum food consumption decreased with water temperatures in Atlantic cod juveniles held at 12, 8 or 5 °C; this decrease in food consumption was independent of fish size and averaged ~45% from 12 to 5 °C.

Finally, these same authors reported a similar temperature-dependent effect for juvenile haddock, with the reduction in maximum food consumption averaging ~10% from 12 to 8 °C.

The mechanism(s) that mediate temperature-dependent changes in food consumption/appetite have only begun to be examined. However, it is likely that neurohormones are involved. For example Kehoe and Volkoff (in press) reported a decrease in food intake when cod were acclimated to 2 °C (~0.25% BM day^{-1}) as opposed to 11 and 15 °C (~0.75% BM day^{-1}), and that this decrease in food consumption was matched by a significant (~40 %) increase in the expression of cocaine and amphetamine regulated transcript (CART). This peptide is known to inhibit feeding in mammals (Vicentic and Jones, 2007) and in fish (Volkoff and Peter, 2000), and to act as a satiety factor in Atlantic cod (Kehoe and Volkoff 2008). In addition, it has been suggested that CART has a strong link with thermogenesis in mammals, and that cold exposure results in increased CART levels in the brain of rats (Kong *et al.*, 2003). Thus, in the present study, it is likely that an increase in CART levels played a significant role in the reduction in food consumption in both Atlantic cod and haddock between 6 and 2 °C.

2.5.3 Specific Dynamic Action (SDA): Duration

In our study, when juvenile Atlantic cod and haddock were fed to satiation, metabolic rate peaked (at approx. 35 - 50% above RMR) shortly after feeding, and declined slowly to baseline levels (i.e. over approx 55 to 80 h; Figure 1). This pattern of change in SDA has been described in other studies on gadoids (Jobling, 1981a;

Blaikie and Kerr, 1996; Hunt von Herbing and White, 2002; Peck *et al.*, 2005). However, the SDA_{DUR} in this study was much longer than in other studies on juvenile gadoids where values for SDA_{DUR} of 8-12 h were typically reported (Hunt von Herbing and White, 2002; Peck *et al.*, 2005). Although the ration size was smaller in this study [e.g. ~ 2.0% body mass at 11 °C vs ~7.5% body mass at 10 °C in Hunt von Herbing and White (2002)], and thus SDA_{DUR} would be expected to be shorter, the size of the fish used in this study (approx. 30-40g vs. < 10 g in the other studies) may have contributed to the longer SDA_{DUR} as several authors have reported that the time for gastrointestinal evacuation increases with fish size (Jobling, 1981a; Peck *et al.*, 2003a; 2005). Nonetheless, differences in fish size could not have accounted for the approx. 6-7 fold longer SDA_{DUR} measured in this study. Interestingly, Blaikie and Kerr (1996) reported SDA_{DUR} values of 51 – 100 h for actively swimming adult Atlantic cod that had been fed a ration equivalent to 2.5% of their body mass, and that SDA_{DUR} increased with swimming speed. This latter study suggests that elevated levels of activity by the fish in our tank respirometers (in agreement with our visual observations), as compared to previous studies where swim-tunnels or individual respirometers with stagnant flow were used, resulted in the prolonged SDA_{DUR}. Furthermore, when the data of the present study and Blaikie and Kerr (1996) are combined, it appears that previous studies using traditional respirometers have significantly underestimated the time required for juvenile Atlantic cod and haddock to completely digest and assimilate the food they consume, and thus, that this data should not be used to estimate or model parameters such as SDA_{DUR} under aquaculture conditions.

Dietary protein level did not have a significant effect on SDA_{DUR}, and this result is consistent with the findings of LeGrow and Beamish (1986). However, the duration of SDA was highly temperature dependent (Fig. 2.5). The effect was dramatic for $SDA_{DUR\ ENER}$ where values increased from 2-3 to 8-12 h g^{-1} food, but less so when SDA was expressed simply in h. This is because food consumption decreased from > 2.0 % body mass at 11 °C to approximately 0.6 % body mass at 2 °C. The increase in $SDA_{DUR\ ENER}$ at the lowest temperature may be partially explained by an increase in gastrointestinal evacuation time, since the mechanical components of SDA include peristaltic movements involved in the passage of food through the alimentary canal (Tandler and Beamish, 1979) and SDA duration with low temperature has been correlated with low rates of digestion and gastric evacuation (Jobling and Davies, 1979; Jobling and Davies, 1980). For example, gastric evacuation rates decreased linearly in brook trout (*Salvelinus fontinalis*) with decreasing temperature from 12.1 to 4.3 °C (Sweka *et al.*, 2004), and in plaice (*Pleuronectes platessa* L.) the effect of temperature on gut evacuation was exponential from 20 to 2 °C (from ~20 – 160 h) (Edwards, 1971).

Species had no effect on food consumption or $SDA_{DUR\ ENER}$, however, the effect of temperature on SDA_{DUR} was different for Atlantic cod and haddock. For example, SDA_{DUR} increased or remained the same when Atlantic cod were exposed to lower temperatures, whereas it decreased by approximately 20 h when the acclimation temperature of haddock was lowered from 11 to 2 °C. The cause of this difference in SDA_{DUR} between the two species is unknown.

2.5.4 Specific Dynamic Action (SDA): Magnitude of SDA and Peak Oxygen Consumption

There was a highly significant temperature effect on the magnitude of SDA, with SDA dropping substantially (by ~ 62% for Atlantic cod and ~75% for haddock) between 11 and 2 °C. This drop in SDA was primarily due to the decrease in food consumption that was concomitant with acclimation to colder water temperatures, as changes in SDA mirrored those for food consumption (compare Figures 2.2C and 2.3A) and there was no effect of temperature on SDA when measured as a percentage of ingested energy (Figure 2.3B). The cost of digestion/assimilation ranged from 3.3 – 5.2 % of ingested energy, and was not affected by the protein level of the diet. While our values of SDA_{ENER} are very similar to values reported by Peck *et al.* (2003a; 2005) (3-4%), the lack of an effect of dietary protein level on SDA and SDA_{ENER} was surprising given earlier work on this topic. For example, Jobling and Davies (1980) and LeGrow and Beamish (1986) indicate that increasing dietary protein level increases SDA in plaice (*Pleuronectes platessa*) and rainbow trout, respectively, and it is estimated that protein synthesis in fishes accounts for between 40 and 50% of the costs associated with SDA (Houlihan *et al.*, 1988; Lyndon *et al.*, 1992; Owen, 2001). However, this difference in the effect/lack of an effect of protein level on SDA between this study and previous studies is likely to be largely explained by the isoenergetic nature of the diets used in the present study. This is because total digestible energy of the diet can have a major effect on the magnitude of SDA (Jobling and Davies, 1980; Jobling, 1981a), and Eliason *et al.* (2007; 2008) recently showed that post-prandial oxygen consumption (SDA) in rainbow trout fed isoenergetic diets

was not affected by dietary protein content (range 35 – 55%). Alternatively, it has been shown that higher lipid content diets can improve the assimilation of protein into muscle, and thus growth, by allowing the fish's energetic demand to be fulfilled by the increased availability of dietary lipid (Morais *et al.*, 2001; Bendiksen *et al.*, 2003). Thus, it is also possible that the metabolic costs associated with an increased protein assimilation efficiency in fish fed the 42% protein:16% lipid diet may have partially offset the elevation in SDA that can be associated with feeding a high protein diet.

Maximum post-feeding oxygen consumption ranged from 33 – 51% above baseline (pre-feeding) levels (Fig. 2.3). This increase in metabolic rate is comparable to values reported for juvenile haddock (44%; (Peck *et al.*, 2003a; 2005), Atlantic cod (54%; (Peck *et al.*, 2003a; 2005) and turbot (*Scophthalmus maximus*) (29-50%; (Mallekh and Lagardere, 2002), and was independent of temperature when expressed in these units (*i.e.* as a percentage increase). At 2 °C, maximum post-feeding oxygen averaged 1.3 and 1.4 times RMR for the Atlantic cod and haddock, respectively, and since the factorial metabolic scope calculated by Claireaux *et al.* (2000) for Atlantic cod is 3.3 at this temperature, it is highly unlikely that food intake was constrained at this temperature due to a limited metabolic capacity. This information makes it even more likely that neurohormonal mechanisms were responsible for the large (60-70%) reduction in appetite/food consumption in haddock and Atlantic cod acclimated to 2 as compared with 6 or 11 °C.

Overall, this study showed that: 1) acclimation to 2 °C had a large depressive effect on food consumption (the fish's level of satiation), and that digestion/assimilation of the amount of food that was consumed was greatly prolonged; and 2) there was no effect of dietary protein and lipid level on any of the

measured parameters, and little difference between species with respect to the magnitude and duration of SDA. Thus, these results indicate that switching to a reduced protein content feed at low water temperatures, while cost effective, is unlikely to result in improved growth of these two species. Finally, our results provide a number of important insights with regards to the metabolic physiology of gadoids. Specifically, food consumption is probably not reduced at cold temperatures because metabolic scope constrains digestive function, that the low routine metabolic rate of juvenile haddock as compared to Atlantic cod may partially explain their enhanced growth rate at ration levels below satiation, and it appears that measurements on individually confined fish greatly underestimate the duration of SDA under typical aquaculture conditions.

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Chapter 3. Effect of Feed Composition and Temperature on Food Consumption, Growth and Gastric Evacuation of Juvenile Atlantic Cod (*Gadus morhua* L.) and Haddock (*Melanogrammus aeglefinus* L.).

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3.1 Abstract

During sea-cage rearing, Atlantic cod and haddock are fed commercial diets that have high protein content (~50–58%) and are designed for optimum growth at temperatures of 10 - 12 °C. However, in Atlantic Canada these fish spend several months at temperatures below 5 °C and data on other fish species (e.g. salmonids) suggest that growth could be enhanced at low water temperatures by feeding a diet with lower protein (LP) content and higher lipid. Thus, we explored how dietary protein and lipid (P/L) levels of 42/16 (LP diet) and 55/11 (high protein, HP diet; both diets isoenergetic) and water temperature (11, 6 and 2 °C) influenced the specific growth rate (SGR), hepatosomatic index (HSI) adjusted specific growth rate (SRG_A), food consumption (FC), gross conversion efficiency (GCE) and gastric evacuation (GE) of juvenile (35 – 59 g) Atlantic cod and haddock. Several temperature and species-specific effects were observed after the 8 week growth trialS. Significant decreases in SGR, SGR_A and FC (range 60–96%) were evident in both species when reared at 2 vs. 11 °C. While the SGR and SGR_A of haddock were significantly (2-fold) higher than for Atlantic cod when reared at 11 °C, this effect was reversed when they were reared at 2 °C. With regards to the effects of dietary protein level, it was found that while feed composition did not affect growth rate (with the exception of haddock at 11 °C) or gastric evacuation, GCE was significantly lower in fish fed the LP diet. These results indicate that 1) growth rate differences between the two species are temperature dependent, with juvenile haddock growing faster and slower than Atlantic cod at 11 °C and 2 °C, respectively; and 2) based on the similar growth rates but lower GCE values, there is no advantage to feeding a LP diet even at cold temperatures.

3.2 Introduction

Due to the decline in the wild fisheries and sustained consumer demand, Atlantic cod (*Gadus morhua* L.) and haddock (*Melanogrammus aeglefinus* L.) are two species that have been identified as promising candidates for marine aquaculture (Morais *et al.*, 2001; Nanton *et al.*, 2001; Rosenlund and Skretting, 2006). The development of culture techniques suitable for these two species has been ongoing for many years in countries such as Norway, Iceland, the United Kingdom, the United States and Canada (Le François *et al.*, 2002; Aiken, 2003; Rosenlund and Skretting, 2006; Treasurer *et al.*, 2006). Larvae and juveniles are typically produced and reared in land-based facilities where conditions can be controlled. However, juveniles must then be transferred to sea-cages for grow out where they are exposed to changes in various environmental factors. For example, sea-cage reared fish are exposed to cold temperatures (< 5 °C) in Atlantic Canada for many months during the winter and these temperatures have a significant negative impact on fish appetite (Brown *et al.*, 1989; Bendiksen *et al.*, 2002), growth (Brown *et al.*, 1989) and activity (Clark *et al.*, 1995). Furthermore, cultured Atlantic cod and haddock are usually fed high protein and low lipid diets that are formulated for optimal growth at temperatures between 10 - 12 °C and to prevent the development of "fatty livers" (Lall *et al.*, 2003).

Protein is the most abundant nutrient in the natural diet of these species (Kim and Lall, 2001) and, because it is typically supplied by high-quality fish meals, accounts for the most expensive constituent of commercial diets (Watanabe, 2002). Thus, it is clear that feed composition must be optimized to achieve significant growth of these species under winter-like conditions and to decrease the costs associated with

feeding. Currently, there is insufficient data on which to base feeding protocols or diet formulations for use at cold temperatures. For example, studies that provide relevant information on the relationship between Atlantic cod husbandry and growth (Lambert and Dutil, 2001) or detailed analyses on the optimization of diets for these species (Lie *et al.*, 1988; Kim and Lall, 2001; Morais *et al.*, 2001; Nanton *et al.*, 2001; Tibbetts *et al.*, 2005) have rarely been conducted at temperatures below 8 °C. Further, although Pérez-Casanova *et al.* (submitted; Chapter 2) showed that there was no benefit to feeding Atlantic cod and haddock a low protein diet at 2 °C based on the cost of digestion (i.e. magnitude and duration of specific dynamic action, SDA), no long-term growth studies have been performed with low protein diets at cold temperatures, and there is no information available on how dietary protein content affects food consumption, growth or gastric evacuation time. This latter parameter may be important since gastric emptying rate has been positively related with return of appetite and food intake in fish species such as the rainbow trout, *Salmo gairdneri,* (Grove *et al.*, 1978), winter flounder, *Pseudopleuronectes americanus,* (Huebner and Langton, 1982), rockfish, *Sebastes schlegeli,* (Lee *et al.*, 2000), dab, *Limanda limanda,* (Gwyther and Grove, 1981) and turbot, *Scophthalmus maximus,* (Grove *et al.*, 1985); and gastric evacuation rate is affected by various factors, including water temperature (Edwards, 1971; Jobling, 1980b; Singh-Renton and Bromley, 1996; Sweka *et al.*, 2004), fish size (Bromley, 1987) and diet composition (Storebakken *et al.*, 1999; Naik *et al.*, 2000).

To better understand the interaction between rearing temperature, dietary protein and lipid levels and cage-site performance of juvenile Atlantic cod and haddock, we measured growth, food consumption, food conversion efficiency,

hepatosomatic index and gastric evacuation rate during an 8 week feeding trial with isocaloric diets containing protein/lipid levels of either 42/16 or 55/11 and at temperatures of 11, 6 or 2 °C. These temperatures were chosen because 11 °C is within the optimum temperature range for growth of juvenile Atlantic cod (Bjornsson *et al.*, 2001a; Peck *et al.*, 2003b), 2 °C poses significant challenges for these species in cage-culture (Brown *et al.*, 1989; Clark *et al.*, 1995) and 6 °C is intermediate between the two temperatures.

3.3 Materials and Methods

3.3.1 Fish Stock and Rearing Conditions

All studies were conducted in accordance with the guidelines published by the Canadian Council on Animal Care, and approved by the Animal Care Committee at Memorial University of Newfoundland (protocol 04-01-KG).

Juvenile haddock used in these experiments were reared from eggs at the Institute for Marine Biosciences, National Research Council (IMB-NRC) Marine Research Station (Ketch Harbour, NS, Canada) following the techniques described in Frantsi *et al.* (2002). Juveniles were initially held in tanks supplied with 10 ± 1 °C seawater (air saturation >90%), and fed twice daily (0800 and 1500 h) at a rate of 1.5% of body mass per day (BM day^{-1}) with a commercial diet (Zeigler Bros., Inc., Gardners, PA, USA; 50% protein, 15% lipid). Thereafter, the juvenile haddock were transported to the Aquaculture Research and Development Facility (ARDF) of the Ocean Sciences Centre (Memorial University of Newfoundland) prior to

experimentation. At the ARDF they were initially held in two 4000 L holding tanks supplied with seawater (temperature 11 ± 1 °C; oxygen saturation >90%, and 12h light:12h dark photoperiod) and fed twice daily (0900 and 1600 h) at a rate of 1.5% BW day^{-1} with a commercial diet (EWOS Canada Ltd, Surrey, BC, Canada; 55% protein, 15% lipid).

Juvenile Atlantic cod used in these experiments were reared in production tanks following standard rearing protocols in place at the ARDF. Juveniles were held in 6000 L tanks supplied with filtered and oxygenated seawater (temperature 11 ± 1 °C; air saturation >90%, and a photoperiod of 12h light:12h dark) and were fed EWOS commercial feed twice daily (0900 and 1600 h) at a rate of 1.5% BM day^{-1}.

3.3.2 Acclimation

Two months before the start of the experiments, the fish were acclimated to 11, 6 or 2 °C. However, no 6 °C experiments were performed with haddock, as insufficient numbers of fish were available to conduct experiments at all temperatures. During this period both species were maintained on a 12h light:12h dark photoperiod and offered the EWOS diet at a rate of 1.5% BM every second day. This change in protocol was implemented in an attempt to ensure that the fish did not grow too large before all experiments including those in Perez-Casanova *et al.* (submitted; Chapter 2) could be completed, and to minimize size differences between fish held at the different temperatures. However, at the end of the acclimation period, the juvenile haddock used in the 2 °C experiments were significantly heavier (by ~ 22 g) as compare to the temperature-matched Atlantic cod (Table 3.1).

Table 3.1. Initial body mass values for the different groups of juvenile Atlantic cod and haddock used during the growth trials. Values are means ± S.E. Different letters indicate significant differences.

		Initial Weights (g)		
		11 °C	6 °C	2 °C
Atlantic cod	HP Diet	40.19 ± 1.17^{a}	37.97 ± 1.30^{a}	37.57 ± 0.75^{a}
	LP Diet	39.15 ± 0.96^{a}	37.43 ± 0.89^{a}	37.48 ± 0.38^{a}
Haddock	HP Diet	36.22 ± 0.48^{a}	ND	59.64 ± 0.50^{b}
	LP Diet	35.07 ± 0.43^{a}	ND	58.97 ± 0.60^{b}

HP = High Protein Diet; LP = Low Protein Diet; ND = Not Determined

3.3.3 Growth Experiment

One week prior to the beginning of the growth experiments, 50 temperature acclimated (2, 6 or 11 °C) juvenile Atlantic cod or haddock were placed into eight 500 L, conical bottom tanks (1.0 m diameter and 0.8 m deep) to allow the fish to acclimate to their new conditions and to recover from handling/netting stress: four tanks were randomly assigned to the control group (high protein diet, HP) and four to the experimental group (low protein diet, LP). For the first 5 days, the fish were fed to satiation once a day with the experimental diets (see below) and were maintained on a 12h light:12h dark photoperiod. All groups were food deprived for 2 days prior to the initial measurement of wet mass and standard length. Both diets fed to the juvenile Atlantic cod and haddock were prepared as 4 mm pellets. The HP diet was similar in protein level to that currently used at Atlantic cod aquaculture cage-sites in Newfoundland and Labrador (protein and lipid levels of 55 and 11%, respectively), while the LP diet contained protein and lipid levels of 42 and 16%, respectively. These diets were isocaloric and contained krill hydrosylate (30 g kg^{-1}) to promote food consumption. See Perez-Casanova *et al.* (submitted; Chapter 2) for further details on diet composition and proximate analysis. All diets were formulated and prepared at the IMB-NRC in a manner similar to Tibbetts *et al.* (2005).

3.3.3.1 Feeding

During the 8 week experiments, the fish were manually fed one of the two experimental diets twice daily (0900 and 1600 h) until apparent satiation was reached: satiation was determined as the point when fish stopped actively feeding and pellets

remained at the bottom of the tanks for more than 2 min. Uneaten pellets were then siphoned out of the tanks and immediately placed in a drying oven for 24 h at 70 °C. The amount of food consumed during each meal was calculated as the difference between the dry mass of the food offered and that of the uneaten food after drying. Food consumption (FC) was calculated as:

$$FC\ (g\ day^{-1}) = (g\ food\ consumed * days^{-1})$$

and gross conversion efficiency (GCE) for each group was calculated as:

$$GCE = (gain\ in\ fish\ wet\ mass * g\ of\ food\ consumed^{-1})$$

3.3.3.2 Growth

On the first day of the experiment and every two weeks thereafter, the wet mass and standard lengths of all fish were measured after brief anaesthesia in 0.15 g l^{-1} tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd, Vancouver, BC, Canada). Fish wet mass was then used to calculate specific growth rate (SGR; % body mass day^{-1}) using the formula:

$$SGR\ (\%\ BM\ day^{-1}) = 100 * (\ln Final\ Mass - \ln Initial\ Mass) * days^{-1}$$

To account for the large percentage of mass that can be comprised of the liver in gadoids, we also calculated an 'adjusted growth rate' (SGR_A) by subtracting liver

mass from body mass prior to calculating growth rate. To calculate both growth rates, a subsample of 50 fish of each group were sacrificed at the beginning of the experiments by an overdose of tricaine methanesulfonate (0.3 g l^{-1}), and weighed before and after their livers were removed. This same procedure was performed at the end of the experiment to obtain values for SGR and SGR_A using the above formulas. The wet mass of the liver was also used to determine hepatosomatic index (HSI):

$$HSI\ (\%) = (\text{liver mass} * \text{fish mass}^{-1}) * 100$$

3.3.4 Gut Evacuation

After obtaining the wet mass and standard length of all of the fish at the end of the growth experiment, the fish were returned to their experimental tanks and deprived of food for 4 - 6 days to ensure that their gastrointestinal tracts were empty. Thereafter, they were fed to apparent satiation with one of the two experimental diets and sampled (2 per tank, 8 fish in total per treatment) at 0, 3, 6, 9, 12, 24, 48, 72, 96 and 120 h post-feeding. This approach was taken, rather than feeding a standardized amount, because of the extremely low food consumption of both species when maintained at 2 °C (Chapter 2). At each time period, fish were carefully captured with a net, killed by a blow to the head (cerebral percussion) followed by insertion of a cotton swab through the mouth and into the oesophagus and stored at –20 °C until analyses were performed. This procedure avoided the loss of stomach contents that can occur when fish are killed by an anaesthetic overdose (which was observed in a preliminary experiment). To process the fish, they were partially thawed, and the

livers were removed and weighed for calculation of HSI (see above). The stomach was then removed and the contents transferred into a previously weighed aluminum tray (W1) by scraping the stomach and washing it with distilled water. The aluminum trays with the total stomach contents were then dried at 80 °C, and then re-weighed (W2). The dry weight of food contained in the stomach was then calculated by subtracting W1 from W2. This data was then used to calculate gastric evacuation rate (GE, g food h^{-1}; calculated using the stomach content of fish sampled immediately after feeding and the first time point when the stomach was completely empty), and to examine if gastric evacuation was affected by dietary protein content, temperature, or differed between the two species.

3.3.5 Statistical Analyses

A general linear model (Method 1) was used to analyze the effects of diet, temperature and species on SGR, SGR_A, FC, GCE, HIS and GE of cod at 11, 6 and 2 °C and haddock at 11 and 2 °C. However, when significant interactions were encountered using this model the data was then analyzed, for each species, using a 2-way ANOVA (Method 2, main effects temperature and diet; Table 1). When main effects were identified as significant ($P < 0.05$) Student Newman-Keuls post-hoc tests were performed to identify where significant differences were for each of these parameters. Throughout the text, and in figures and tables, values are reported as means $\pm$ S.E. Statistical analyses were performed using STATISTICA (StatSoft Inc., Tulsa, OK, USA).

3.4 Results

3.4.1 Food Consumption, Growth Rate and Conversion Efficiency

Food consumption was not influenced by dietary protein/lipid content in either species at any of the tested temperatures. However, FC decreased dramatically (by ~ 80%) in both cod and haddock when temperature was decreased from 11 to 2 °C and was significantly higher in haddock at both 11 and 2 °C (by approx. 2-fold) (Fig. 3.1A).

In Atlantic cod, protein content of the diet had no effect on SGR and SGR_A, and the effect of temperature on these two parameters largely mirrored those for FC (Fig. 3.1B). For example, SGR and SGR_A fell from approx. 0.8 % BM day^{-1} at 11 °C, to ~ 0.6% BM day^{-1} at 6 °C, and finally to ~ 0.3% BM day^{-1} at 2 °C. In the haddock, however, both diet and temperature affected these two parameters, and there was a significant diet × temperature interaction (see Table 3.1). This was because, although the growth rate of haddock (both SGR and SGR_A) fed the reduced protein diet was approx. 15% lower than in those fed the high protein diet at 11 °C, there were no diet related differences in growth rate at 2 °C (Figs. 3.1B and 3.2A). With regards to temperature, there was a marked decrease in haddock SGR and SGR_A with values at 2 °C ~ 95% lower than measured at 11 °C ($P< 0.001$). In addition, because of the greater decrease in these two parameters in haddock vs. Atlantic cod between 11 and 2 °C (~ 95% vs. 65%, respectively), species also had a profound effect on temperature-dependent growth rates: i.e. although SGR and SGR_A were approximately 2-fold

Parameter	Method	Species	Source	*F*	d.f.	*P*
FC	1	Cod and Haddock	Sp	1418.405	1	<0.001
			Diet	1.489	1	0.234
			Temp	693.205	1	<0.001
			Sp*Diet	3.343	1	0.079
			Sp*Temp	794.282	1	<0.001
			Diet*Temp	0.392	1	0.536
			Sp*Diet*Temp	3.650	1	0.068
	2	Cod	Diet	1.049	1	0.319
			Temp	41.469	2	<0.001
			Diet*Temp	1.418	2	0.267
		Haddock	Diet	2.945	1	0.111
			Temp	3575.500	1	<0.001
			Diet*Temp	0.522	1	0.483
SGR	1	Cod and Haddock	Sp	329.268	1	<0.001
			Diet	9.276	1	0.005
			Temp	1711.152	1	<0.001
			Sp*Diet	8.538	1	0.007
			Sp*Temp	448.832	1	<0.001
			Diet*Temp	2.799	1	0.107
			Sp*Diet*Temp	9.773	1	0.004
	2	Cod	Diet	0.469	1	0.502
			Temp	119.949	2	<0.001
			Diet*Temp	0.936	2	0.410
		Haddock	Diet	11.686	1	0.005
			Temp	1283.932	1	<0.001
			Diet*Temp	7.558	1	0.018
GCE	1	Cod and Haddock	Sp	1.581	1	0.220
			Diet	7.118	1	0.013
			Temp	18.075	1	<0.001
			Sp*Diet	0.228	1	0.637
			Sp*Temp	44.256	1	<0.001
			Diet*Temp	0.486	1	0.492
			Sp*Diet*Temp	3.364	1	0.079

(continued)

Parameter	Method	Species	Source	*F*	d.f.	*P*
	2	Cod	Diet	5.248	1	0.034
			Temp	2.982	2	0.076
			Diet*Temp	1.984	2	0.166
		Haddock	Diet	2.912	1	0.114
			Temp	35.005	1	<0.001
			Diet*Temp	0.380	1	0.549
SGR_A	1	Cod and Haddock	Sp	145.957	1	<0.001
			Diet	5.494	1	0.028
			Temp	1382.118	1	<0.001
			Sp*Diet	6.894	1	0.015
			Sp*Temp	348.724	1	<0.001
			Diet*Temp	13.464	1	0.001
			Sp*Diet*Temp	6.097	1	0.021
	2	Cod	Diet	1.589	1	0.223
			Temp	101.113	2	<0.001
			Diet*Temp	1.415	2	0.268
		Haddock	Diet	12.578	1	0.015
			Temp	967.855	1	<0.001
			Diet*Temp	7.466	1	0.004
HSI	1	Cod and Haddock	Sp	2031.341	1	<0.001
			Diet	20.360	1	<0.001
			Temp	15.682	1	<0.001
			Sp*Diet	8.736	1	0.003
			Sp*Temp	142.941	1	<0.001
			Diet*Temp	12.126	1	<0.001
			Sp*Diet*Temp	1.870	1	0.172
	2	Cod	Diet	2.419	1	0.122
			Temp	2.876	2	0.060
			Diet*Temp	1.102	2	0.335
		Haddock	Diet	51.334	1	<0.001
			Temp	233.159	1	<0.001
			Diet*Temp	21.650	1	<0.001

(continued)

Parameter	Method	Species	Source	*F*	d.f.	*P*
Gastric Evacuation	1	Cod and Haddock	Sp	5.057	1	0.028
			Diet	1.118	1	0.294
			Temp	2.190	1	0.144
			Sp*Diet	2.796	1	0.100
			Sp*Temp	0.062	1	0.803
			Diet*Temp	0.107	1	0.745
			Sp*Diet*Temp	0.107	1	0.745
	2	Cod	Diet	21.405	2	<0.001
			Temp	0.760	1	0.388
			Diet*Temp	0.637	2	0.534
		Haddock	Diet	0.264	1	0.611
			Temp	1.056	1	0.312
			Diet*Temp	0.000	1	1

Method 1 = 2 sp, 2 temperatures, 2 diets; Method 2 = Species analyzed indicidually: Atlantic cod, 3 temperatures, 2 diets and haddock, 2 temperatures, 2 diets.

higher in the haddock than Atlantic cod at 11 °C, this difference was reversed at 2 °C (Fig. 3.1B).

In contrast to FC, a significant effect of dietary protein content was evident for GCE (P= 0.013; see Table 3.1), with diminished conversion efficiencies observed for Atlantic cod (P= 0.03; by 3, 22 and 28% at 11, 6 and 2°C, respectively) and in haddock [by 20 and 22% at 11 and 2 °C respectively, although not significant (P= 0.11)] when fed the LP diet (Fig. 3.1C, Table 3.1). Further, it is clear that GCE was a major contributor to the reversal in growth rates of Atlantic cod and haddock between 11 and 2 °C. The GCE of haddock was 46 and 31% (for HP and LP diets, respectively) higher than measured for Atlantic cod at 11 °C, wheras the values were 35 and 40% lower (for HP and LP diets, respectively) at 2 °C.

3.4.2 Hepatosomatic Index

HSI for Atlantic cod juveniles (2.7 – 4.6 %) was much lower than for haddock, with values ranging from 9.8 - 13.6% depending on the diet/temperature combination (Fig. 3.2B). Although HSI values for Atlantic cod did not vary between the treatments, both water temperature and diet had significant effects (P< 0.001) on HSI of haddock (Table 3.1). HSI values at 11 °C were significantly higher than those at 2 °C (by 16% for the HP group and 25% for the LP group), and HSI was significantly higher for 11 °C haddock fed the LP diet (Figure 3.2B).

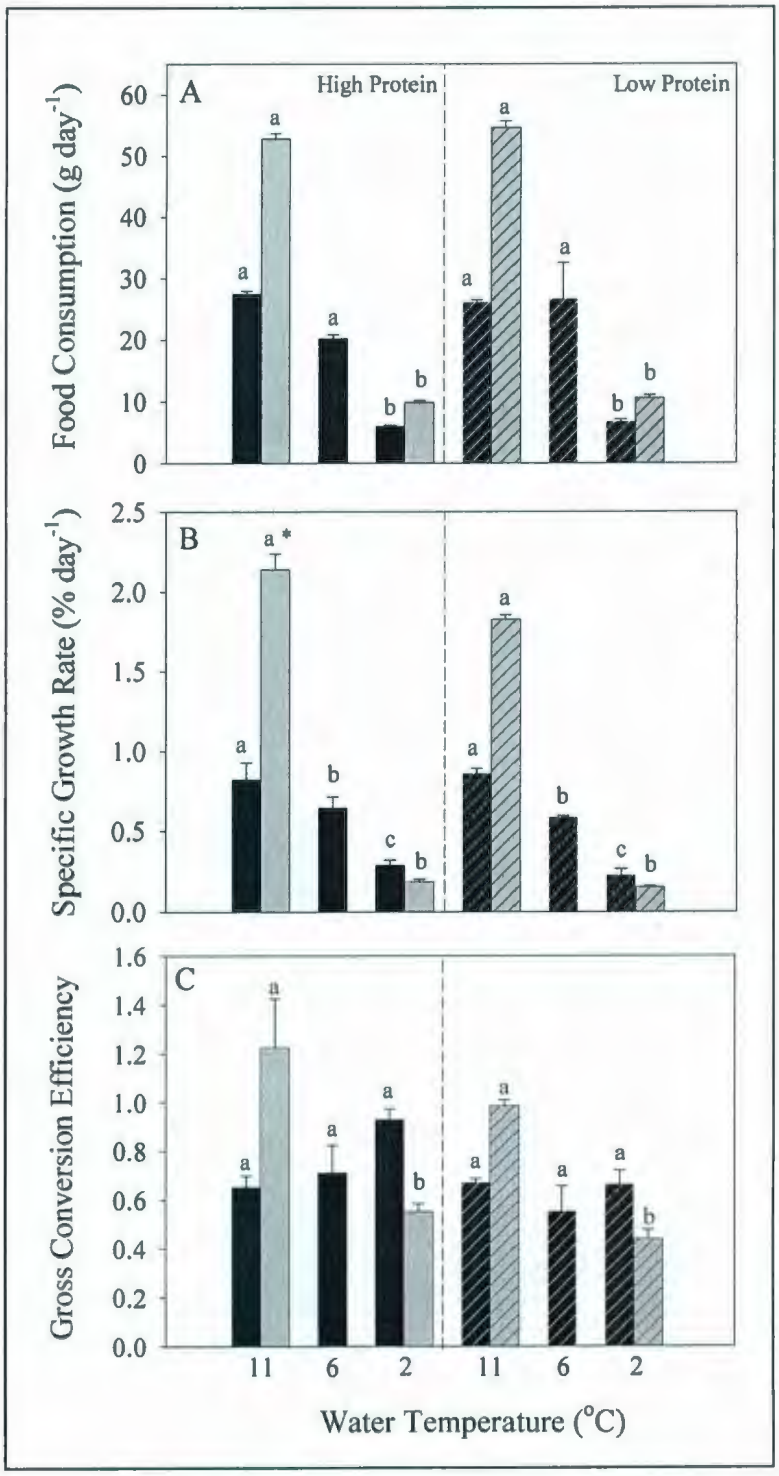

Fig. 3.1. The effects of dietary protein and lipid levels and water temperature on the food consumption rates (A), specific growth rates (B) and gross conversion efficiency (C) of Atlantic cod (dark columns) and haddock (light columns). Values are means ± S.E. (n = 8). Within each diet, Atlantic cod and haddock values without a letter in common are statistically different ($P < 0.05$) and an * indicates a significant difference between the HP and LP groups at a particular temperature.

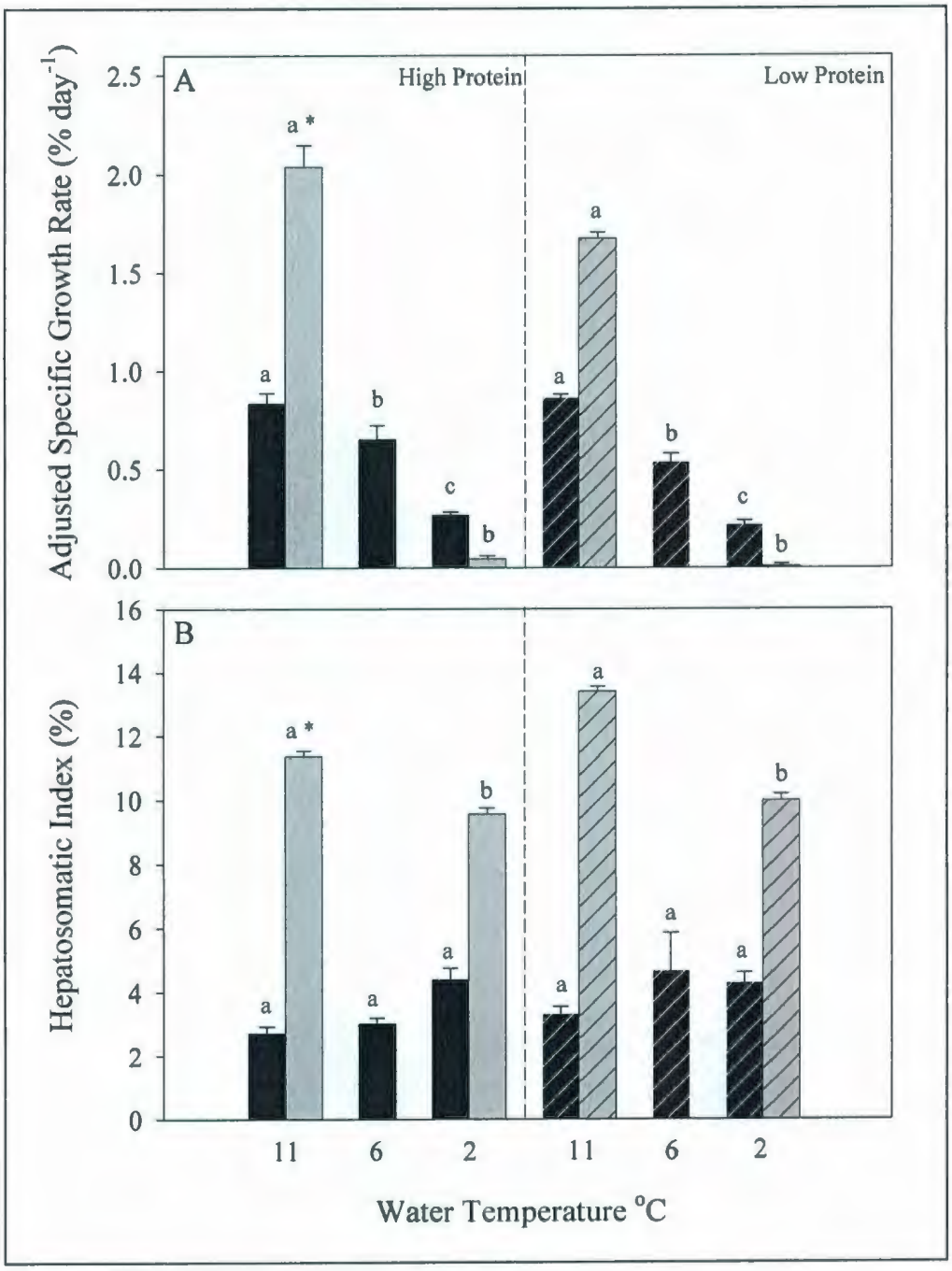

Fig. 3.2. The effects of dietary protein and lipid levels and water temperature on the adjusted specific growth rate (i.e. SGR after removal of the liver) (A) and hepatosomatic index (% body mass; B) of Atlantic cod (dark columns) and haddock (light columns). Values are means $\pm$ S.E. (n = 8). Within each diet, Atlantic cod and haddock values without a letter in common are statistically different ($P< 0.05$) and a * indicates a significant difference between the HP and LP groups at a particular temperature.

3.4.3. Gastric Evacuation

Although the experimental design (i.e. feeding tanks of fish, and the relatively long inter-measurement periods after 24 h post-feeding) precluded us from precisely measuring GE, the reported values for post-feeding stomach content (Fig. 3.3) and GE (Table 3.2) reveal important information with regards to the effects of diet and temperature on gastric evacuation. First, dietary protein content did not have an effect on gastric evacuation time, as evidenced by the extensive overlap in stomach content values between individuals fed the high and low protein diets (Fig. 3.3) and similar ($P > 0.05$) values for GE. Second, gastric evacuation time increased considerably with reduced rearing temperature. For example, estimated values for GE (Table 3.2) fell from 0.14 – 0.32 g h^{-1} at 11 °C to 0.001 – 0.004 g h^{-1} at 2 °C. Finally, based on the fact that cod required ~ 72 h to evacuate their stomachs as compared to ~ 48 h for haddock, and GE was slightly (HP) or significantly (LP) faster in haddock at 11 °C, it appears that GE is faster in haddock than in Atlantic cod.

3.5 Discussion

3.5.1 Food Consumption, Growth Rate, and Conversion Efficiency: Effect of Temperature

In the present study, exposure to low water temperatures resulted in decreased feeding levels; e.g. haddock FC decreased from ~50 g day^{-1} at 11 °C to 10 g day^{-1} at 2 °C, and in Atlantic cod FC decreased from ~25 g day^{-1} to ~5 g day^{-1} at the same

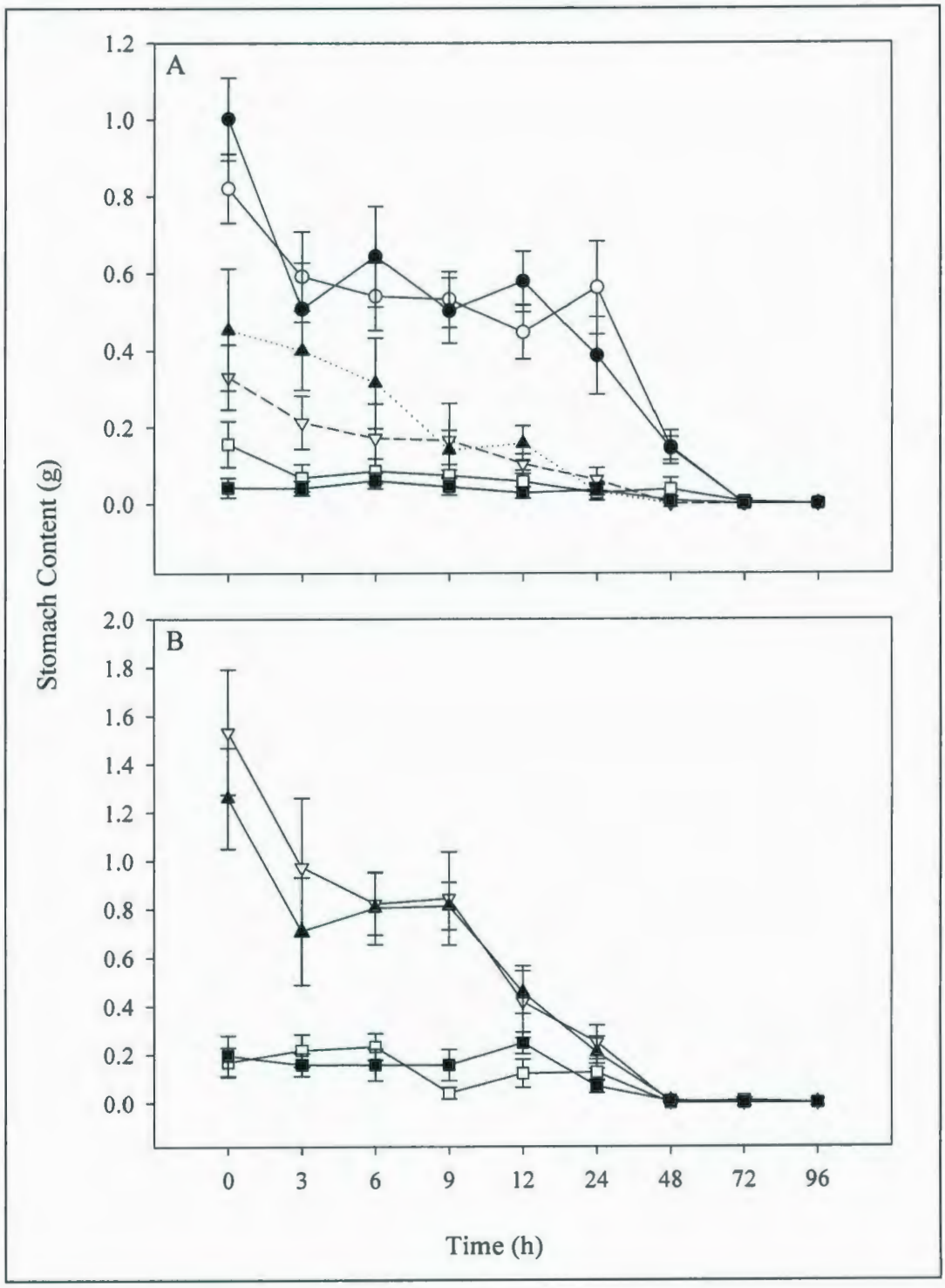

Fig. 3.3. The patterns of gastric evacuation for Atlantic cod (A) and haddock (B) fed a HP diet (closed symbols) vs. a LP diet (open symbols) at 11 °C (triangles), 6 °C (circles) and 2 °C (squares). Each point represents mean values ± S.E. (n = 8).

Table 3.3. Gastric evacuation (g of food h^{-1}) for Atlantic cod and haddock at various diet – temperature combinations. Values are means ± S.E. (n = 8). Within each diet, Atlantic cod and haddock values without a letter in common are statistically different ($P < 0.05$), and a * indicates a significant difference between species.

		11 °C	6 °C	2 °C
Atlantic Cod	HP Diet	0.018 ± 0.007^{a}	0.020 ± 0.002^{a}	0.001 ± 0.001^{b}
	LP Diet	0.014 ± 0.004^{a}	0.017 ± 0.002^{a}	0.002 ± 0.001^{b}
Haddock	HP Diet	0.021 ± 0.004^{a}	ND	0.004 ± 0.001^{b}
	LP Diet	$0.032 \pm 0.005^{a*}$	ND	0.002 ± 0.001^{b}

HP = High Protein Diet; LP = Low Protein Diet; ND = Not Determined

temperatures. Decreases in food consumption, concomitant with reductions in water temperature, have been reported for various fish species. For example, Atlantic salmon (*Salmo salar* L.) post smolts held at 6 °C had significantly lower food intake (by ~35 and 50%) than fish held at 10 and 14 °C, respectively (Handeland *et al.*, 2008), Atlantic salmon parr held at 2 °C had a significant lower (by ~75%) food intake than fish held at 8 °C regardless of feed composition (low or high lipid diet) (Bendiksen *et al.*, 2002) and the food consumption of juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.) at 6 °C (~0.6% day^{-1}) was significantly lower than that of juveniles kept at 12 °C (~1% day^{-1}) (Jonassen *et al.*, 2000). Further, similar decreases in food consumption at low water temperatures have been previously described for Atlantic cod. Brown *et al.* (1989) reporting that Atlantic cod held at 0.6 °C had significantly lower food intake as compared with fish held at either 4.5 °C (~37% reduction) or 8.3 °C (~58% decrease). Likewise, Peck *et al.* (2003b) showed that the maximum food consumption of Atlantic cod was reduced by ~45% between 12 and 5 °C.

Although the mechanism(s) that regulate temperature-dependent changes in food consumption and appetite have received little attention in fishes, it is likely that neurohormones such as CART (cocaine and amphetamine regulated transcript) played a significant role in mediating the reduction in FC in both Atlantic cod and haddock between 11 and 2 °C. For example, when juvenile Atlantic cod were acclimated to 15, 11 and 2 °C, a 66% decrease in food consumption was reported (from 0.75 to 0.25% BM day^{-1}) across this temperature range, and this decrease in FC was linked to a ~40% increase CART gene expression (Kehoe and Volkoff, 2008). CART is a peptide known to inhibit feeding in mammals (Hunter *et al.*, 2004; Vicentic and Jones, 2007) and in fish (Volkoff and Peter, 2000; Volkoff *et al.*, 2005) and it is thought to act as a

satiety factor in Atlantic cod (Kehoe and Volkoff, 2008). Furthermore, it appears that in mammals, CART has a strong link with thermogenesis and cold stress (Koylu *et al.*, 2006) and that in rats, exposure to low temperatures results in increased CART levels (Kong *et al.*, 2003).

The SGR at 11 °C reported in the present study for juvenile Atlantic cod (0.8% BM day^{-1}) is lower than the maximal values reported and predicted by some authors (~ 1.3 to 2.6%). However, the data for Atlantic cod SGR in the literature are quite variable due to the different sizes of fish used, as well as diet formulations and experimental conditions (e.g. temperature). In this study, given that the mean mass of the fish increased from ~ 40 to 66 g over the 8 week experimental period, and thus the average fish mass was approximately 50g, we would have expected a maximum growth rate of ~ 1.5 % BM day^{-1} according to the data of Bjornsson *et al.* (2001a). However, FC was only ~ 0.8% BM day^{-1} and thus growth would not be expected to be maximal. Further, when only the data for their largest juvenile Atlantic cod (~7.3 g) reported in Peck *et al.* (2003b) are considered, growth rate at the temperature and levels of FC reported here, would only have resulted in a growth rate of approx. 0.6% day^{-1}. In the present study, the growth rate for the 11 °C reared haddock was ~ 1.8 – 2.1% BM day^{-1} which is in between those described by other authors. For example, although Treasurer *et al.* (2006) reported SGR for juvenile haddock of 64 – 67 g initial mass of ~1.10 % BM day^{-1}, SGR values of ~2.3 – 2.8% BM day^{-1} have been reported for juveniles between 6.8 – 13.5 g initial mass (Kim and Lall, 2001; Nanton *et al.*, 2001; Tibbetts *et al.*, 2005).

The reductions in juvenile Atlantic cod growth rate observed at colder temperatures in this study (~60%) are in agreement with those reported by other

authors. For example, Brown *et al.* (1989) report a decrease in growth rate of 58% between 8.3 and 0.6 °C for age 0+ fish, Purchase and Brown (2001) found a decrease in growth rate of 60% between 12 and 3 °C (3.6 – 8.8 g initial mass) and Bjornsson *et al.* (2001a) showed a decrease in growth rate of 73% (initial mass 12 g) between 12 and 4 °C. When we compared the SGR values for Atlantic cod and haddock at 11 °C, the SGR for haddock was approximately twice that measured for Atlantic cod. This was not unexpected as Peck *et al.* (2003b) report that haddock grow faster than Atlantic cod (by ~3× at 12 °C) and the results for FC and GCE for haddock are also approximately twice of those measured for Atlantic cod at this temperature. However, we were surprised to find that the growth rate of haddock at 2 °C was only ~7% of that measured at 11 °C and ~65% (~ 0.15 vs. 0.25% BM day^{-1}) of that measured for Atlantic cod given that the values for FC were not significantly different between the two species at 2 °C. This latter result shows that the effects of temperature on growth rate are very different in these two species (11 vs. 2.7-fold decrease from 11 to 2 °C) and that species differences in growth rate are highly temperature dependent over the range of temperatures that these two fish would experience in cage-culture. This temperature-related species difference was also observed when we examined the cause of the decreased growth rates in the two species from 11 to 2 °C. The decrease in Atlantic cod growth rate was directly related to the decrease in FC, as GCE did not change significantly when temperature was reduced from 11 to 2 °C. In contrast, the reduction in haddock growth rate was associated with both reductions in FC (80%) and GCE (40%).

Previous studies examining the relationship between GCE and temperature in Atlantic cod and haddock are contradictory, as some authors have reported that GCE

increases with decreasing temperature and have suggested that this is an adaptive physiological characteristic of these predominantly cold-water species (Brown *et al.*, 1989; Peck *et al.*, 2003b). Whereas other authors have reported that GCE is higher at elevated temperatures (e.g. 13 vs 2 °C) in Atlantic cod populations from different stocks (Purchase and Brown, 2001). In the present study, the reason why GCE was insensitive (Atlantic cod) or fell (haddock) substantially when temperature was lowered from 11 to 2 °C is unknown, but it is unlikely to be related to FC since Peck *et al.* (2003b) showed that GCE (calculated as GCE=SGR/FC) is higher at lower temperatures, irrespective of FC. Furthermore, the GCE values reported at both 11 and 2 °C (approx. range 0.5 – 1.0) are comparable to, or slightly above, those reported in the literature by other authors for these two species at comparable temperatures (Brown *et al.*, 1989; Purchase and Brown, 2001; Peck *et al.*, 2003b).

3.5.2 Food Consumption, Specific Growth Rate, and Conversion Efficiency: Effect of Diet

In the present study, although varying levels of dietary protein and lipid had no effect on the FC or SGR of juvenile Atlantic cod at any of the tested temperatures, a significant benefit (i.e. ~15% greater SGR and SGR_A) was associated with feeding haddock the HP diet at 11 °C. The results for Atlantic cod are in agreement with those reported for rainbow trout (*Onchorhynchus mykiss*) (Eliason *et al.*, 2007) and Atlantic cod. For example, Grisdale-Helland *et al.* (2008) found no significant differences in growth rate when juvenile Atlantic cod were fed 14 different protein/lipid combinations for 8 weeks. No significant difference in the final growth rate of juvenile

Atlantic cod (440 g initial mass) was reported by Grisdale-Helland *et al.* (2007) when fish were fed diets containing 60% protein and 17% lipid vs. 53% protein and 28% lipid, and Morais *et al.* (2001) reported similar growth rates for Atlantic cod juveniles (233 g initial mass) fed 4 different diets (protein 48 vs. 58% and lipid 12 vs.16%) over 16 weeks. While growth rates are often unaffected by feeding diets of varying protein level, many of the same studies on Atlantic cod reared at 8 – 11 °C have recommended that this species be fed a diet with a protein content of 48-55% protein based on measurements of nitrogen (protein) retention and conversion efficiency (Morais *et al.*, 2001; Rosenlund *et al.*, 2004; Karlsen *et al.*, 2006; Grisdale-Helland *et al.*, 2008). In agreement with these studies, the GCE of Atlantic cod in the present study was significantly lower when fed the LP diet and this difference was ~ 22 and 28% at 6 and 2 °C (Figure 1B and 2A). Thus, our results strongly suggest that there is no benefit to feeding Atlantic cod LP diets even at cold temperatures (i.e. < 6 °C) despite the reduced cost of these diet formulations. In the case of haddock, the higher growth rate for the HP group at 11 °C is in contrast to the results of Kim and Lall (2001) who found no significant difference in the growth of smaller juveniles (6 g) reared at 13 °C and fed isoenergetic diets containing different amounts of protein (45, 50, 55 and 65%) for a six week period. This difference is probably not related to GCE since this parameter was reduced in both studies when dietary protein was lowered, but instead, a difference in the effect of feeding LP vs. HP diets on FC. In Kim and Lall (2001) FC showed an inverse relationship with dietary protein level, whereas we report that FC was similar in haddock fed the two diets. The reason for the lack of an impact of diet on SGR at 2 °C is unknown, but may be explained by the extremely low feeding rates exhibited at this temperature.

3.5.3 Hepatosomatic Index

Values for HSI in the present study ranged between 3-5% and 9-13% for juvenile Atlantic cod and haddock, respectively and were not affected by the protein: lipid ratio with the exception of an increased HSI for the haddock fed the LP diet at 11 °C. The HSI values obtained in the present study for haddock are consistent with those reported by other authors. For example, Tibbetts *et al.* (2005) reported HSI values between 9.3 – 12.6% for haddock fed diets similar to those used in the present experiment, and Nanton *et al.* (2001) reported HSI values between 9.8 – 12.1% for fish fed isonitrogenous diets with four different lipid levels (14, 16, 19, 22%). It is known that gadoids accumulate dietary lipids in the liver, and that in haddock, there is limited transport of lipoproteins from the liver to the muscle (Lall *et al.*, 2003) and limited β-oxidation of lipids in this tissue (Nanton *et al.*, 2003). Furthermore, this can lead to the development of fatty liver syndrome, an undesirable condition in which fish do not utilize dietary energy efficiently (Tibbetts *et al.*, 2005). Values of HSI for Atlantic cod in the literature are variable, for example, low values between 3 – 6% have been reported for wild juveniles caught and kept in captivity (Couture *et al.*, 1998) in laboratory-cultured juveniles (Grant *et al.*, 1998) and in adults reared under different feeding frequencies, stocking densities and size-grading protocols (Lambert and Dutil, 2001). Furthermore, higher HSI values of 6 – 11% have also been reported for juvenile Atlantic cod fed diets varying in protein:lipid ratios (Lie *et al.*, 1988; Morais *et al.*, 2001; Hamre and Mangor-Jensen, 2006; Grisdale-Helland *et al.*, 2008) or subjected to different feeding frequencies (Rosenlund *et al.*, 2004; Solberg *et al.*, 2006). The HSI

values obtained in the present study are in the range of 2 – 6% reported for normal wild Atlantic cod by Jobling (1988), however the reason(s) for the low HSI value in our study is unclear as Roselund *et al.* (2004) performed diet trials where the amount of protein and lipid were similar to those used in this experiment. However, the FC of our Atlantic cod was lower than that reported in several other studies (see above), and HSI is regulated not only by dietary lipid and protein content, but also the interrelationship between the dietary energy-yielding nutrients, namely protein, lipid and carbohydrate (Jobling *et al.*, 1991; Tibbetts *et al.*, 2005).

The effect of water temperature on the HSI of haddock was considerable, as HSI decreased by 18% in the HP group and by 26% in the LP group when temperature was reduced from 11 to 2 °C. In contrast, no effect of temperature on HSI was observed for Atlantic cod. This species difference is likely related to the very large decreases in FC and GCE for haddock (as compared with Atlantic cod) between 11 and 2 °C. It is difficult to compare the results of the present study with the literature, since to our knowledge, only one other study has made measurements of HSI at low temperatures. Further, Purchase and Brown (2001) report a significant temperature-population interaction for the effects of exposure to cold temperatures on HSI. For example, Atlantic cod from the Gulf of Maine had HSI values of ~9.8 and 8.3% at 13 and 2 °C, respectively, whereas, the HSI of Atlantic cod from the Grand Banks increased from ~6.8 to 8.1% when temperature was lowered from 13 to 2 °C.

3.5.4 Gastric Evacuation

In the present study, temperature had a strong negative effect on the gastric evacuation time of both species. This was not surprising as dos Santos and Jobling (1995) showed that the Q_{10} for Atlantic cod gastric emptying was 3.7 (i.e. it increases/decreases by 3.7 × for a change in 10 °C), and studies on numerous other fish species have shown GE to be highly temperature dependent: brown trout (*Salmo trutta*; Q_{10} = 3.0) (Elliott, 1972); whiting, (*Merlangius merlangus*; Q_{10} = 2.2) (Andersen, 1999); plaice (*Pleuronectes platessa*; Q_{10} = 2) (Basimi and Grove, 1985). Although the main purpose of the research on gastric evacuation was to determine whether varying dietary protein and lipid content would increase the rate of gastric evacuation, and thus feeding frequency/consumption (Grove *et al.*, 1978; Grove *et al.*, 1985; Bromley, 1987) at 2 °C, the results indicate that protein:lipid ratio had no significant effect on evacuation time. This agrees with published data on Atlantic salmon (Sveier *et al.*, 1999), but is in contrast with data on Indian major carp (Naik *et al.*, 2000). Based on our results for Atlantic cod and haddock, it appears that there is no advantage to feeding gadoids diets with low protein content with respect to its effects on GE. However, varying the dietary energy could be an alternative, since dietary energy content has been related to gastric evacuation rate in different species, including three different gadoids, the whiting, saithe (*Pollachius virens*) and Atlantic cod (Andersen, 2001), as well as the plaice (Jobling, 1980a) and rainbow trout (Grove *et al.*, 1978).

Although differences in the quantity of food eaten (FC) and the interval between sampling periods makes interpretation of the GE data difficult, our data (Fig.

3.3 and Table 3.2) suggest that GE in haddock is considerably faster than in cod. This may be the primary, or one of, the reasons why FC and thus SGR were significantly greater in juveniles of this species as compared to Atlantic cod at 11 °C, and warrants further investigation. For example, understanding the mechanisms that control appetite and determine gastric emptying/the rate of digestion may be key to the economic viability of Atlantic cod aquaculture.

3.6 Conclusion

In summary, the present study shows that low rearing temperature (2 °C) has a negative impact on FC, SGR and SGR_A, in both Atlantic cod and haddock juveniles, and on GCE of haddock, and indicates that feeding a low protein diet during the winter months would not be beneficial. This is because cod and haddock fed the low protein diet (42/16) in this study experienced a considerable reduction in GCE, and gastric emptying (which is known to affect appetite) was not affected by varying dietary protein and lipid content. Finally, we show that while haddock has a higher SGR at 11 °C, GCE is negatively influenced by cold temperatures in this species, and this contributes to reduced growth in haddock, as compared to Atlantic cod, at 2 °C.

3.7 Acknowledgments

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Chapter 4. The Stress and Metabolic Responses of Juvenile Atlantic Cod (*Gadus Morhua* L.) to an Acute Thermal Challenge.

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4.1 Abstract

Survival, oxygen consumption (MO_2), total plasma cortisol and glucose levels, and gill heat shock protein 70 (hsp70) expression were measured in 10 and 50 g juvenile Atlantic cod *Gadus morhua*, during an acute temperature increase (2 °C h^{-1}) to their critical thermal maximum. Ninety three percent of the fish in both size classes survived to 24 °C; however, mortality was 100% within 15 minutes of reaching this temperature. MO_2 for both size classes increased significantly with temperature, reaching peak values at 22 °C that were approximately 2.8-fold those of control (10 °C) fish. Resting plasma cortisol and glucose levels were lower in 10 g as compared to 50 g fish. Plasma glucose levels were highly variable in both size classes, and significant increases were only seen at > 22 °C for the 10 g fish. In contrast, plasma cortisol showed an exponential increase with temperature starting at 16 °C in both size classes, and reached maximum levels at 22 °C that were 19-fold (10 g fish) and 35-fold (50 g fish) higher than their respective control groups. Both the constitutive (73 kDa) and inducible (72 kDa) isoforms of hsp70 were detected in both size classes using the widely utilized mouse monoclonal antibody. However, expression of these isoforms did not change when cod were exposed to elevated temperature, and the 72 kDa isoform was not detected using salmonid-specific antibodies. These results indicate that juvenile cod are very sensitive to acute increases in water temperature. In addition, they: 1) show that MO_2 and plasma cortisol, but not plasma glucose or gill hsp 70 levels, are sensitive indicators of thermal stress in cod; and 2) support previous reports that the upper critical temperature for this species is 16 °C.

4.2 Introduction

Although free-swimming Atlantic cod *Gadus morhua* L., held in thermally stratified water are known to move to preferred temperatures (Claireaux *et al.*, 1995), sea-caged fish are limited in their movements in the water column. Thus, they can be exposed to large seasonal (~ 0 to 20 °C) and daily (± 10 °C, *e.g.* inversion of the thermocline) (Gollock *et al.*, 2006) temperature fluctuations. Thermal biology and tolerance are important physiological traits that may determine whether wild or cultured Atlantic cod survive temperature challenges, and define suitable areas for cage-site aquaculture production. A large number of studies have investigated the thermal limits of salmonids and other freshwater species, and the thermal tolerance zone and optimum temperatures for many species are known [*e.g.* see Cherry *et al.*, (1977); Jobling, (1981b); Beitinger *et al.*, (2000)]. Further, there is accumulating evidence that the thermal tolerance of marine organisms (including fishes) is limited by blood oxygen transport and aerobic scope, and that temperature dependent constraints on these physiological processes translate into alterations in population dynamics and biogeography (Portner *et al.*, 2001; Portner, 2002; Portner *et al.*, 2004; Portner *et al.*, 2006; Portner and Knust, 2007; Wang and Overgaard, 2007). The thermal biology of Atlantic cod has also received considerable attention, but most of this work has focused on determining temperatures where growth and food conversion efficiency are optimal (Jobling, 1988; Otterlei *et al.*, 1999; Bjornsson *et al.*, 2001a), on thermal preferenda (Despatie *et al.*, 2001; Petersen and Steffensen, 2003; Lafrance *et al.*, 2005), or on the magnitude of temperature effects on metabolism (Saunders, 1963; Schurmann and Steffensen, 1997; Claireaux *et al.*, 2000; Peck *et al.*, 2003a,b).

The few studies on cod thermal tolerance have revealed considerable variation with regards to this species' upper thermal limit (24 °C, (McKenzie, 1934); 15.5 – 18 °C for yolk-sac larvae acclimated to 7 – 9 °C, (Yin and Blaxter, 1987); 16 – 22 °C depending on acclimation temperature, (Bøhle, 1974); 19 – 22 °C with sublethal effects appearing around 13 – 16 °C, (Sartoris *et al.*, 2003); 22.2 °C with sublethal effects appearing at 17-18 °C, (Gollock *et al.*, 2006). However, the applicability of some of these data to cage-culture or to the impact of global warming on wild fisheries is questionable. First, the upper thermal limits reported by Yin and Blaxter (1987) and Bøhle (1974) are upper incipient lethal temperatures (*i.e.* determined by rapidly exposing acclimated fish to a novel temperature, and recording the temperature at which 50% die after 24 hours of exposure). Second, McKenzie (1934) and Sartoris *et al.*, (2003) used adult wild fish, and Rodnick *et al.* (2004) indicate that the thermal tolerance of wild fish may be different than those reared under aquaculture conditions.

Given the limited available data (Jobling 1988) on ontogenetic differences in thermal tolerance in Atlantic cod, it is apparent that size (age)-specific data on the thermal tolerance (sub-lethal and maximal) of Atlantic cod will be required before: 1) decisions can be made about where to locate cage-site operations or what size to stock fish during their first summer; and 2) accurate predictions on the influence of climate change on Atlantic cod populations can be made (*e.g.* see Portner and Knust 2007). However, it is presently unclear which physiological parameters are most appropriate for routinely assessing thermal stress in Atlantic cod, and comprise the hierarchical order of responses to temperature stress in this species. For example, although increases in cellular heat shock protein (hsp) concentrations in response to elevated temperatures have been reported for many fish species (Basu *et al.*, 2002), cod and

haddock, *Melanogrammus aeglefinus* L., apparently do not show any increase in hsp70 levels when exposed to acute temperature increases (from 11 to 16.5 °C and 10 to 15 °C, respectively) (Zakhartsev *et al.*, 2005; Afonso *et al.*, 2008). Further, the lack of an hsp70 response in these two species may be the result of maximum temperatures not exceeding their upper critical temperature (~ 16 °C in Atlantic cod; Sartoris *et al.*, 2003; Lannig *et al.*, 2004).

To further the understanding of Atlantic cod thermal biology and to identify appropriate indicators of thermal stress, survival, oxygen consumption (MO_2), plasma cortisol and glucose, and gill hsp70 levels were measured in 10 and 50 g juvenile Atlantic cod (sizes typically stocked into sea cages) exposed to an acute increase in water temperature (2 °C h^{-1} from 10 to 24 °C).

4.3 Materials and Methods

These studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol # 05-07-KG).

Fish for these experiments were obtained from the Aquaculture Research and Development Facility (ARDF) of the Ocean Sciences Centre, Memorial University of Newfoundland. All fish were obtained from a single communal spawning and were reared in the same production tank following standard rearing protocols in place at the ARDF. These experiments were performed using two size classes of Atlantic cod (10 g and 50 g) and two different sets of tanks: 4 tank respirometers for the measurement of MO_2; and 8 uncovered tanks of similar size for the measurement of plasma cortisol

and glucose, and gill hsp70 levels. Fish in both the respirometers and the experimental tanks were randomly assigned to 1 of 2 treatments: 1) control, where the water temperature remained constant at 10 °C; and 2) experimental, where the fish were exposed to an increase in water temperature of 2 °C every hour until loss of equilibrium.

4.3.1 Oxygen Consumption

Four custom built 279-litre conical bottom tank respirometers were used in this experiment (Fig. 4.1). These tanks had a Plexiglass cover fitted with an oxygen electrode (Trioximatic® 701, WTW Instruments, Weilheim, Germany), and water flow into each tank was automatically controlled by a solenoid valve in conjunction with a laptop computer running custom software (Memorial University of Newfoundland Tech Services). This software switched water flow into the tank respirometers on and off at set intervals without disturbing the fish. Further, two small 115 V submersible pumps (ZooMed Laboratories Inc, San Luis Obispo, CA, USA) effectively mixed the water in each tank, one placed at the bottom of the tank and one placed mid-depth on the side of the tank. This latter pump also provided sufficient flow past the oxygen electrode to permit accurate recordings of tank water oxygen content. Each tank respirometer had a watertight hatch in the Plexiglass cover through which the fish could be fed during the acclimation period. A drain valve at the bottom of the tank respirometers allowed for the removal of uneaten pellets and feces. One week prior to the beginning of the experiment, groups of fish (approx. 500 g total wet mass for the 10 g fish and 1000 g for the 50 g fish) were placed into each one of the tank

respirometers to allow the fish to acclimate to their new conditions and to recover from handling/netting stress. Two tank respirometers were assigned to the control group (constant 10 °C) and two to the experimental group (increase in temperature by ~ 2 °C h^{-1}). During this acclimation period, the fish were fed a commercial cod diet (EWOS Canada Ltd, Surrey, BC, Canada) at a ration of 1% of body mass day^{-1} and were maintained on a 12 h light:12 h dark photoperiod. However, fish were food deprived for 48 h prior to the beginning of the experiment. On the day of the experiment, water flow into the 4 tank respirometers was turned off during the last 20 minutes of each hour by the control system, and during this time water oxygen concentration was measured every minute using a RS-232 Interface Module (WTW Instruments Inc.) connected to the computer. Routine oxygen consumption (MO_2) was calculated according to Crocker and Cech Jr. (1997) as:

$$MO_2 = (O_{2i} - O_{2f})\,(V)\,kg^{-1}\,t^{-1}$$

where O_{2i} = initial water oxygen concentration (mg l^{-1}), O_{2f} = final water oxygen concentration (mg l^{-1}), V = volume of tank respirometers (l) – volume displaced by fish (1 kg = 1 litre of water), kg = fish mass (kg) and t = time (h). Although background measurements of oxygen consumption were made on the respirometers at 10 °C after removing the fish, MO_2 during these periods was negligible, and thus adjustments to the MO_2 of the fish was not required. This experiment was repeated four times per size class, thus providing eight measurements of oxygen consumption per temperature/fish size combination.

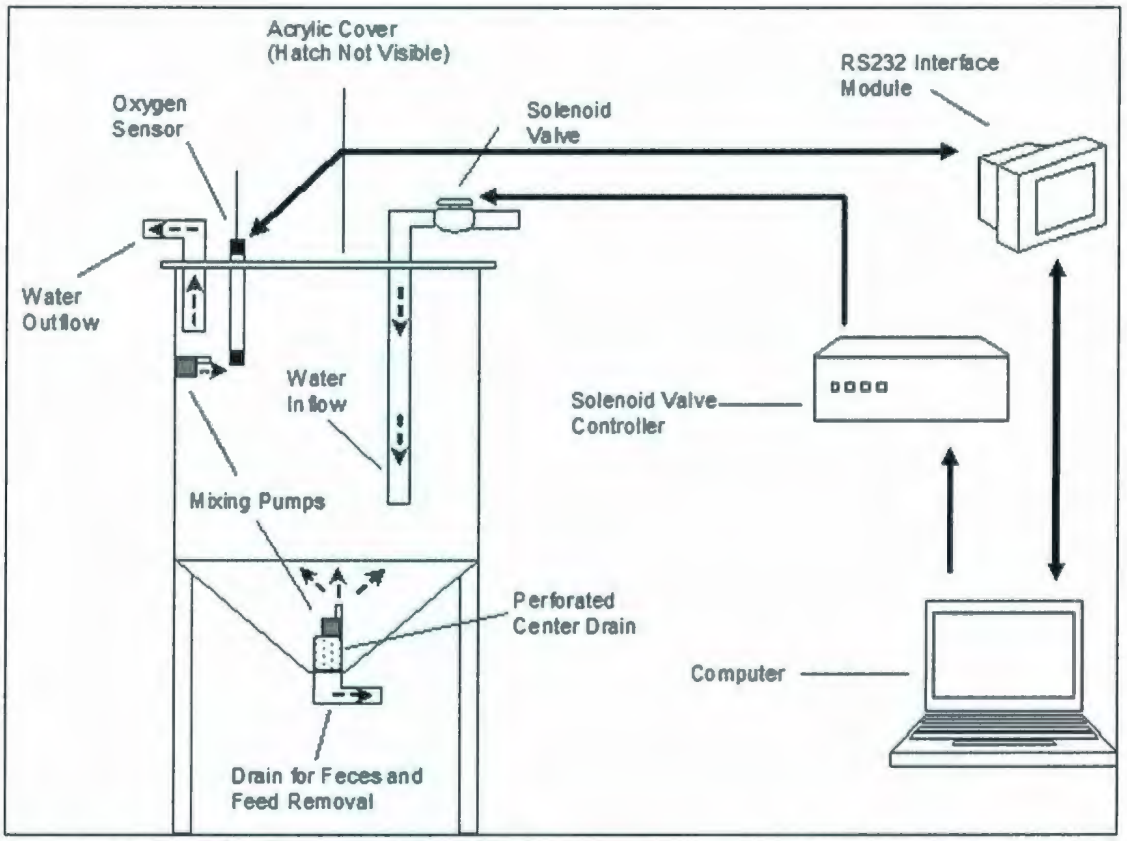


Fig. 4.1. Schematic diagram of the 279 L respirometer used for the measurement of oxygen consumption in groups of 50 and 10g cod. The computer controlled the opening/closing of the solenoid valve and made continuous measurements of water oxygen content at scheduled intervals.

4.3.2 Plasma Glucose, Plasma Cortisol and Gill HSPs

4.3.2.1 Blood and Tissue Sampling

Groups of 40 fish were randomly placed into the uncovered tanks 1 week prior to the beginning of the experiment to allow for acclimation to their new conditions and for recovery from handling/netting stress. Four tanks were randomly assigned to the control group (constant 10 °C) and four to the experimental group (increase in temperature by ~ 2 °C h^{-1}). Conditions for the fish during the acclimation period were similar to those in the previous experiment. Coincident with the MO_2 measurements, and at every 2 °C increment in water temperature, two fish per uncovered tank (8 fish per treatment) were carefully netted, anaesthetized with an overdose (300 mg l^{-1}) of tricaine methanesulfonate (TMS) (Syndel Laboratories Ltd, Vancouver, BC, Canada) and quickly sampled as follows. Blood samples (100-300 µl by caudal puncture) were taken within 2 minutes of netting using previously heparinized (100 U ml^{-1} heparin, Sigma Aldrich, St. Louis, MO, USA) 1cc U-100 syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Blood samples were then transferred to 1.5 ml centrifuge tubes and stored on ice prior to centrifugation. Plasma was separated by centrifugation at 10,000 rcf for 5 minutes, transferred to new 1.5 ml centrifuge tubes, frozen in liquid nitrogen and stored at -80 °C until analysis. Gill tissue (filaments; approx. > 100 mg) for the measurement of hsp70 levels was quickly excised, placed in 2 ml cryovials, flash frozen in liquid nitrogen and stored at -80 °C until analysis.

4.3.2.2 Measurement of Plasma Glucose and Cortisol

Plasma glucose levels were measured in triplicate using a modified Trinder (1969b) enzymatic assay available in kit form (Diagnostic Chemicals Ltd. Oxford, CT, USA) (Hosoya *et al.*, 2007). Plates were read at 505 nm on a VERSAmax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA), and intra- and inter-assay coefficients of variation were always less than 5%. Total plasma cortisol levels were determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN CORP. Lexington, KY, USA), and the VERSAmax microplate reader at an absorbance of 650 nm (Basu *et al.*, 2001). Intra- and inter-assay variation never exceeded 10%.

4.3.2.3 Determination of Gill hsp70 Levels – SDS-PAGE and Immunodetection

To date, the evidence for a lack of a significant temperature-related hsp70 in cod (gadids) is based on the use of a mouse monoclonal antibody (clone BRM-22). Thus, I also examined the effect of the acute temperature challenge on gill hsp70 levels using recently available fish (salmonid) primary antibodies that are specific for both constitutive and inducible forms of hsp70 (Agrisera, Vännäs, Sweden).

During preliminary experiments to optimize procedures, it was noted that gill tissue samples displayed the most consistent bands for hsp70. Therefore, in this study, only gill tissue was examined. Gill tissue was thawed on ice and a sub-sample of approx. 50 mg was homogenized in ice-cold lysis buffer following the procedure

outlined in Hosoya *et al.* (2007). After homogenization, samples were centrifuged at 13,000 rcf for 3 minutes. A 10 µl sample of supernatant was taken for protein determination using the bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) as a standard (Smith *et al.*, 1985). A 1:1 solution of supernatant and SDS-sample dilution buffer (Laemmli, 1970) was then boiled for three minutes and frozen at -80 °C, until hsp70 analysis. Samples were subjected to discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). Proteins were resolved with a 4% stacking and 12% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA) using two different settings: 1) 75 V for 15 minutes, then 150 V for 45 minutes (for analysis of single hsp70 bands using Agrisera salmonid antibodies) or 2) 75 V for 15 minutes, then 150 V for 150 minutes (resulting in the separation of hsp72 and hsp73 bands labelled with the Sigma mouse antibody). Molecular weight markers (MagicMark™ XP Western Protein Standards, Invitrogen, Carlsbad, CA, USA) were loaded onto every gel and each lane was loaded with 30 µg of protein. Following electrophoretic separation, levels of hsp70 were determined following the methods given in Basu *et al.* (2001). Briefly, proteins were transferred onto nitrocellulose membranes (at 100 V for 60 minutes). After blotting, the membranes were incubated with either: 1) a monoclonal anti-hsp70 antibody produced in mouse (clone BRM-22, Sigma-Aldrich, St. Louis, MO, USA) that recognizes both the constitutive (hsp73) and inducible (hsp72) forms of hsp70 in a number of fish species (De Boeck *et al.*, 2003; Rendell and Currie, 2005; Zakhartsev *et al.*, 2005); or 2) a polyclonal anti-hsp70 antibody produced in rabbit (Agrisera, Vännäs, Sweden) that is specific for the salmonid

(cognate) form of constitutive hsp70; or 3) a polyclonal anti-hsp70 antibody produced in rabbit (Agrisera, Vännäs, Sweden), which is specific for the salmonid form of inducible hsp70 (Rendell *et al.*, 2006). Liver, gill and red blood cell samples were used to optimize the concentration of antibodies used for immunodetection. A concentration of 1:1000 for the primary antibody and 1:2000 for the secondary antibody produced the best results. Following 35 min. incubation in the primary antibody, membranes were incubated for 35 min. with secondary antibody: an anti-mouse IgG peroxidase antibody produced in goat (Sigma-Aldrich, St. Louis, MO, USA) for membranes incubated with the monoclonal anti-hsp70 mouse antibody; or an anti-rabbit IgG peroxidase antibody produced in goat (Nventa Biopharmaceuticals Corporation, Victoria, BC, Canada) for the salmonid-specific hsp antibodies.

All antibodies were diluted with 2% skim milk powder in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5). Hsp70s were detected by chemiluminescence (ECL™ Western Blotting Analysis System, Amersham Biosciences UK Lmt, Buckinghamshire, UK) on Kodak BioMax Light film (Eastman Kodak Company, Rochester, NY, USA). Bands were scanned using a HP Scanjet 4570C (Hewlett-Packard, Palo Alto, CA., USA), and quantified using Quantity One software (Version 4.5.1., Bio-Rad Laboratories, Hercules, CA., USA) (Hosoya *et al.*, 2007). Finally, the values were expressed relative to the band intensity of a positive control (a liver sample from heat shocked Atlantic salmon, *Salmo salar* L., that were run concurrently, 3 times, on each gel.

4.3.3 Statistical Analyses

All data are presented as means ± S.E. n = 8 for oxygen measurements and n = 4 for plasma cortisol and glucose, and gill hsp70 levels [the value for each replicate (tank) equal to the average of the fish sampled from that tank]. Data were initially submitted to a 2-way repeated measures analysis of variance (RM-ANOVA): temperature as the repeated variable (because tank was the unit sampled), and treatment/control and fish size as main effects. However, there were significant interactions because none of the parameters in the control (10 °C) fish were affected by the sampling protocol (*i.e.* repeated netting of fish from the tanks). Thus, separate one-way RM-ANOVAs were performed (with size as the main effect) for the treated and control groups. In all analyses, the Student-Newman-Keuls test was applied to discern differences among means when main effects were significantly different. Whenever necessary, the data were log-transformed to achieve either a normal distribution or equal variance.

4.4 Results

4.4.1 Survival

Because these experiments were performed on groups of fish, it was not possible to determine the mean critical thermal maximum (CTM) values for these juvenile Atlantic cod. However, it was noticed that although > 93% of fish of both sizes survived to 24 °C, all fish lost equilibrium within 15 minutes of this temperature being reached.

4.4.2 Oxygen Consumption

At 10 °C, MO_2 for both fish size classes was around 180 mg O_2 kg^{-1} h^{-1}. In both the 10 g and the 50 g cod, MO_2 increased significantly (RM-ANOVA, n= 4, P < 0.05) with the first 2 °C increment in water temperature (to 12 °C), and continued to increase with increasing temperature. Peak values were reached at 20 and 22 °C, respectively, and these values were approximately 2.8-fold higher than those measured in time-matched control fish (Fig. 4.2).

4.4.3 Plasma Glucose and Total Cortisol

In the control groups plasma glucose values for 10 g Atlantic cod at 10 °C were significantly lower (RM-ANOVA, n= 4, P < 0.05) when compared to the 50 g juveniles (approx. 75 vs. 110 mg dl^{-1}) (Fig. 4.3). For the 50 g juveniles there were no significant differences in plasma glucose levels with temperature. However, significantly higher levels (RM-ANOVA, n= 4, P < 0.05) of plasma glucose levels were found in 10 g Atlantic cod, at temperatures of 22 °C (114.82 ± 4.74 mg dl^{-1}) and 24 °C (113.30 ± 5.11 mg dl^{-1}) when compared to values at 10 °C. These temperatures are just below the temperature at which they lost equilibrium.

In contrast to the plasma glucose response, the cortisol response was robust and highly temperature sensitive (Fig. 4.4). Plasma cortisol showed an exponential increase with temperature within each size class, starting at approx. 16 °C (*i.e.* ~ 6-8 °C prior to their CTM). Although a significant size x temperature interaction precluded a

statistical evaluation of whether size class influenced maximum plasma levels, values were 2-fold higher in 50 g cod (195.1 ± 35.1 ng ml^{-1} vs. 448.6 ± 64.8 ng ml^{-1}); values were 19-fold and 35-fold higher as compared to time-matched control fish.

4.4.4 Gill HSP70

Using the mouse monoclonal hsp70 antibody, 2 bands with molecular masses of approximately 72 and 73 kDa were detected in both the control and stressed groups (only data for 10 g fish shown, Fig. 4.5). Based on data from mammalian and avian studies (see Sigma-Aldrich product information), these bands correspond to the inducible and constitutive forms of hsp70, respectively. Exposure to elevated temperatures had no effect on the band intensity of either hsp70 isoform. Further, no difference in hsp protein levels was detected between size classes.

Using the antibody specific for the constitutive form of salmonid hsp70, a single protein band at approximately 73 kDa was detected in both groups. However, analysis of the band intensities did not reveal a significant difference in expression at any temperature (Fig. 4.6). Surprisingly, the inducible form of hsp70 could not be detected using the salmonid specific antibody (data not shown), and thus, I was not able to evaluate whether hsp72 was affected by exposure to increased temperatures using this antibody.

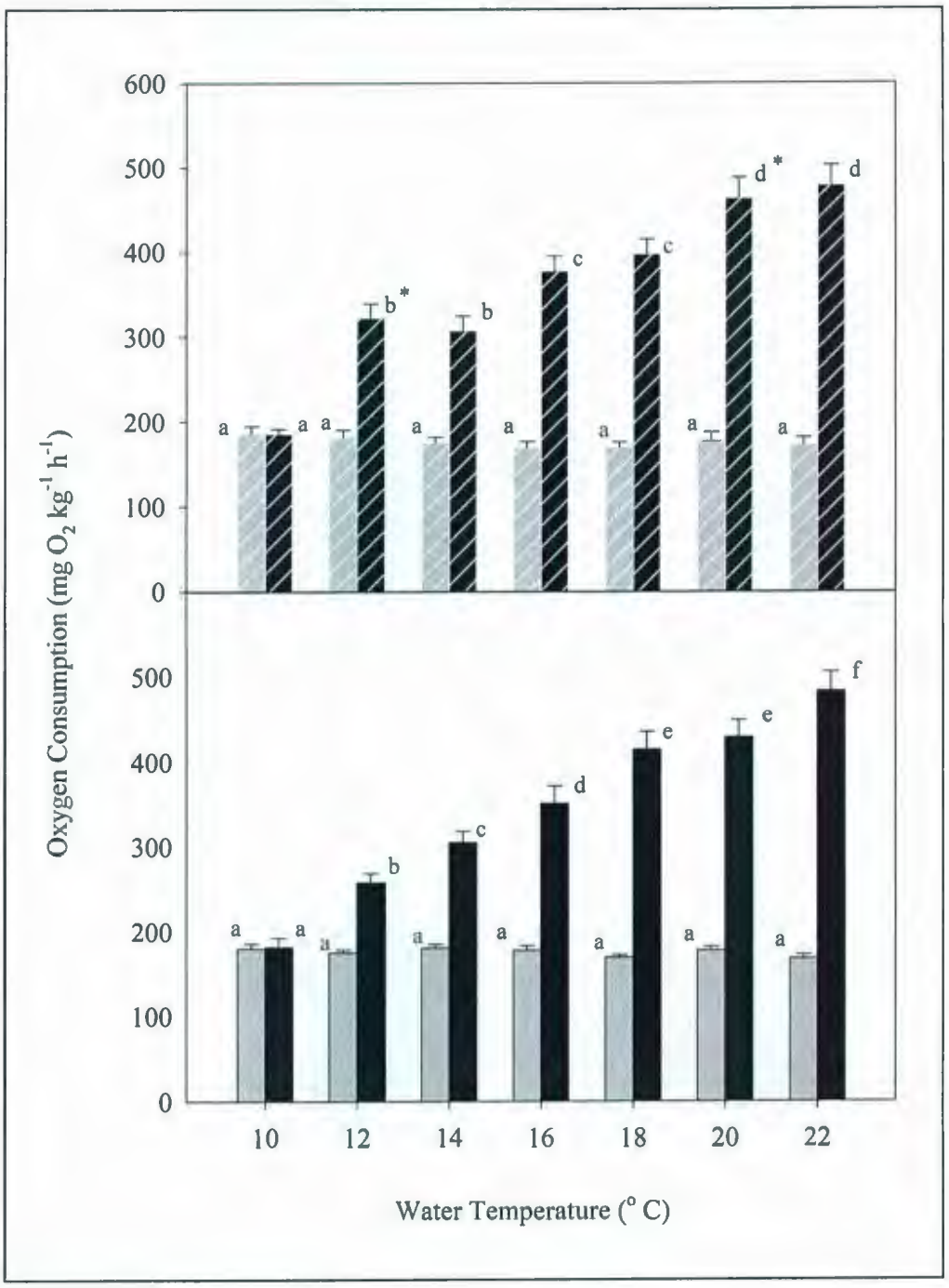


Fig. 4.2. Oxygen consumption (mean ± S.E., $n = 4$) for 10 g (▨ Control, ▨ Stressed) and 50 g (▭ Control, ■ Stressed) Atlantic cod subjected to an acute temperature challenge (2 °C h^{-1}) until their critical thermal maximum. Dissimilar letters indicate values within the control (maintained at 10 °C) and experimental groups that were significantly (RM-ANOVA, $P < 0.05$) different from each other. * Indicates significant differences between sizes of the stressed group at the same temperature.

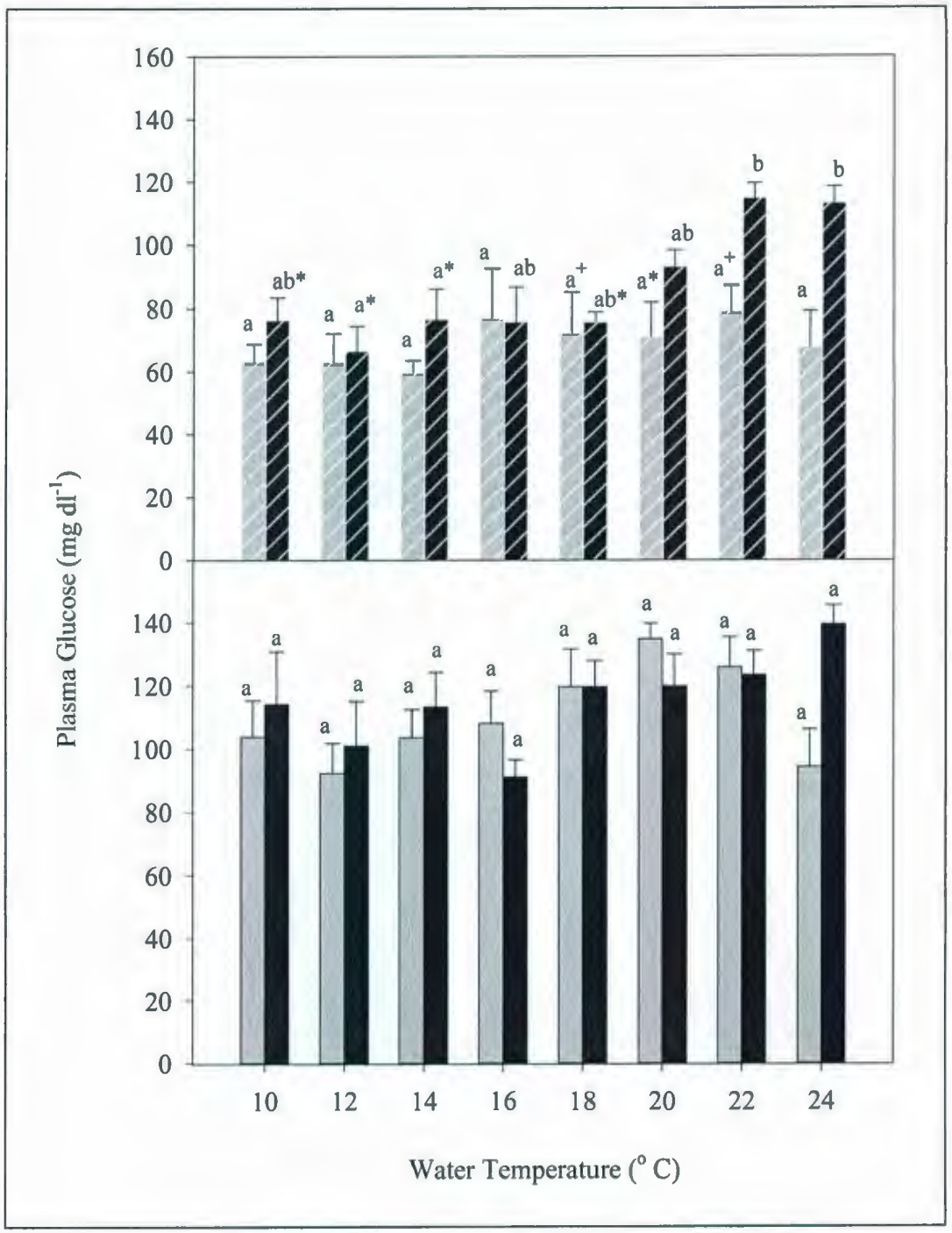


Fig. 4.3. Plasma glucose concentrations in 10 g (▨ Control, ▨ Stressed) and 50 g (▭ Control, ■ Stressed) juvenile Atlantic cod subjected to an acute thermal stress (2 °C h^{-1}) until their critical thermal maximum. Dissimilar letters indicate values within the control (maintained at 10 °C) and experimental groups that were significantly (RM-ANOVA, $P < 0.05$) different from each other. Values are means + S.E., $n = 4$.
* Indicates significant differences between size classes at the same temperature.

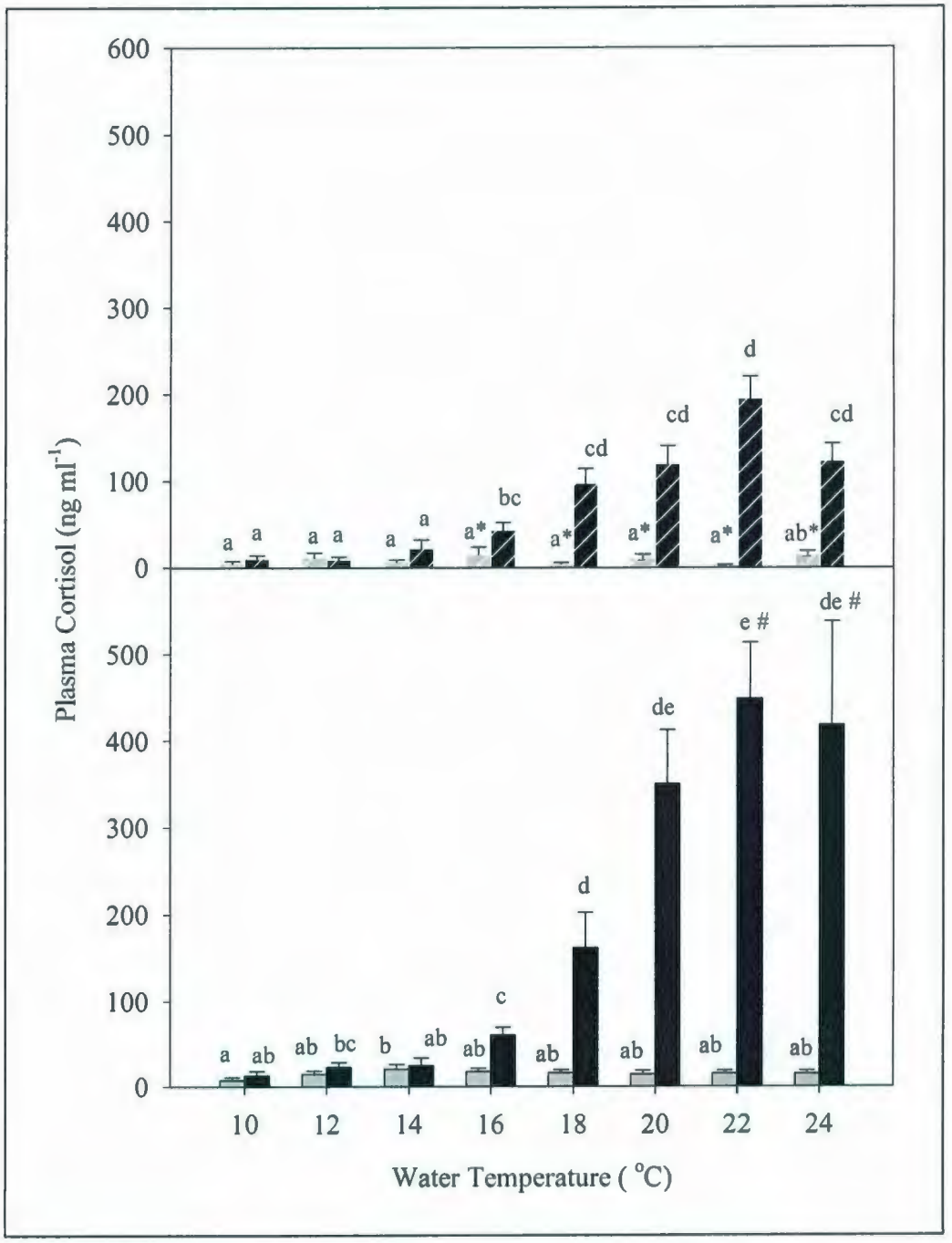
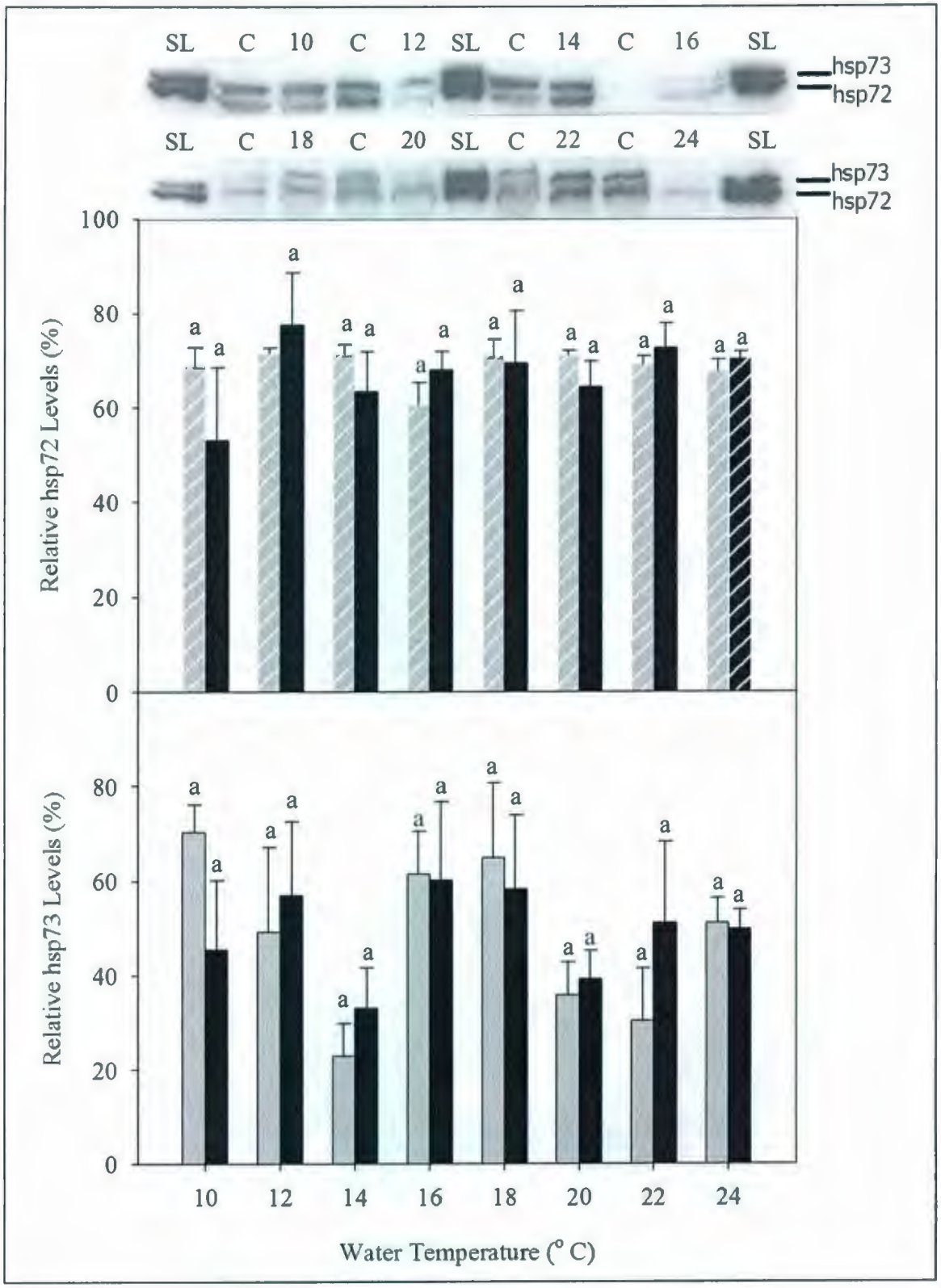


Fig. 4.4. Plasma cortisol concentrations in 10 g (Control, Stressed) and 50 g (Control, Stressed) juvenile Atlantic cod subjected to an acute thermal stress (2 °C h^{-1}) until CTM. Dissimilar letters indicate values within the control (maintained at 10 °C) and experimental groups that were significantly (RM-ANOVA $P < 0.05$) different from each other. Values are means ± S.E., $n = 4$. # = $P < 0.1$. * Indicates significant differences between size classes at the same temperature.


SL C 10 C 12 SL C 14 C 16 SL
hsp73
hsp72
SL C 18 C 20 SL C 22 C 24 SL
hsp73
hsp72
Relative hsp72 Levels (%)
Relative hsp73 Levels (%)
0
20
40
60
80
100
10 12 14 16 18 20 22 24
Water Temperature (° C)

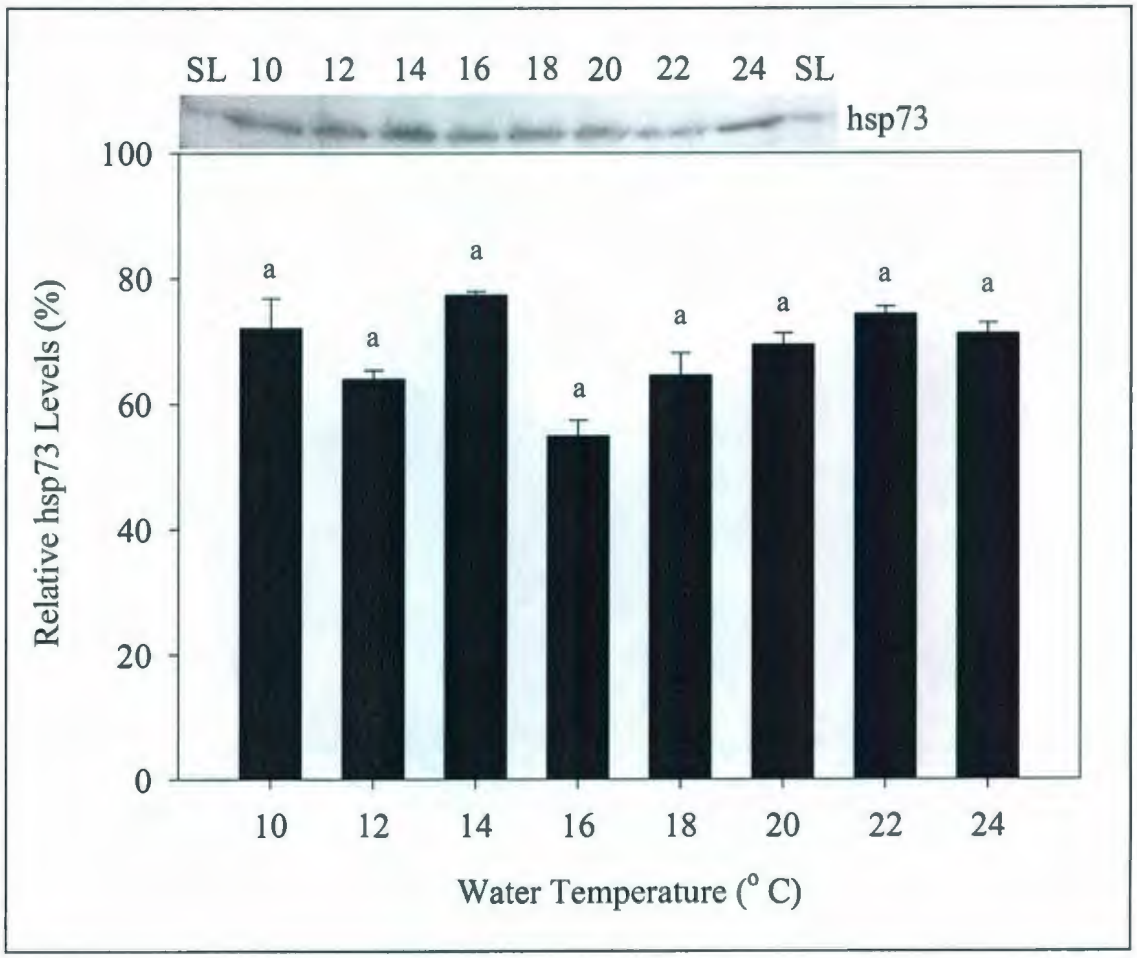


Fig. 4.6. Gill hsp73 (constitutive) levels in 10 g temperature stressed Atlantic cod relative to the positive control. Heat shock protein levels were quantified using a polyclonal anti-hsp70 antibody produced in rabbit that is specific for salmonid hsp73. Values are means $\pm$ S.E., $n = 4$. No significant elevation in hsp73 was detected at any temperature. Above the data figure is a representative image showing the bands that were used to quantify the effect of water temperature on gill hsp73 expression. The numbers correspond to experimental temperature. Note: No hsp72 bands could be detected using a polyclonal anti-hsp70 antibody produced in rabbit that is specific for salmonid hsp72 (data not shown).

4.5 Discussion

At 10 °C, MO_2 was approximately 180 mg O_2 kg h^{-1} in both size classes of cod. This value is high as compared to the majority of routine oxygen measurements made on juvenile Atlantic cod at similar temperatures (*e.g.* Soofiani and Hawkins, 1982: 131 mg O_2 kg h^{-1}; Peck *et al.*, 2003a: ~ 120 mg O_2 kg h^{-1}) even after the allometric scaling of standard/routine metabolism with body mass is considered (b ~ 0.8 - 0.85; Soofiani and Priede, 1985; Claireaux *et al.*, 1995; (Killen *et al.*, 2007). This difference is likely related to the use of groups of fish in tank respirometers, where MO_2 would be increased by the fish's activity and interactions with conspecifics, experimental conditions that are a more realistic representation of aquaculture conditions. Oxygen consumption increased by 2.63-fold when temperature was increased from 10 – 24 °C (Q_{10} = 2.23; Fig. 2), an increase very similar in magnitude to that observed by Gollock *et al.* (2006) (2.56-fold) when individual adult Atlantic cod were subjected to a temperature increase of ~ 1.7 °C to CTM. In contrast to the study of Gollock *et al.* (2006) and Gamperl and Canada (unpublished), however, a plateau in oxygen consumption was not observed in either size class as temperature approached the fish's CTM. This finding suggests that MO_2 measurements on groups of fish are not as useful as those on individuals for assessing when the ability of the fish's cardiorespiratory system to deliver oxygen becomes limiting.

As previously mentioned, it was not possible to determine the mean CTM values for the juvenile Atlantic cod used in these experiments because the studies were carried out on groups of fish. However, all fish lost equilibrium within 15 minutes of reaching 24 °C. This finding agrees with Gamperl and Canada (unpublished), where

CTM measurements were performed on 12 °C acclimated juvenile Atlantic cod in swim-tunnel respirometers, and their average CTM was 23.0 $\pm$ 0.3° C. Further, this study did not detect a size-related (10 vs. 50 g) difference in the thermal tolerance of juvenile Atlantic cod. This lack of a size effect on Atlantic cod acute thermal tolerance is consistent with the swim-tunnel respirometry experiments performed by Gamperl and Canada (unpublished) and Gollock *et al.* (2006) on juvenile (approx. 65g; 12 °C acclimated) and adult (> 800 g; 10 °C acclimated) Atlantic cod, respectively. When acclimation temperature is taken into account (Gamperl and Canada, unpublished), no significant difference in critical thermal tolerance is evident between these two very different size classes (65 g cod, 23.0 $\pm$ 0.3 ° C; > 800 g cod, 22.2 $\pm$ 0.2 °C).

The lack of a size (age) effect on cod CTM is in agreement with Rodnick *et al.* (2004) who report that redband trout differing in body mass by 35-fold (40 – 1400 g) had similar CTM values (~ 29.5 °C), and Carline and Machung (2001) who showed that neither length or condition influenced the T_{crit} of wild and domestic strains of brown, *Salmo trutta* L., and rainbow trout. However, the data for cod CTM should not be used to conclude that juveniles and adults of this species have similar thermal sensitivities. For example, oxygen limitation models predict that temperature-dependent aerobic limits are experienced earlier by larger than smaller individuals (Portner *et al.*, 2004). Lafrance *et al.* (2005) showed that the thermal preferendum (T) of Atlantic cod is very size dependent (T = 7.23 – 0.054 * fork length). Finally, Björnsson *et al.* (2001b) reported that long-term exposure to 16 °C greatly increases the mortality of large (1.5 kg) but not small (< 6 g) cod, and showed that the optimum temperature for growth in Atlantic cod decreases from 17.2 °C for 2 g fish to 7 °C at 2.2 kg.

In this study, juvenile Atlantic cod of both size classes had peak plasma cortisol levels (195.06 ± 35.08 ng ml^{-1} and 448.58 ± 64.77 ng mL^{-1} in 10 g and 50 g fish, respectively) that are much higher than previously reported for this species following a single acute stressor. For example, when juvenile Atlantic cod were subjected to a 30 second air stressor, King V and Berlinsky (2006) and King V *et al.* (2006) reported that peak values of plasma cortisol were ~100 ng ml^{-1} and ~80 ng ml^{-1}, respectively. Further, studies with haddock, a closely related gadid, report plasma cortisol values of 40 – 50 ng ml^{-1} following an acute net/handling stress (Hosoya *et al.*, 2007; Afonso *et al.*, 2008). The 2-10 fold higher total plasma cortisol levels reported in the present study are likely due to the cumulative effect of exposure to increasing elevated temperatures (Wendelaar Bonga, 1997; Barton, 2002). Such a cumulative effect of a stressor has been reported in salmonids. For example, Barton *et al.* (1986) exposed juvenile Chinook salmon *Oncorhynchus tshawytsch* (Walbaum) to repeated 30 second air exposures, and found that there was a stepwise increase in plasma cortisol and glucose levels with each additional handling. Interestingly, however, Staurnes *et al.* (1994) and Herbert and Steffensen (2005) report levels of plasma cortisol for cod of 71 ng ml^{-1} and ~110 ng ml^{-1} after exposure to 48 hours of simulated transport and graded hypoxia down to 4 kPa, respectively. Thus, the current results suggest that an acute increase in water temperature is a much more severe stressor for Atlantic cod than prolonged culture procedures or other environmental perturbations. Further, the finding that plasma cortisol levels begin to increase at ~ 16 °C supports previous data indicating that this temperature is the upper critical temperature (Portner, 2002) for this species. For example, Sartoris *et al.* (2003) and Lannig *et al.* (2004) report that venous PO_2 falls dramatically when 10 °C acclimated cod are acutely exposed to 15-17 °C,

and cardiac arrhythmias first appear at 16 – 18 °C (Lannig *et al.*, 2004; Gollock *et al.*, 2006).

The finding that the older (50 g) Atlantic cod had higher levels of post-stress cortisol is also in contrast to the generalization that younger fish are more sensitive to stressors such a temperature (Wendelaar Bonga, 1997). The reason(s) for the size-related difference in cortisol levels are unknown. However, the current finding that plasma cortisol is a sensitive indicator of thermal stress is consistent with the data of Afonso *et al.* (2008) for haddock, and research on a number of other fish species (Gamperl *et al.*, 1994; Wendelaar Bonga, 1997; Mommsen *et al.*, 1999).

The increase in plasma cortisol levels associated with stress results in the production and release of glucose by the liver (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999), a response that ensures the fish has adequate circulating energy substrate to restore/maintain its internal homeostasis (Ackerman *et al.*, 2000). In the present study, plasma glucose values for 10 g cod at 10 °C were considerably lower than for 50 g cod (approx. 75 vs. 110 mg dl^{-1}; Fig. 4). Irrespective of this difference between the two size classes, plasma glucose levels were not very informative with respect to thermal stress. Plasma glucose levels were highly variable; no significant changes were found for 50 g cod, and significant increases were only recorded for 10 g cod at temperatures of 22 and 24 °C (*i.e.* just before they lost equilibrium). Although plasma glucose levels have been used as an indicator of stress in numerous fish species (Barton and Iwama, 1991), the results of the present work agree with those of Herbert and Steffensen (2005) and Afonso *et al.*(2008). These authors subjected adult Atlantic cod to progressive hypoxia and juvenile haddock to a handling or thermal stress (rapid

increase from 10 to 15 °C), respectively, and report that these stressors did not elicit a glucose response in these two gadids.

Consistent with previous studies on mammals *e.g.* (Manzerra *et al.*, 1997) and fish (Rendell and Currie, 2005; Zakhartsev *et al.*, 2005), basal levels of both hsp70 isoforms (presumably hsp72 – inducible and hsp73 – constitutive) could be detected in control and experimental fish at 10 °C (*i.e.* in unstressed fish) using the mouse antibody (clone BRM-22). Further, in agreement with data for the Atlantic cod (Zakhartsev *et al.*, 2005) and haddock (Afonso *et al.*, 2008), no changes in gill hsp70 levels were detected when cod were exposed to elevated temperatures. To explain the lack of a change in cod hsp70 levels with exposure to increased temperature, Zakhartsev *et al.* (2005) proposed that levels of constitutive hsp70 in Atlantic cod and other gadids are high enough to overcome the potentially harmful effects of temperature within their physiological range. However, in this study, there were no changes in constitutive or inducible hsp70 levels even after the cod juveniles were exposed to temperatures well above their upper critical temperature (~ 16 °C). The lack of an hsp70 response to heat stress in Atlantic cod and haddock is in contrast to that observed in most teleost species (Iwama *et al.*, 1998; Hightower *et al.*, 1999; Basu *et al.*, 2002). However, it is not unique. For example, Hofmann *et al.* (2000): 1) showed that, emerald rockcod (*Trematomus bernacchii* Boulenger) acclimated to 5 °C for 22 days (the preferred temperature range for this species is 0.3 to -1.86 °C) did not increase gill hsp70 levels; and 2) did not find evidence of hsp70 synthesis when *T. bernacchii* hepatocytes were subjected to a temperature increase from 5 to 15 °C *in vitro* (*i.e.* exposed to temperatures ~ 15 °C above their temperature range). Further, Buckley *et al.* (2004) examined several steps in the hsp70 expression pathway in *T.*

bernacchii (transcription factor activity, hsp70 mRNA production and protein synthesis) during thermal stress, and report that inducible and constitutive hsp70 mRNA expression in hepatocytes was unaltered after exposure to thermal stress.

At the moment it is still unclear why some fish do not present a heat shock response after thermal stress. For example, the lack of an hsp70 response in Atlantic cod cannot be explained by the high cortisol levels seen when they were exposed to temperatures greater than 16 °C (see Fig. 4.4). High cortisol levels diminished (by approx. 30%), but did not eliminate, the hsp70 response to an acute heat shock in tilapia and rainbow trout (Basu *et al.*, 2001). Further, the biological significance of this lack of response is intriguing, considering the important chaperoning activities of the heat shock proteins during thermal stress (Feder and Hofmann, 1999).

In this study, the inducible form of cod hsp70 was not detected using the salmonid specific antibody. The reason(s) for this are unknown. However, several isoforms of inducible hsp70 exist in mammals (Manzerra *et al.*, 1997), and thus it is possible that there are important sequence differences in the inducible form of hsp70 between salmonids and gadids. Alternatively, it is possible that there are two isoforms of constitutive hsp70 in the cod gill, and this is what we were detecting with the mouse monoclonal antibody. Clearly, efforts should be made to obtain the DNA sequences for cod hsp70s and to develop antibodies for further investigations of the importance of heat shock proteins in gadids. For example, these tools will be required to examine whether: 1) the mouse monoclonal antibody (BRM-22) is indeed detecting inducible hsp70 in Atlantic cod; 2) pre-stress levels of hsp70s are sufficient to protect cod tissues from temperature-related damage; and 3) whether the lack of an increase in hsp72 (as determined with the mouse antibody) was related to a lack of hsp72 transcription, or

translation as would be suggested by the work of Hofmann *et al.* (2005) for the black cod *Notothenia angustata* (Hutton).

To summarise, this study: 1) shows that although juvenile Atlantic cod are highly sensitive to acute variations in water temperature they can tolerate short-term exposure to temperatures exceeding 20 °C; 2) supports previous data indicating that the upper critical temperature for cod is ~ 16 °C; and 3) indicates that oxygen consumption and plasma cortisol are sensitive indicators of thermal stress in this species. In contrast to most fish species, juvenile Atlantic cod did not show either a consistent glucose or hsp70 response when subjected to an acute thermal challenge. While the interpretation of the glucose data is clear, further research is required to ascertain why Atlantic cod (and other gadids) do not increase hsp70 levels during an acute temperature challenge.

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Chapter 5. The Immune and Stress Responses of Atlantic Cod to Long-Term Increases in Water Temperature.

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5.1 Abstract

Sea-caged cod are limited in their movements in the water column, and thus can be exposed to large seasonal (~ 0 to 20 °C) temperature fluctuations. To investigate the physiological response of Atlantic cod to summer-like increases in temperature, we exposed 10 °C acclimated juvenile cod to a graded thermal challenge (1 °C increase every 5 days) and measured: 1) plasma cortisol and glucose levels; 2) the respiratory burst activity of blood leukocytes; and 3) the expression of specific immune-related genes [MHC Class I, Interleukin-1β (IL-1β), β_2-microglobulin (β_2-M), Immunoglobulin M (IgM) -light (L) and heavy (H) chains] in the blood using Quantitative Reverse Transcription – Polymerase Chain Reaction (QRTPCR). The experiment was stopped at 19.1 °C, with 26.7% percent of the fish surviving to this point. Plasma glucose levels increased slightly at 16 and 18 °C (by 1.39 and 1.74 fold, respectively), in contrast, cortisol levels were elevated significantly (by 2.9 fold) at 16 °C but returned to control levels thereafter. The effect of increasing temperature on the expression of immune related genes in blood cells (leukocytes) was variable and depended on the gene of interest. The expression of IgM-H remained stable for the duration of the experiment. In contrast, IL-1β expression was increased significantly (by ~25 fold) at 19 °C as compared to time-matched control fish, and changes in the expression of β_2-M, MHC Class I and IgM-L followed a pattern similar to that seen for cortisol: increasing at 16 °C (by 4.2, 5.3 and 17 fold, respectively), but returning to pre-stress levels by 19 °C. Interestingly, increasing temperatures had no effect on respiratory burst activity. This study is the first to examine the effects of a chronic regimen of increasing temperature on the stress physiology and immunology of a

marine teleost, and suggests that immune function is influenced by complex interactions between thermal effects and temperature-induced stress (elevated circulating cortisol levels).

5.2 Introduction

Atlantic cod (*Gadus morhua L.*) aquaculture is a growing industry in Canada, the United States and Europe (Gollock *et al.*, 2006; Kjesbu *et al.*, 2006), and there are significant efforts underway in several countries to identify cod with economically beneficial production traits and to initiate selective breeding programs (e.g. Atlantic Cod Genomics and Broodstock Development Project; www.codgene.ca). When cultured in sea-cages, Atlantic cod are limited in their movement in the water column, and thus can be exposed to water temperatures that exceed their thermal preferenda (Gollock *et al.*, 2006). Given that temperature has a considerable influence on fish biochemistry, physiology and behaviour (Beitinger *et al.*, 2000), and elevated temperatures can have a negative influence on fish health and lead to decreased growth and increased mortality (Dominguez *et al.*, 2004), it is important to understand the effects of elevated water temperatures on cod biology so that: 1) appropriate cage-sites can be selected, and 2) breeding programs can identify genotypes/phenotypes that are best suited for cage-site locations where high summer temperatures are a concern.

Over the past 4 decades, there has been a considerable amount of work on cod thermal biology. However, most of this work has focused on determining preferred temperatures (Despatie *et al.*, 2001; Petersen and Steffensen, 2003; Lafrance *et al.*, 2005), optimal temperatures for growth and feed conversion (Jobling, 1988; Otterlei *et al.*, 1999; Bjornsson *et al.*, 2001a), or the effects of temperature on metabolism (Saunders, 1963; Schurmann and Steffensen, 1997; Claireaux *et al.*, 2000; Peck *et al.*, 2003a; Peck *et al.*, 2003b). Interestingly, very few studies have focused on the effects of temperature on the immune system of Atlantic cod, and those that did failed to

address the potential impact of high temperatures (i.e. > 15 °C). For example, Magnadottir *et al.* (1999a) only exposed fish to constant temperatures of 14 °C or below, and compared immune parameters in wild cod captured at 0 - 4 °C vs. 6 -10 °C (Magnadottir *et al.*, 1999b).

Studies on cod immune function are clearly needed, as while it is generally accepted that high temperatures enhance the specific immune response of fishes (Alcorn *et al.*, 2002; Nikoskelainen *et al.*, 2004), measurements of circulating IgM in the Nile tilapia *(Oreochromis niloticus)* (Dominguez *et al.*, 2004) suggest that some species may have an optimal thermal range for immune function. Further, the effect that elevated water temperature has on the fish's innate immune system is quite variable. For example, while respiratory burst was reported to be more intense at higher acclimation temperatures when rainbow trout (*Oncorhynchus mykiss*) were reared between 5 to 20 °C (Nikoskelainen *et al.*, 2004), Le Morvan *et al.* (1997) reported that high temperatures resulted in decreased respiratory burst activity in common carp (*Cyprinus carpio*), and Ndong *et al.* (2007) found that respiratory burst increased but lysozyme activity decreased when Mozambique tilapia (*Oreochromis mossambicus*) were acclimated to temperatures between 19 and 35 °C.

In addition to the paucity of published studies pertaining to the impact of temperature on cod immune function, the only data on cortisol levels in cod during exposure to high temperatures (15 °C or greater) have been generated using acute experimental protocols (Afonso *et al.*, 2008; Pérez-Casanova *et al.*, 2008). Information on the effects of chronic high temperature on this hormone are potentially critical to managing cod aquaculture operations because cortisol is the predominant corticosteroid in teleost fish (Mommsen *et al.*, 1999), and exerts a modulatory effect

on a diversity of fish immune parameters. For example: 1) *in vitro* studies on winter flounder (*Pseudopleuronectes americanus*) indicate that long-term cortisol administration reduces the primary immune response of lymphocytes (Carlson *et al.*, 1993); 2) gilthead seabream (*Sparus aurata* L.) head-kidney leukocytes exposed to high doses of cortisol have decreased respiratory burst activity (Esteban *et al.*, 2004); and 3) intraperitoneally injected cortisol increases circulating IgM levels in gilthead seabream (Cuesta *et al.*, 2006) and can suppress head kidney leukocyte IL-1β expression in common carp and rainbow trout (Zou *et al.*, 2000; Engelsma *et al.*, 2001; Engelsma *et al.*, 2002).

Given the lack of information on the responses of Atlantic cod immunology and stress physiology to chronically high (> 15 °C) temperatures, we examined the impact of a chronic regimen of slowly increasing water temperature (which mimicked changes in seawater temperatures previously recorded at Newfoundland sea-cage sites) on this species' stress response and various immune parameters. The cod's stress response to chronically elevated temperatures was assessed by measuring plasma cortisol and glucose levels. Effects on components of the innate and adaptive immune system were examined by measuring blood leukocyte respiratory burst activity, as well as the expression of MHC Class I, Interleukin-1β (IL-1β), β_2-microglobulin (β_2-M), and Immunoglobulin M (IgM) -light and heavy chains using Quantitative, Real-Time Reverse Transcription – Polymerase Chain Reaction (QRTPCR).

5.3 Materials and Methods

These studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol # 05-07-KG).

5.3.1. Animals

Fish for these experiments were obtained from the Aquaculture Research and Development Facility (ARDF) of the Ocean Sciences Centre, Memorial University of Newfoundland, and were from a single spawning of Cod Genomics Project broodstock. These fish were selected for study based on their excellent growth and low rates of mortality, and were reared (at 10-11 °C) in the same production tank following standard rearing protocols in place at the ARDF (Puvanendran and Brown 1998, 1999, 2002; Puvanendran et al. 2004). Interestingly, despite their high levels of performance in culture, some of the fish in this population were later identified as being carriers of nodavirus (by PCR and SSN-1 cell culture). However, we are confident that their carrier state had no influence on their critical thermal maximum (CTM), temperature-related physiology or immune function. These fish never showed any clinical signs of the disease [erratic swimming behaviour, floating belly up due to over-inflation of the swim bladder, lethargy, change of pigmentation, etc., (Johnson *et al.*, 2002; Samuelsen *et al.*, 2006), and it has recently been shown that nodavirus carrier status does not influence the constitutive expression of immune-relevant genes, or their expression following polyriboinosinic polyribocytidylic acid (pIC) injection in cod (as assessed by QRTPCR analysis)(Rise *et al.*, 2008).

Experiments were performed using juvenile Atlantic cod of ~ 40 g initial average wet mass and six 250 L tanks. The experimental tanks were randomly assigned to 1 of 2 treatments: 1) control, where the water temperature remained constant at 10 °C; and 2) temperature stressed (TS), where the fish were exposed to an increase in water temperature of ~ 1 °C every 5 days from 10 to 19 °C. In order to obtain the desired water temperatures for the TS group, submersible heaters (accuracy $\pm$ 1 °C) were placed in each tank, and in the header tank supplying water to these fish.

Groups of 40 fish were randomly placed into each of the experimental tanks 1 week prior to the beginning of the experiment to allow them to acclimate to their new conditions, and to recover from handling/netting stress. During this period, and throughout the experiment, the fish were fed a commercial cod diet (EWOS Canada Ltd., Surrey, BC, Canada) to satiation twice a day. Diet composition was as follows: 55% protein, 15% lipids, 1.5% fibre, 3.0% Calcium, 2.0% phosphorus, 1.0% sodium and 5000, 3000 and 2000 IU of vitamins A, D and E, respectively. Any uneaten pellets were siphoned out of the tank after each feeding, and then dried and weighed to obtain accurate measurements of feed consumption. At temperatures above 16 °C, the fish in the TS group reduced their food consumption or stopped eating altogether. Thus, to maintain similar conditions between the 2 groups, control fish were not fed more than the TS group consumed. Water temperature was monitored several times daily using digital thermometers placed in each of the tanks, and water oxygen concentration was maintained above 95 % over the duration of the experiments. Further, the condition of the fish was monitored at least twice daily (more often at the highest temperatures), and any moribund or dead fish were immediately removed from the experiment. Moribund fish were euthanized by a rapid blow to the head.

5.3.2. Plasma Glucose and Cortisol

5.3.2.1. Blood and Tissue Sampling

At 0, 10, 20, 30, 40 and 45 days after the beginning of the experiment, 3 fish per tank (9 fish per treatment) were carefully netted, anaesthetized with an overdose (300 mg l^{-1}) of tricaine methanesulfonate (TMS) (Syndel Laboratories Ltd, Vancouver, BC, Canada) and quickly sampled. Blood samples (600 - 1000 µl by caudal puncture) were taken within 2 min of netting using previously heparinized (100 U ml^{-1} heparin, Sigma Aldrich, St. Louis, MO, USA) 1cc U-100 syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the majority of the blood was transferred to 1.5 ml centrifuge tubes and stored on ice. This blood sample was subsequently used to obtain plasma (by centrifugation at 10,000 rcf for 5 min at 4 °C) for cortisol and glucose analysis, while the remaining blood cells were resuspended in 4 volumes of RNAlater for subsequent analysis of immune-related gene expression. All of these samples were stored at -80 °C until further processing. For each fish sampled, the blood remaining in the syringe (approx. 100 µl) was briefly stored on ice (~ 20 min) prior to the measurement of respiratory burst.

5.3.2.2. Measurement of Plasma Glucose and Cortisol

Plasma glucose levels were measured in triplicate using a modified Trinder (1969a) enzymatic assay that is commercially available as a kit (Diagnostic Chemicals

Ltd. Oxford, CT, USA.) (Hosoya *et al.*, 2007). Plates were read at 505 nm using a Bio-Tek Synergy HT microplate reader and the KC4 KinetiCalc software for windows version 3.3 Rev. 10 (Bio-Tek Instruments, Inc. Winooski, VT, USA). The intra- and inter-assay coefficients of variation were always less than 5%. Total plasma cortisol levels were determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN CORP. Lexington, KY, USA), and the same microplate reader and software described above, at an absorbance of 650 nm (Hosoya *et al.*, 2007). Intra- and inter-assay variation never exceeded 10%.

5.3.3. Respiratory Burst

The production of reactive oxygen species by Atlantic cod blood leukocytes was measured by chemiluminescence following the methods described by Marnila (1995) and Nikoskelainen *et al.* (2004; 2006). Briefly, whole blood (2 µl ml^{-1} final concentration) was added to a mixture of 1 mM luminol (5-amino-2,3-dihydro 1,4-phthalazinedione, Sigma, St. Louis, MO, USA) in 0.2 M sodium borate and phosphate buffered saline. Two hundred and twenty-five µl of this solution was then added into each of 3 replicate wells of a 96-well white cliniplate (Cat No. 28298-610, VWR International, Mississauga, Ont.), and 25 µl of 20 mg ml^{-1} zymosan A from *Saccharomyces cerevisiae* (Sigma, St. Louis, MO, USA) was added into each well. Chemiluminescence was then measured over 40 min using the microplate reader and software described above; during this time, a curve of the luminescence counts per second (LCS) was obtained, and peak values were taken to represent the respiratory burst of the each sample.

5.3.4. Expression of Immune-Related Genes

5.3.4.1. RNA Extraction and cDNA Synthesis

This analysis was only performed at three temperatures (10, 16 and 19 °C), or at equivalent time points for the control fish, using 6 randomly selected samples from each temperature/treatment combination. Sixteen °C was selected because our data (Figure 2A) and that of Perez-Casanova *et al.* (2008) show that this is the temperature where cod become stressed (as assessed by plasma cortisol), and 19 °C was chosen because this was the maximum temperature to which cod were exposed before the experiment was terminated due to ethical considerations.

Blood samples stored in RNAlater were thawed on ice and centrifuged at 1,000 x g for 10 min. The RNAlater solution was then removed and the pellet washed three times with 1 ml of phosphate buffered saline (PBS). Total RNA was extracted from the pellets using TRIzol Reagent (Invitrogen Canada Inc., Burlington, Ont.) following the manufacturer's instructions. Individual total RNA samples were then treated with 6.8 Kunitz units of DNaseI (RNase-Free DNase Set, QIAGEN Inc., Mississauga, ON) at room temperature for 10 min to remove any residual genomic DNA, and column purified using the RNeasy MinElute Cleanup Kit (QIAGEN Inc., Mississauga, ON). One µl of the final elution was then diluted 70 fold in RNase free water and the concentration of RNA was measured spectrophotometrically using a GeneQuant Pro RNA/DNA calculator (Biochrom LTd., Cambridge, UK).

5.3.4.2. Expression of Immune-Related Genes in Blood Evaluated by Quantitative Reverse Transcription – Polymerase Chain Reaction (QRTPCR)

While whole blood RNA samples were utilized for gene expression analyses, the immune-relevant genes selected for QRTPCR are expressed predominantly or exclusively in leukocytes (Rodrigues *et al.*, 1998). Therefore, we refer to the immune gene QRTPCR results as leukocyte data.

Transcript (mRNA) expression levels of β-2-microglobulin (β_2-M), major histocompatibility complex class I (MHC Class I), Interleukin-1 beta (IL-1β), Immunoglobulin M light and heavy chains (IgM-L and IgM-H) and elongation factor 1α (EF-1α, used as a normalizer gene) in the RNAlater preserved blood samples were quantified by QRTPCR using SYBR Green I dye chemistry and the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). EF-1α was selected as the normalizer gene because it is commonly used for this purpose (Hansen and Strassburger, 2000; Kales *et al.*, 2007), and was found by QRTPCR to be stably transcribed at all temperatures.

The sequences of the primers used in gene expression analysis were based upon Atlantic cod sequences for β_2-M, MHC Class I, IL-1β, IgM-L, IgM-H and EF-1α that were obtained from GenBank. Primer sequences and GenBank Accession Numbers are presented in Table 1. Amplification efficiency was calculated for one random sample from both the experimental and control groups using a 5 point 1:4 dilution series starting with 5 ng of cDNA, with the reported value (Table 5.1) being the average of the two. Multiple candidate QRTPCR primer pairs were tested for genes of interest,

and primer pairs with the best performance (e.g. single PCR product, no primer dimer, high amplification efficiency) were selected for use.

First-strand cDNA was synthesized from 0.5 μg of DNAse-treated, column purified, total RNA using 250 ng of random primers (Invitrogen, Burlington, ON) and M-MLV Reverse Transcriptase (200 units) (Invitrogen, Burlington, ON), and an incubation temperature of 37 °C for 50 min. PCR amplification was performed with the 7300 PCR Detection System (Applied Biosystems, Foster City, CA) in a 25 μl reaction using 1 μl (5 ng) of cDNA, 50 nM each of forward and reverse primer and 1X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The real-time analysis program consisted of 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. On each plate, for every sample, the target gene (gene of interest) and endogenous control (normalizer gene: Elongation Factor 1 alpha) were tested in duplicate. Then expression levels of the genes of interest were normalized to EF-1α, and the fluorescence threshold cycle (C_T) determined using the 7300 PCR Detection System SDS Software Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems, Foster City, CA). The relative starting quantity (RQ) of each transcript was determined using the comparative C_T method for relative quantification (Livak and Schmittgen, 2001), using the individual with the lowest gene of interest expression (highest C_T value) as calibrator. Fold regulation for each group was calculated as [(average RQ)/(average RQ for the appropriate control sample)].

Gene of Interest	Primers	GenBank Acc. No.	Nucleotide Sequence (5' → 3')	Amplification Efficiency (%)	Amplicon Size (bp)
Elongation Factor 1α	EF1α Forward	CO541820	GATGCACCACGAGTCTCTGA	92.38	171
	EF1α Reverse		GGGTGGTTCAGGATGATGAC		
β-2-microglobulin	β2M Forward	AJ132752	CTTCGACAAGACCTGGCACTTC	80.47	101
	β2M Reverse		TCCGTTTGCCGATGTGTGT		
Major Histocompatibility Complex class-1α	MHC I Forward	AF414219	CTAGCGTGGGACCTGAAGAC	91.74	171
	MHC I Reverse		CAGAGTGCTCTTCCCGTAGG		
Interleukin 1β	IL-1β Forward	AJ535730	AGCAGCATGAAGCAAACAACCT	95.99	107
	IL-1β Reverse		TTGACGACTTGCCTCATGGTC		
Immunoglobulin M light chain	IgM-L Forward	X76517	CACTACAGCTGGAGCAGCAC	101.30	154
	IgM-L Reverse		CCATGCTGGAGCCTCTCTAC		
Immunoglobulin M heavy chain	IgM-H Forward	X58870	GGTGAGGTGTTATCCGTGCT	83.00	196
	IgM-H Reverse		GCAGATAAACGGATGGAGGA		

5.3.5. Statistical Analyses

All data are presented as means ± S.E., n = 3 (i.e. 3 tanks each with 3 subsamples) for plasma cortisol, plasma glucose and respiratory burst, and n = 6 for the analysis of gene expression. Data for plasma glucose and cortisol, and respiratory burst, were initially submitted to a 1-way repeated measures analysis of variance (RM-ANOVA): temperature as the repeated variable (because tank was the unit sampled), and treatment/control as the main effect. In contrast, data for immune-related gene expression was subjected to a two-way ANOVA (with temperature and experimental groups as main effects). In all analyses, the Student-Newman-Keuls test was applied to identify differences among means when main effects were significant ($P < 0.05$).

5.4 Results

5.4.1. Mortality

Cumulative mortality (CM) for the TS group increased with temperature (Fig 5.1). For example, at 16 °C, 30 days after the beginning of the experiment, CM had reached 31.2%, at 18 °C (~ 40 days) it was 61.9%, and at the termination of the experiment (at 19.1 °C) CM had reached 73.3%. Overall, the CM for the control group was lower at all time points. However, a water flow interruption prior to reaching 14 °C (20 days) resulted in the loss of 9 fish in this group, and this is reflected in the CM values over the duration of the experiment (i.e. mortality is an overestimation by approx. 8%).

5.4.2. Plasma Glucose and Cortisol

Plasma glucose values for the control group remained relatively constant throughout the experiment (approx. range 60 – 100 mg dl^{-1}) with no significant differences seen at any time point. In the TS group, plasma glucose levels were significantly elevated ($P < 0.05$) as compared to 10 °C (initial) values at 16 and 18 °C [1.39 fold (122.21 ± 10.92 mg dl^{-1}) and 1.75 fold (134.53 ± 15.86 mg dl^{-1}), respectively, as compared to time-matched controls]. However, by 19 °C, plasma glucose had returned to levels comparable to initial values (Fig 5.2A).

As with plasma glucose, no significant changes in total plasma cortisol were detected in the control group. In contrast, plasma cortisol in TS cod showed a biphasic response pattern. Plasma cortisol levels were 2.9-fold higher (to 51.84 ± 18.30 ng ml^{-1}) at 16 °C as compared to the control group, but had returned to initial/control levels by 18 °C (Fig. 5.2B).

5.4.3. Respiratory Burst

There was no significant difference in the respiratory burst activity of whole blood between the control and TS groups over the duration of the study. Luminescence counts per second (LSC) remained around 1000 for fish in both groups (Fig. 5.3).

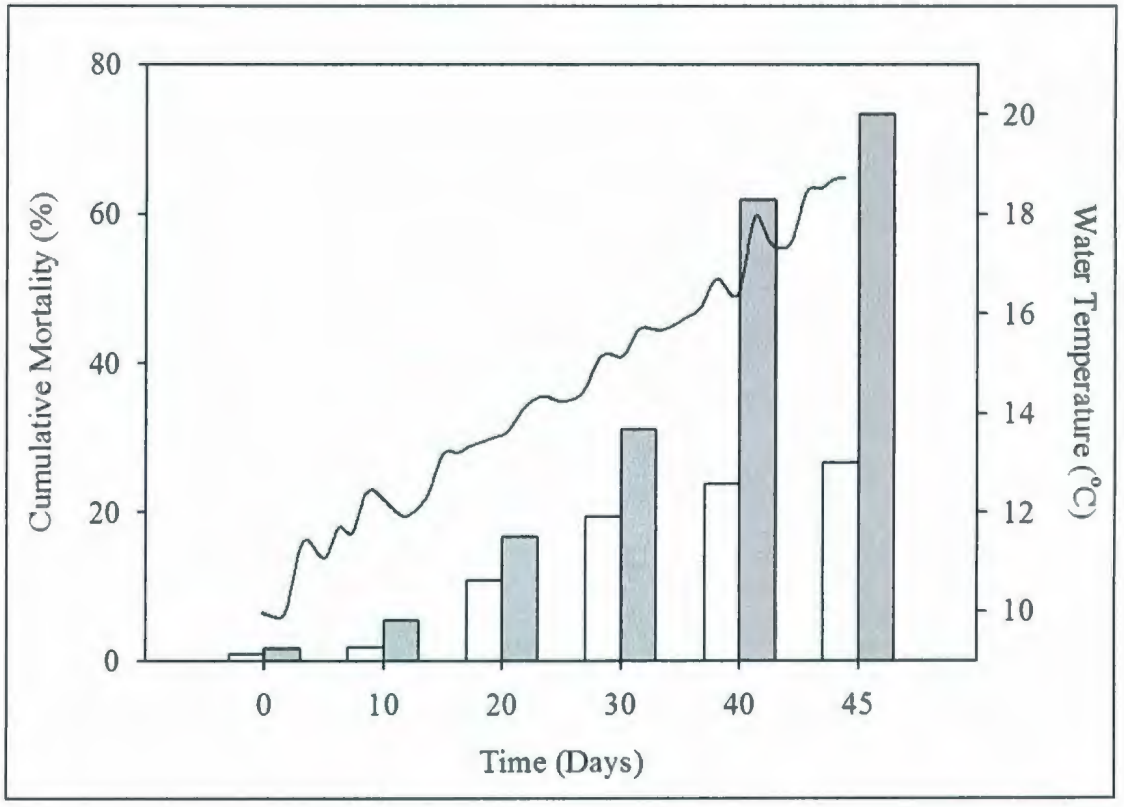


Fig. 5.1. Cumulative mortality of juvenile Atlantic cod subjected to a regimen of chronically increasing temperature (1 °C every 5 days). White columns represent the control group (held at 10 °C throughout the experiment) and the gray columns represent the temperature-stressed group. Solid line represents the water temperature during the experiment.

5.4.4. Expression of Immune-Related Genes

No significant ($P > 0.05$) changes in gene expression were seen in the control group over the duration of the experiment. In contrast, changes in the expression of four of the 5 immune-related genes studied by QRTPCR were detected in TS fish. IL-1β mRNA was elevated slightly (by 5.6-fold) at 16 °C, and greatly elevated at 19 °C (approx. 25-fold; Fig 4A) relative to time controls (i.e. cod held at 10 °C and sampled at the same time as the temperature-stressed cod). The expression of B_2-M (Fig 5.4B), MHC Class I (Fig 4C) and IgM-L (Fig 5.4D) followed a biphasic pattern similar to that seen for total plasma cortisol. For B_2-M and IgM-L, although significant increases in expression were seen at 16 °C (4.3 and 16.9-fold, respectively, relative to time controls), mRNA levels had returned to, or moved back towards, control and initial levels by 19 °C. The expression of MHC class I was significantly elevated at both 16 and 19 °C (by 5.3 and 4.7-fold, respectively) as compared with time control fish. Interestingly, although temperature had no effect on IgM-H expression in the TS fish, this group had consistently lower IgM-H mRNA levels as compared with control fish (Fig. 5.4E).

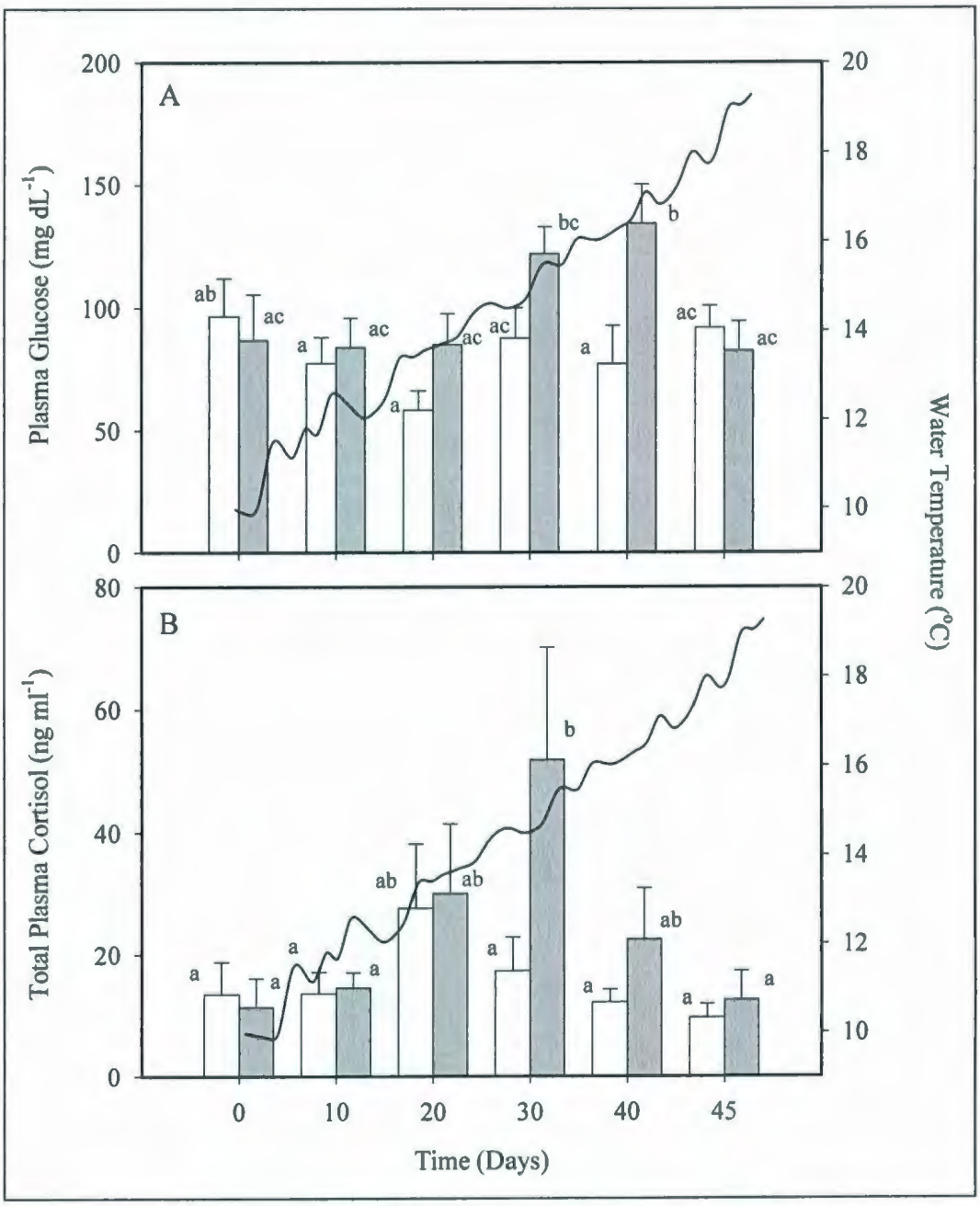


Fig. 5.2. Plasma glucose (A) and total plasma cortisol (B) concentrations in juvenile Atlantic cod subjected to a regimen of chronically increasing temperature (1 °C every 5 days). White columns represent the control group (held at 10 °C throughout the experiment) and the gray columns represent the temperature-stressed group. Solid line represents the water temperature during the experiment. Dissimilar letters indicate values within the control andtemperature stressed groups that were significantly (RM-ANOVA $P < 0.05$) different from each other. Values are means ± standard error (n = 3).

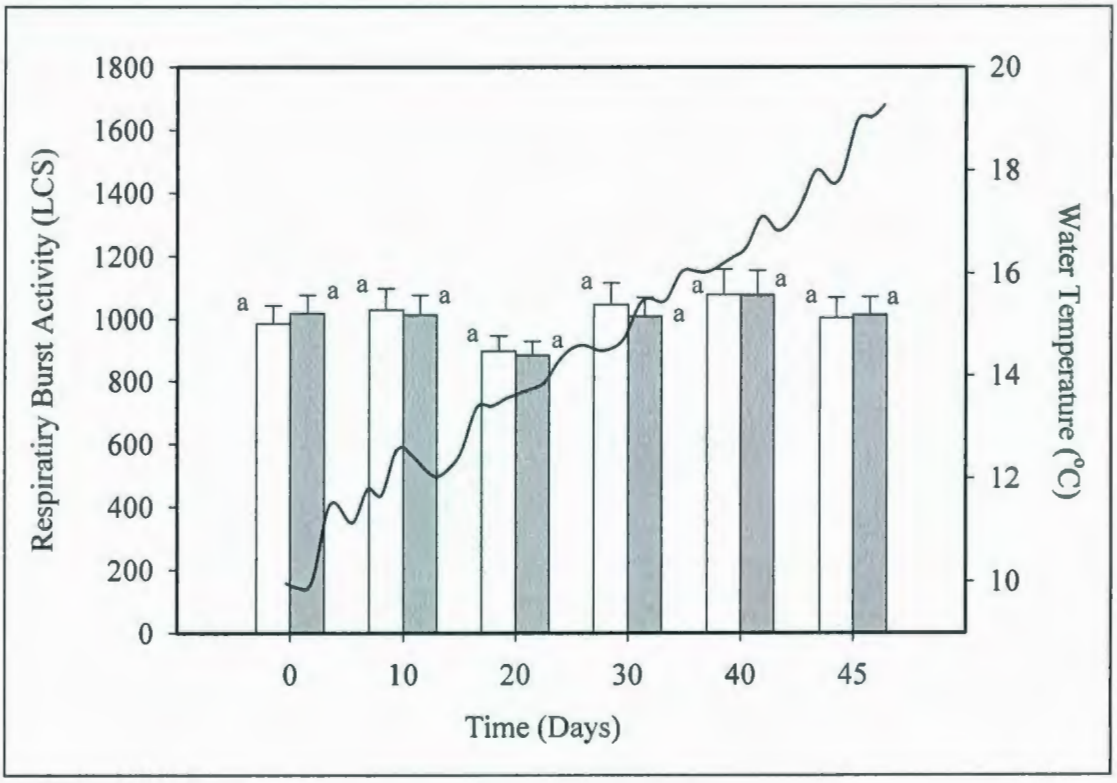
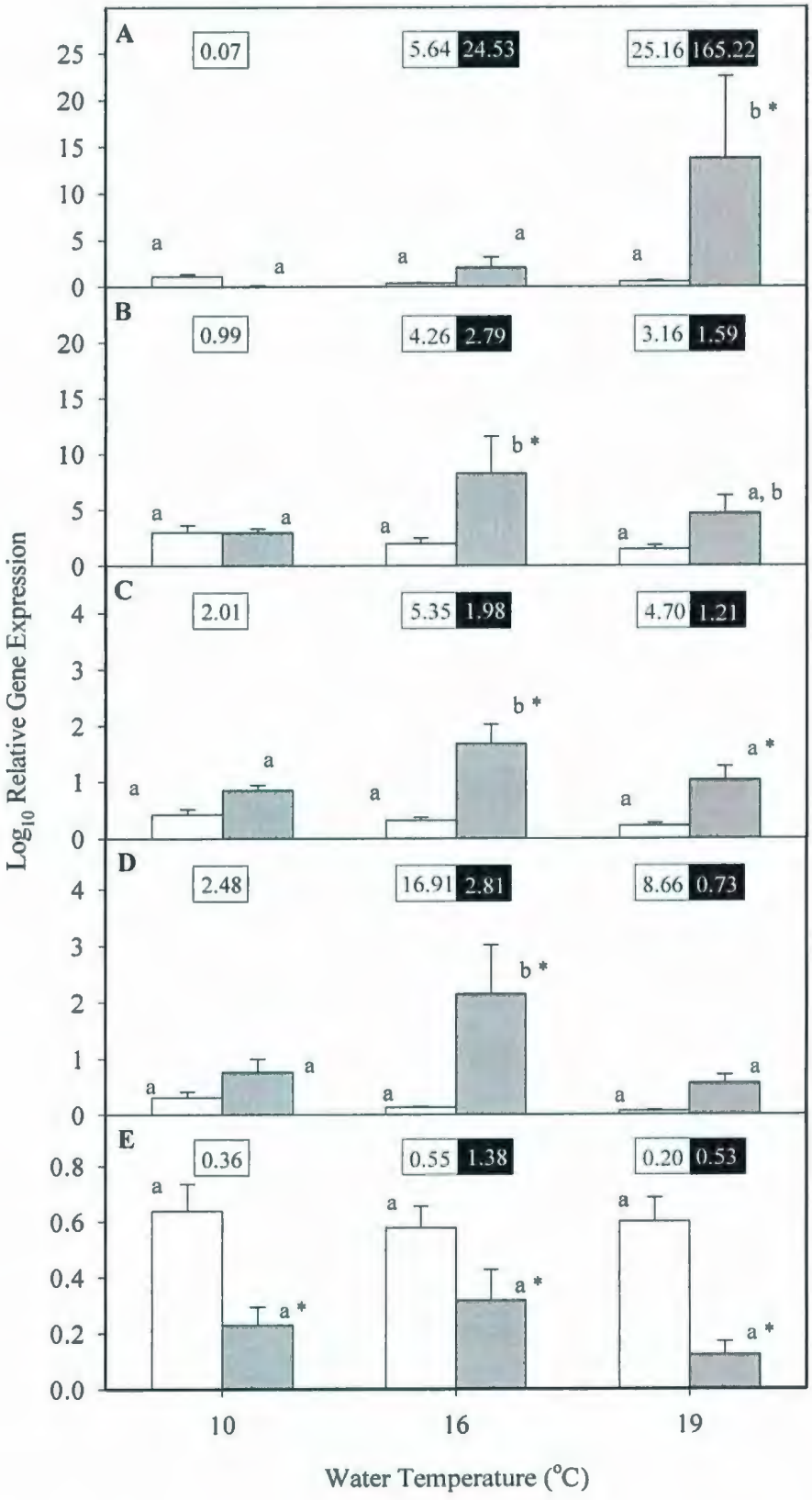


Fig. 5.3. Respiratory burst activity (luminescence counts per second, LCS) of blood leukocytes of juvenile Atlantic cod subjected to a regimen of chronically increasing temperature (1 °C every 5 days). White columns represent the control group (held at 10 °C throughout the experiment), while the gray columns represent the temperature stressed group. Solid line represents the water temperature during the experiment. There were no significant differences in LCS across time (temperatures) or between groups at a specific temperature (RM-ANOVA $P > 0.05$). Values are means ± standard error (n = 3).


A
0.07
5.64
24.53
25.16
165.22
b *
B
0.99
4.26
2.79
3.16
1.59
b *
a, b
C
2.01
5.35
1.98
4.70
1.21
b *
a *
D
2.48
16.91
2.81
8.66
0.73
b *
E
0.36
0.55
1.38
0.20
0.53
a *
a *
Log10 Relative Gene Expression
Water Temperature (°C)
10
16
19

5.5 Discussion

In this study, I used a chronic regimen of steadily increasing temperature to mimic seasonal (spring to summer) changes in water temperature observed at cod cage-sites in Newfoundland. Cumulative mortality in the experimental group increased with water temperature, reaching 31.2% at 16 °C and 73.3% by the time the experiment was terminated (at ~ 19 °C)(Fig. 1). Although ~ 27% of the fish in the experimental group were still alive at 19 °C, I chose to terminate the experiment at this temperature because of ethical concerns relating to undue stress on the fish, and because the aim of the study was not to determine the average CTM for these cod. The CTM of Atlantic cod when exposed to an acute thermal challenge (2 °C h^{-1}) has been found to range from 22 – 24 °C (Sartoris *et al.*, 2003; Gollock *et al.*, 2006; Pérez-Casanova *et al.*, 2008), Gamperl and Canada unpublished). Thus, although 19 °C is an underestimate of the cod's CTM when exposed to a chronic regimen of steadily increasing temperatures, the data suggest that the upper thermal tolerance for Newfoundland cod exposed to summer temperatures is lower than their acute CTM. This result may seem surprising given that the upper thermal tolerance of most fishes is highly dependent upon acclimation temperature (Beitinger *et al.*, 2000). However, the Atlantic cod exposed to the chronic regimen of increasing temperature were above their upper critical temperature [~ 16 °C: (Sartoris *et al.*, 2003; Lannig *et al.*, 2004; Gollock *et al.*, 2006; Pérez-Casanova *et al.*, 2008)] for at least 10 days before termination of the experiment, and they had stopped feeding at 38 days into the experiment (at ~17 °C).

In the present study, total plasma cortisol was significantly elevated at 16 °C (51.84 ± 18.30 ng ml^{-1}), but then decreased to baseline levels thereafter. The elevation of cortisol levels at 16 °C is consistent with the results of Perez-Casanova *et al.* (2008; Chapter 4) who challenged cod of two different size classes (~ 10 and 50g) with an acute thermal stress (2 °C h^{-1}) until their CTM. Thus, in combination, these two studies suggest that 16 °C is stressful for Atlantic cod, and that this effect is independent of the rate of temperature increase. The maximum cortisol values measured in this study are also in agreement with most other studies on cod and other fish species. For example, Hosoya *et al.* (2007) measured total plasma cortisol levels of 42 ng ml^{-1} in juvenile haddock (*Melanogrammus aeglefinus*) subjected to long-term handling stress, Afonso *et al.* (2008) reported total plasma cortisol levels of 40 – 50 ng ml^{-1} when juvenile haddock were exposed to handling and heat shock, and King V *et al.* (2006) found that post-air exposure cortisol levels in cod and haddock acclimated to temperatures from 4 – 14 °C ranged from 60-70 and 30-40 ng ml^{-1}, respectively. Furthermore, in rainbow trout, Basu *et al.* (2001) reported levels of plasma cortisol between 40 -50 ng ml^{-1} following heat shock. Our results, however, are in contrast with those described by Perez-Casanova *et al.* (2008). These authors report that cortisol levels in cod exposed to acute thermal stress (2 °C h^{-1} until CTM) were highest at 22 °C, and that cortisol levels were between 4 and 9 fold higher than determined in the present study (195.06 ± 35.08 ng ml^{-1} and 448.58 ± 64.77 ng mL^{-1} in 10 g and 50 g fish, respectively). This difference in the cortisol response of Atlantic cod subjected to acute vs. chronic thermal challenges is likely due to a negative-feedback effect of chronically elevated cortisol on the hypothalmus-pituitary interrenal (HPI) axis (Pickering and Pottinger, 1987; Haukenes and Barton, 2004). However, it cannot be

ruled out that the cod in the current study became desensitized to the chronic stressor (elevated temperature) or that corticosteroid clearance rates increased over the duration of the experiment (Redding *et al.*, 1984; Barton *et al.*, 1987).

In the present study, plasma glucose values for both the control and the stressed groups were highly variable and only significantly different in the experimental group at 16 and 18 °C (Figure 2A). Although changes in plasma glucose have been used as an indicator of stress in fish (Barton and Iwama, 1991), previous studies carried out on gadids have yielded disparate results. For example, Perez-Casanova *et al.* (2008) reported no significant changes in plasma glucose in 50 g Atlantic cod exposed to acute thermal changes in water temperature from 10 to 24 °C, and Hosoya *et al.* (2007) reported highly variable levels of plasma glucose in juvenile haddock exposed to long-term handling stress. On the other hand, King V *et al.* (2006) reported significantly elevated plasma glucose levels in Atlantic cod and haddock when given an acute air exposure. The variability observed between these studies may be due to differences in methodology, the type of stressor, and or the size/age of the fish. However, when all the present data on plasma glucose in gadids are considered, it appears that plasma glucose is not a reliable indicator of stress in this taxon.

In this study, I measured the zymosan-induced respiratory burst of Atlantic cod leukocytes by means of luminol-enhanced chemiluminescence, and found no significant differences between control and temperature-stressed fish, or as compared to initial (10 °C) values. This lack of change in respiratory burst (RB) with increasing ambient temperature is in contrast to data on other fish species. For example, RB increased with acclimation temperature (5 - 20 °C) in rainbow trout (Nikoskelainen *et al.*, 2004). Verma *et al.* (2007) report decreased RB in carp fingerlings warmed from

their acclimation temperature (26 °C) to 31, 33 and 36 °C and held at these new temperatures for 4 weeks. Finally, Nadong *et al.* (2007) showed that RB decreased when 27 °C acclimated Nile tilapia were exposed to 31 and 35 °C over 24 and 96 h, respectively. The lack of change in the RB of Atlantic cod with increasing temperature is interesting, and may be another way, in addition to some aspects of specific immunity (Israelson *et al.*, 1991; Pilström and Petersson, 1991; Pilström *et al.*, 2005; Lund *et al.*, 2006; Lund *et al.*, 2007) and the absence of a heat shock protein response to temperature stress (Zakhartsev *et al.*, 2005; Afonso *et al.*, 2008; Pérez-Casanova *et al.*, 2008), in which gadids appear to differ from the generalized model for teleosts. However, I cannot rule out alternative explanations as to why RB was unaffected by the imposed regimen of increasing temperatures. First, RB may have remained relatively constant because temperature-dependent effects on RB were offset by those of elevated cortisol. Increased plasma cortisol levels can have a negative influence on phagocytic activity (Yin *et al.*, 1995; Law *et al.*, 2001; Esteban *et al.*, 2004), and it has been suggested that the negative effects of cortisol on phagocytic activity are long lasting (Yin *et al.*, 1995). Second, it is plausible that the blood phagocytes of both experimental groups were activated by the microplates used in the RB assay (Nikoskelainen *et al.*, 2006), thus eliminating any potential differences in RB between groups or temperatures, or that assaying RB at a common temperature (~20 °C) influenced the peak RB values measured in cod collected at the different temperatures. Although, the latter is unlikely as Nikoskelainen *et al.* (2004; see their Fig. 4A) showed that there is little difference in peak RB values when RB from trout acclimated to temperatures between 10 and 20 °C are assayed over the same temperature range.

In this study, it was found that the effect of increasing temperature on the mRNA expression of 5 immune related genes produced by blood leukocytes was quite variable. With regards to cytokine IL-1β mRNA, although no significant increase in expression was seen at 16 °C, a 25 fold increase was observed relative to the time control group when water temperature reached 19 °C (Fig. 5.4A). This increase in IL-1β mRNA observed in heat-stressed cod in this study is consistent with data obtained for mammals which suggests that cytokines (including IL-1β) play important physiological and immunological roles during/following prolonged heat exposure and other stressors. For example, elevated expression of IL-1β (by ~ 1.7-fold) was reported in the brain of rats during thermal stress (Michel *et al.*, 2007) and elevated plasma concentrations of IL-1β (by ~ 3-fold) were reported in mice during recovery from thermal stress (Leon, 2006). Elevated IL-1β plasma levels (by ~ 13 fold) are thought to contribute to morbidity/mortality observed during heat stroke in rats (Lin *et al.*, 1997). Finally, increases (2-3-fold) in IL-1β mRNA levels were reported in several tissues including hypothalamus, hippocampus, pituitary, and spleen when rats were exposed to inescapable tail shock (O'Connor *et al.*, 2003), and in the preoptic area of rats (2.5 fold) exposed to cold shock (Tanabe *et al.*, 2000). With regards to other fish species Metz *et al.* (2006) detected a significant increase (2.0-fold) in the expression of IL-1β in common carp head kidney after a 24 h restraining stress, as well as in other non-immune tissues such as the pituitary (~3.5-fold) and the nucleus preopticus (~2.0-fold) of the hypothalamus. Further, daily handling stress (during 30 days), increases the expression of IL-1β by Atlantic salmon head kidney macrophages after 1, 2 and 4 weeks (Fast *et al.*, 2006). However, the magnitude of the increase in response to the present chronic temperature stressor was much greater than seen in these other studies.

It is possible that this difference is tissue-related (i.e. that the response of blood leukocytes to stressors may be enhanced as compared to other immune or non-immune tissues) or that increases in the expression of this cytokine's mRNA are highly dependent upon species and the nature of the stressor. However, whether one or more of these potential explanations is correct awaits further study.

The stress response in fishes involves the activation of the sympathetic nervous system and the (HPI) axis [the equivalent of the hypothalamus-pituitary-adrenocortical (HPA) axis in mammals], and it has recently been established that there is bidirectional communication between the fish's HPI axis and its immune system (Engelsma *et al.*, 2002; Holland *et al.*, 2002; Metz *et al.*, 2006). Thus, based on this, and the fact that cortisol has been shown to inhibit IL-1β expression in both in common carp (Engelsma *et al.*, 2001) and rainbow trout (Zou *et al.*, 2000), one might speculate that IL-1β expression was suppressed by the elevation in cortisol levels at 16 °C, and then increased dramatically at 19 °C because circulating cortisol levels returned to control (and pre-stress) levels. However, it is unclear whether the maximum cortisol levels measured in the cod could have suppressed IL-1β expression because cortisol effects on this gene appear to be dose-, tissue- and species-dependent in fishes. For example, cortisol (circulating level 36 ng ml^{-1}) did not suppress lipopolysaccharide (LPS)-induced IL-1β expression in head kidney leukocytes in the common carp (Engelsma *et al.*, 2001), whereas 29 and 52% reductions were observed in the rainbow trout at 100 and 320 ng ml^{-1}, respectively (Zou *et al.*, 2000). Further, while Engelsma *et al.* (2001) showed that exposure to 36 ng ml^{-1} could suppress the constitutive expression of IL-1β transcript by carp kidney, Metz *et al.* (2006) showed that IL-1β expression in head

kidney was elevated two fold when carp were restrained for 24 h (cortisol levels ~ 56 ng ml^{-1}).

In contrast to IL-1β, the expression of β_2-M, MHC and IgM-L mRNA in the temperature stressed group showed a pattern of change very similar to that of cortisol: significant increases (4 - 17 fold relative to time controls) at 16 °C, but a return to, or towards, pre-stress (10 °C) levels by 19 °C. It is difficult to interpret the data for β_2-M and MHC Class I because, to our knowledge, only one study has investigated the effects of temperature (Rodrigues *et al.*, 1998), and no studies have examined the effect of stress (elevated cortisol levels), on the expression of these two immune-related genes in fishes. Further, the pattern of change in blood leukocyte IgM-L expression (increase, then return to basal levels) can be explained by both a modulatory effect of cortisol and the range of temperatures to which the cod were exposed. For example, Dominguez *et al.* (2004) showed that in Nile tilapia acclimated to temperatures of 18.4 (control), 23, 28 and 33 °C, plasma IgM was elevated at 28 °C but had returned to control levels by 33 °C, and suggested that fish may have an optimal thermal range for immune function. Cuesta *et al.* (2006) showed that intraperitoneal cortisol implants, which produced levels of circulating cortisol (50–70 ng ml^{-1}) similar to those measured in the present study, resulted in a 30% increase in circulating IgM levels in the gilthead seabream. Suzuki *et al.* (1996) report that plasma IgM levels in goldfish (*Carassius auratus*) closely mirror the seasonal temperature cycle (5-25 °C). Finally, Saha *et al.* (Saha *et al.*, 2002) showed that plasma IgM levels in male carp were maximal at a temperature (~ 23 °C) intermediate between seasonal minimum (10 °C) and maximum (30 °C) temperatures, and that cortisol was at its highest values (70 ng ml^{-1}) at this time.

In contrast to the other four immune-relevant genes involved in the QPCR study, no significant heat-induced change in gene expression was seen for IgM-H. Further, the expression of IgM-H mRNA in the temperature-stressed group was lower relative to the control group at all temperatures. The lack of a significant change in IgM-H mRNA in response to heat stress is interesting given that the expression of IgM-L was significantly elevated at 16 °C. However, at this point, I have no explanation for this discrepancy in results. With regards to the lower IgM-H mRNA expression levels at 10 °C in the experimental group, tank effects (e.g. changes in tank conditions before the first sampling point) cannot be ruled out as potential contributors to the difference in expression between the control and temperature stressed fish. However, I believe that the observed difference in IgM-H expression between blood samples from fish in the control tanks (2 fish from each triplicate tank, n=6 total) and blood samples from fish in the experimental tanks (2 fish from each triplicate tank, n=6 total) may also reflect random sampling error arising from the relatively small numbers of biological replicates used in this experiment. If this experiment were to be repeated, it might be useful to: 1) use data loggers to constantly monitor water conditions in all tanks, potentially enabling identification of water quality changes between tanks that could influence gene expression data; and 2) increase the numbers of biological replicates used in the study (e.g. to 4 fish from each triplicate tank, n=12 total), decreasing the potential influence of random sampling error on the gene expression results.

For all five genes involved in the QPCR study, gene expression of the fish from the temperature stressed group (i.e. triplicate tanks dedicated to the "temperature stress" component of the experiment) at 16 and 19 °C was compared with that of fish from the same group at 10 °C (i.e. the same triplicate tanks dedicated to the "temperature stress" component of the experiment, but prior to temperature increase) (see Fig. 5.4). These comparisons of blood gene expression levels in 10 °C, 16 °C, and 19 °C fish sampled from the triplicate tanks dedicated to the temperature stress experiment clearly show that four genes (interleukin-1beta, beta2-microglobulin, MHCI, and IgM-L) responded to the heat stress, whereas one gene (IgM-H) did not respond to the heat stress. Due to the difference in IgM-H expression in the "unstressed" tanks versus the "temperature stress" tanks at 10 °C, it is likely that the 10 °C fish from the "temperature stress" set of replicate tanks are the best controls for the heat stress QPCR data.

In this study, I measured a variety of immune parameters in cod, and showed that while respiratory burst and blood leukocyte IgM-H gene expression did not change when this species was challenged with an increasing temperature regimen mimicking cage-site conditions, the expression of other immune-related genes either: 1) increased dramatically at temperatures near the cod's upper thermal limits; or 2) showed a biphasic response that could be explained by cortisol modulatory effects or the presence of an optimal temperature for immune function in this species. These are very intriguing results which further highlight the complexity of fish immune responses to environmental challenges (Tort *et al.*, 2004a), and suggest that the high mortality experienced at some cod cage-sites at temperatures above 16 °C is not

directly related to compromised immune function. However, the latter hypothesis should be viewed cautiously, and only used as a basis for further investigation. This is because we only measured the mRNA expression of immune genes in blood leukocytes, and temperature effects on the circulating level and function of the proteins for which these genes code, and on other important immune tissues, could be different. Further, we do not know whether changes in gene expression were due to an increase in transcription per leukocyte, a change in the number of leukocytes, and/or alterations in the proportion of the various classes of leukocytes. The latter two responses have been shown by fish to elevated temperatures (Carlson *et al.*, 1995; Houston *et al.*, 1996; Langston *et al.*, 2002), and the exact cause of the increase in gene expression has important implications for fish health.

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Chapter 6. Summary

The goal of my doctoral research was to investigate the effects of high and low temperatures on several aspects of Atlantic cod and haddock biology/physiology, and ultimately to improve the growth and survival of farmed Atlantic cod and haddock during sea-cage rearing. Thus I: 1) explored whether feeding low protein diets at cold temperatures (2 °C) would decrease the metabolic cost of digestion and the time required for digestion/gastric evacuation, thus leaving more available energy for growth and potentially improving the fish's food consumption and gross conversion efficiency; and 2) determined the upper critical temperature for juvenile Atlantic cod, and studied the effects of high water temperatures on aspects of the stress response and immune function of this species.

My results show that low water temperatures (2 °C) significantly reduce food consumption, growth rate, gross conversion efficiency and absolute SDA (mg O_2) in juveniles of both species, and provide a number of important insights with regards to the metabolic and digestive physiology of both haddock and Atlantic cod. For example, gastric evacuation is prolonged greatly at 2 °C, and this very likely contributes to the reduced food consumption of both species at cold temperatures. Feeding low protein diets in the winter is unlikely to be of benefit to gadoid culture operations, as any cost savings associated with the use of low protein diet formulations would be negated by the 20% lower conversion efficiency associated with feeding a low protein diet. The lower routine metabolic rate, greater food consumption and faster gastric evacuation of juvenile haddock as compared to Atlantic cod largely explain their enhanced growth rate. Finally, it appears that measurements of post-feeding

oxygen consumption in individually confined fish underestimates the duration of SDA under aquaculture conditions (e.g. groups of fish held in cages).

With regards to the effects of high temperature, my research identified oxygen consumption and changes in plasma total cortisol levels as reliable indicators of thermal stress in Atlantic cod and showed that although juvenile Atlantic cod are very sensitive to acute variations in water temperature (as demonstrated by their upper critical temperature of 16 °C) they can tolerate short-term exposure to temperatures above 20 °C. Moreover, it was revealed that the stress response differs in Atlantic cod exposed to acute vs. chronic increases in temperature (based on differences in the magnitude and pattern of changes in plasma cortisol), that immune function during chronic heat stress is influenced by complex interactions between thermal effects and temperature-induced stress, and that not all immune-related parameters are affect by temperature. For example, interleukin 1β mRNA expression in the Atlantic cod's blood increased dramatically (by approx. 25 fold) at temperatures close to this species upper thermal limit, whereas β-2-microglobulin, MHC class 1 and IgM-L expression showed a biphasic pattern with temperature similar to what was seen for plasma cortisol (increasing until 16 °C, then decreasing), and respiratory burst activity and IgM-H expression were unaffected by steadily increasing water temperatures. Some of these findings contrast with what has been shown for other teleosts, and challenge the commonly held belief (i.e. 'dogma') that stress/elevated cortisol levels are always associated with immunosuppression in fishes. Further, they illustrate that we have a considerable way to go before we understand why large mortality events associated with summer temperatures (e.g. as occurred at Turnip Cove, NL in 2003) occur. Although my research suggests that basal (constitutive) immune function is not

negatively impacted by exposure to elevated temperatures up to 19 °C, there is presently no data on how high temperatures influence the capacity of Atlantic cod to mount an immune defence against viral (e.g. nodavirus) or bacterial (e.g. *Aeromonas salmonicida*; which causes the disease furunculosis) pathogens, or parasites (e.g. *Loma morhua*). Further, our interpretation of the effects of temperature on stress proteins (no change with temperature) and immune function are based on results obtained with antibodies developed for mammals or other fish species, and on the measurement of the constitutive expression for a very limited number of genes, respectively.

Clearly, there is a need to develop more Atlantic cod (gadoid)-specific molecular tools before we have a comprehensive understanding of the effect of temperature on commercially- (and biologically-) important traits, and how the differential responses of Atlantic cod as compared to other teleost species is due to differences in gene sequences and protein structure/function. Fortunately, the Genome Canada funded Cod Broodstock and Genomics Development Project (CGP; www.codgene.ca) has greatly contributed to characterizing the Atlantic cod transcriptome. For example, the CGP has already sequenced 160,000 ESTs, and is in the process of developing a genetic map for Atlantic cod and a 20,000 gene microarray for use in studies of global gene expression. These tools, and those still to be developed, will permit us to specifically examine whether, and to what extent, the expression of anorexigenic and orexigenic genes relates to differences in feed intake between Atlantic cod and haddock, and the loss of appetite with low temperature (see chapter 2). Further, they will allow for the examination of whether IL-1β (and other immune-related proteins) affect smooth muscle tone in Atlantic cod at high temperature ultimately leading to circulatory collapse, and if so through which

mechanisms. The answer to such questions potentially has significant implications to the Atlantic cod (gadoid) aquaculture industry and/or opens up new and exciting avenues for research on Atlantic cod (fish) biology/physiology.