STRESS-RELATED AND ONTOGENETIC ASPECTS OF METABOLIC DEPRESSION IN CUNNER (TAUTOGOLABRUS ADSPERSUS)

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Stress-Related and Ontogenetic Aspects of Metabolic Depression in Cunner (*Tautogolabrus adspersus*)

by

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Abstract

This thesis examined: 1) how ontogeny affects aspects of metabolic depression in the cunner; and 2) whether the stress response of adult cunner is modified during metabolic depression. The effect of decreasing temperature at 2°C per day on the metabolism of three size classes of ~9°C acclimated cunner was studied. The onset-temperature for metabolic depression in cunner decreased with body size (age), i.e. 7°C in adult fish vs. 6°C and 5°C in small and young-of-the-year (YOY) fish, respectively. In contrast, the extent of metabolic depression was ~80% (Q_{10} = ~15) for YOY fish, ~65% (Q_{10} = ~8) for small fish, and ~55% (Q_{10} = ~5) for adults. Stress hormone levels and steroidogenic transcript levels were measured in adult cunner acclimated at 0°C and 10°C. Fish at 10°C had 9.09 fold and 4.77 fold higher post-stress plasma epinephrine and norepinephrine levels, respectively, and 4.67 fold greater resting plasma cortisol levels (~65 ng ml^{-1}) compared with 0°C fish (~10 ng ml^{-1}). However, cortisol levels did not increase post-stress in 10°C fish, whereas 0°C fish had a 'typical' stress response with cortisol levels reaching ~107 ng ml^{-1} by 2 hours post-stress. Transcript levels of P450*scc* were 3.25 fold higher in the head kidney of 10°C fish compared with 0°C fish. However, there was no significant difference in StAR transcript levels in the head kidney of 10°C fish compared with 0°C fish. GR transcript levels were 1.70 fold and 1.29 fold lower in the head kidney and liver, respectively, of 10°C fish compared with 0°C fish.

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List of Abbreviations and Symbols

°C	temperature in degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
b	scaling exponent
b_b	scaling exponent for basal metabolic rate
BLAST	basic local alignment search tool
b_m	scaling exponent for maximal metabolic rate
b_s	scaling exponent for standard metabolic rate
cDNA	complementary deoxyribonucleic acid
cm	centimetre
C_T	threshold cycle
DDT	dithiothreitol
DHBA	3, 4-dihydroxybenzylamine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E	epinephrine
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E-value	expected value
g l^{-1}	gram per litre
g	gram
GaP	genomics and proteomics

GR	glucocorticoid receptor
h	hour
HPI	hypothalamic-pituitary-interrenal
HPLC	high performance liquid chromatography
HSC	hypothalamic-sympathetic-chromaffin-cell
kg	kilogram
l	litre
M	mass or molar
m	metre
m^3	cubic metre
mg	milligram
mg l^{-1}	milligram per litre
mg O_2 kg^{-1} h^{-1}	milligram of oxygen per kilogram per hour
mg O_2 l^{-1} min^{-1}	milligram of oxygen per litre per minute
min	minute
ml min^{-1}	millilitre per minute
ml	millilitre
mm	millimetre
mM	millimolar
M-MLV	moloney murine leukemia virus
MO_2	oxygen consumption
mRNA	messenger ribonucleic acid
MS-222	tricaine methanesulfonate
n	number
NCBI	national center for biotechnology information
NE	norepinephrine
ng ml^{-1}	nanogram per millilitre
ng	nanogram

nM	nanometre
nr	non-redundant
O_2	oxygen
P	probability
P450*scc*	cytochrome P450, family 11, subfamily A, polypeptide 1 (P45011a1, encoded by CYP11A1 gene)
PCR	polymerase chain reaction
Q_{10}	temperature coefficient
QPCR	quantitative reverse transcription-polymerase chain reaction
RMR	resting metabolic rate
RNA	ribonucleic acid
RQ	relative quantity
rRNA	ribosomal ribonucleic acid
S.E.	standard error of the mean
sec	second
StAR	steroidogenic acute regulatory protein
TAE	tri-acetate-EDTA
U	unit
UV	ultraviolet
V	volume
YOY	young-of-the-year

Chapter One

General Introduction

Temperature can be an enormously important environmental variable because of its large influence on the rate of biological reactions and physiological processes (Rome et al., 1992). Although most animals are specialized for life within a relatively narrow range of temperatures, some may experience marked daily and seasonal temperature fluctuations (Rantin et al., 1998; Guderley and St-Pierre, 2002; Rodnick et al., 2004; Schulte, 2007). These temperature fluctuations affect virtually all levels of biological organization, from the rates of molecular diffusion and biochemical reactions, to membrane permeability, to cellular, tissue and organ function, and finally their integration within the whole organism (Guderley and St-Pierre, 2002). In addition, temperature changes may alter the equilibrium between synthesis and degradation of biological structures, change metabolic requirements, favour certain functions over others and alter trophic interactions (Guderley and St-Pierre, 2002).

The effect of temperature on biological processes can be described using the temperature coefficient (Q_{10}), which is the fold change in the rate of a particular biological process for a 10°C change in temperature (Rome et al., 1992). Chemical and biological processes are extremely temperature sensitive and have Q_{10}s that generally range from 1.8 to 4 (Rome et al., 1992). Given the large impact that temperature has on various functions, many animals regulate their body temperature to cope with the effects of thermal

fluctuations. However, the degree of regulation varies with the animal's thermoregulatory strategy.

1.1. Thermoregulation

Thermoregulation is the ability of an organism to maintain its body temperature within certain boundaries, independent of the temperature of the environment. The thermoregulatory mode of animals can generally be classified into two main categories: 1) *Endotherms* (mainly mammals and birds) produce metabolic heat at high rates, and have a low but variable thermal conductance, and this allows them to regulate their body temperature within relatively narrow limits; and 2) *Ectotherms* (reptiles, amphibians and fish) have body temperatures that vary, often matching the temperature of the immediate environment. They also produce metabolic heat, but at considerably lower rates and have high thermal conductivity (Guderley and St-Pierre, 2002; Guderley, 2004).

Endotherms maintain a stable internal body temperature, which is normally higher than the immediate environment, and they are often referred to as homeotherms (Reynolds, 1979). However, not all homeotherms are endotherms. For example, the body temperature of many Antarctic fish species is relatively constant due to the absence of significant thermal variation in their habitat (Guderley, 2004). In contrast, the majority of ectotherms are considered poikilothermic organisms, meaning that their body temperature is primarily determined by the heat gained from, or lost to, their

environment. For example, aquatic ectotherms generally have body temperatures identical to that of water (Guderley and St-Pierre, 2002) because of the high heat capacity of water. In addition, the high surface area of the gills and the arrangement of the blood vessels in fish, lead to thermal equilibrium with the environment, and oblige their body temperature to conform to habitat temperature (Guderley, 2004; Crockett and Londraville, 2006).

Temperatures in the aquatic environment do not differ to the same extent as in terrestrial habitats. However, temperatures at the surface of the ocean range from 30°C in tropical areas to -2°C in the polar seas, they can exceed 40°C in tropical tidepools (Myers, 1991; Taylor et al., 2005; Eme and Bennett, 2009) and seasonal temperature fluctuations in temperate zones can be significant (e.g. from -1°C to approximately 16°C in Logy Bay, Newfoundland, Canada). Just as the aquatic habitats vary in maximum/minimum temperatures, and in the degree of temperature fluctuation, fish inhabiting these environments also vary in their cold and warm thermal tolerance (Guderley, 2004).

1.2. Strategies to cope with thermal fluctuations

The adaptive response of animals to thermal fluctuations can be broadly classified into two categories: 1) physiological responses, this is when an organism adjusts its physiology to reach a new steady state; and 2) behavioural responses, where animals that are not able to cope with the

physiological stress associated with thermal fluctuations alter their behaviour or remove themselves from the unfavourable environment (e.g. by migrating). Further, to cope with particular environmental stressors, some animals utilize a combination of behavioural and physiological adaptations (Storey, 2001).

When encountering reduced environmental temperatures (e.g. seasonal cooling), non-migratory fish can adjust their physiology in a number of ways (Guderley and St-Pierre, 2002; Guderley, 2004). For example, they can:

1) Submit to the Q_{10} effects, and let their physiological and biochemical processes slow.

2) Use compensatory mechanisms to offset the Q_{10} effects, and thus, retain function/capacity.

3) Enhance the Q_{10} effect by reducing metabolism/physiological processes to a greater extent than what would be considered normal for the change in temperature. This phenomenon is referred to as *metabolic depression*.

1.3. Metabolic depression

Metabolic depression is a reduction in metabolic rate to below normal resting values. It has been recorded in all major animal phyla as a response to environmental stresses including temperature, desiccation (lack of water), hypoxia/anoxia (lack of oxygen), hypersalinity, and food deprivation (Storey and Storey, 1990; Guppy et al., 1994; Hand and Hardewig, 1996; Guppy and

Withers, 1999). Although the triggers stimulating entry into a state of active metabolic depression are many, the effects are similar. Various physical processes (e.g., feeding, movement) are greatly curtailed or stopped, physiological functions (e.g., heart beat, breathing, or ventilation rate) are reduced, non-essential cellular functions (e.g., anabolism and growth) are reduced or suspended, and essential metabolic functions are brought into a new balance (Storey, 1988).

The purpose of metabolic depression is to maximize the survival time of an individual when environmental conditions are unfavourable for normal life (Storey and Storey, 1990). The survival time of an organism during exposure to environmental stresses that limit energy availability is, in general, directly related to the degree of metabolic depression achieved (Hand and Hardewig, 1996). The extent of metabolic depression can vary from 80% of resting metabolic rate to complete absence of measurable metabolism (Storey and Storey, 1990; Guppy et al., 1994; Hand and Hardewig, 1996; Guppy and Withers, 1999; Storey and Storey, 2004).

Metabolic depression is associated with the coordinated inactivation of many cellular processes. One strategy to achieve metabolic depression is a decrease in the generation and utilization of adenosine tri-phosphate (ATP) (Storey, 1988; Storey and Storey, 1990). Cells are capable of achieving this new energetic balance by switching to efficient pathways of energy production (e.g. carbohydrate degradation) and decreasing energy consuming processes (e.g. ion pumping and protein synthesis/degradation) (Guppy et al., 1994; Boutilier, 2001; Storey and Storey, 2004). The first known observations of

metabolic depression were made in the pioneering microscopical work of Van Leeuwenhoek 1702 (cited *in* Guppy, 2004), who identified small desiccated animals (rotifers) in dry sediments that were dormant, but resumed normal activities when rehydrated; a phenomenon called cryptobiosis.

1.4. Metabolic depression in fish

According to Crawshaw (1984) the overwintering response of fish can be broadly categorized into two ecological groups: 1) fish that acclimate biochemically and remain active during the winter (e.g. *Salmonidae*); and 2) fish that are inactive and hidden during the winter, and are assumed to be dormant (e.g. warm water families such as *Centrarchidae* and *Ictaluridae*). The term 'dormancy' is used to describe a behaviourally inactive state characterized by the cessation of feeding and activity (Crawshaw, 1984). However, dormancy is not necessarily associated with metabolic depression (i.e. a regulated decrease in cellular metabolism). For example, although Crawshaw et al. (1982) observed dormancy in the largemouth bass *(Micropeterus salmoides)* and the brown bullhead *(Ictalurus nebulosus)* in response to low temperature, and both species showed decreased metabolic rates, there was no abrupt change in this parameter as temperature fell. In contrast, the American eel *(Anguilla rostrata)*, another teleost that exhibits winter dormancy, has a Q_{10} for metabolic rate of 4.10 between 10°C and 5°C (Walsh et al, 1983). Lungfish, which aestivate to avoid desiccation, reduce oxygen consumption by 85%, and significantly decrease breathing, heart rate

and blood pressure (Guppy et al., 1994). Moreover, the European goldsinny wrasse *(Ctenolabrus rupestris),* a marine teleost that spends winter in a dormant state, shows large decreases in mean oxygen uptake, opercular and heart rates (Q_{10} = 542.01, 6.39 and 24.52, respectively) when acutely exposed to a drop in temperature from 6°C to 4°C (Sayer and Davenport, 1996).

1.5. The cunner

Studies on metabolic depression in fish have focused on decreases in metabolism associated with shortages of water (desiccation) and oxygen (hypoxia/anoxia), or a combination of low oxygen and temperature (Smith, 1930; Van Waversveld et al., 1989; Johansson et al., 1995; Muusze et al., 1998; Nilsson and Renshaw, 2004). In contrast, only a few studies have examined aspects of cold-induced metabolic depression (Walsh et al., 1983; Crawshaw, 1984; Sayer and Davenport, 1996). However, it has recently become clear that the cunner is a species capable of metabolic depression, and that it is a convenient and valuable model for studying various aspects of cold-induced metabolic depression in fish.

1.5.1. Ecology and life history

The cunner (*Tautogolabrus adspersus*, Figure 1.1), a member of the mainly tropical family Labridae (the wrasses), and is distributed along the western Atlantic from Chesapeake Bay, USA to Newfoundland, Canada - their

most northern distribution (Bigelow and Schroder, 1953; Scott and Scott; 1988). Along the coast of Newfoundland, cunner occupy small home ranges and return to them after displacement (Green, 1975). Cunners are omnivorous feeders that consume a wide variety of molluscs, crustaceans and other benthic invertebrates (Bigelow and Schroder, 1953; Scott and Scott; 1988; Green et al., 1984). The spawning season extends from June to August, and the buoyant eggs hatch in about 40 hours at ~21°C and 3 days at ~12-18°C (Bigelow and Schroder, 1953). Cunners reach sexual maturity at 8 - 11 cm in length and grow up to 43 cm long with a mass of 1.4 kg (Scott and Scott; 1988). In Newfoundland, adult fish ranging from ~14 to 27 cm long have been found, and these were between 3 and 10 years of age (Naidu; 1966). This species is active during the day but becomes quiescent at night (Dew, 1976), and remains inshore throughout the year rather than retreating to deeper warmer waters as other coastal Newfoundland fish species (Green and Farwell, 1971).

During the winter, the cunner exhibits winter dormancy in response to the low environmental temperatures of its habitat (Green and Farwell, 1971; Dew, 1976). Green and Farwell (1971) reported that cunner enter a dormant state in late fall/early winter when ocean temperatures fall to 5°C, and arose from this state in the spring when temperatures reach a similar level. Further, these authors reported no cunner mortality in the field or in the laboratory at temperatures below 0°C

1.5.2. Metabolic depression

The first study on cunner metabolism was in 1943, when Haugaard and Irving showed that the metabolic rate of winter (5°C) acclimated cunner was about one fourth of the metabolic rate of summer (20°C - 21°C) acclimated fish, a Q_{10} of about 2.50. However, more recent studies have shown that the cunner is capable of cold-induced metabolic depression. Curran (1992) reported that when seawater temperatures fall from 12°C to 6°C, cunner from Woods Hole (Massachusetts, USA) actively deceased their routine metabolic rate (Q_{10} = 8.50), but maintained a constant metabolic rate as temperature fell further (from 6°C to 3°C). Costa (2007) reported acute and seasonal Q_{10} values for metabolic rate of 8 - 10 between 5°C and 0°C for cunner from Newfoundland. Lewis and Driedzic (2007) showed that protein synthesis in several tissues of Newfoundland cunner decreased by ≥ 65% when seasonal temperature fell from 8°C and 0°C. Finally, Corkum and Gamperl (2009) showed that the ability of cunner to depress their metabolism at cold temperatures (1°C) enhanced their hypoxia tolerance, and that metabolic depression in this species is dependent on other environmental variables such as water oxygen level and photoperiod.

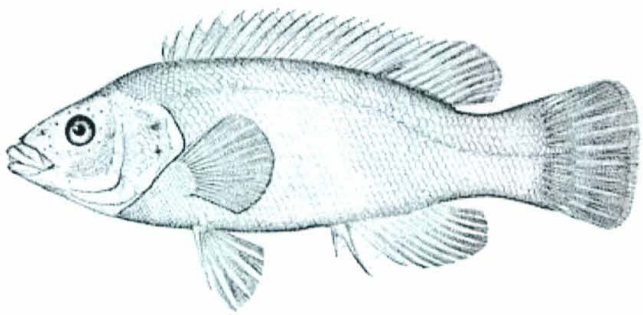

Figure 1.1 – The cunner, *Tautogolabrus adspersus*. Source: http://www.nefsc.noaa.gov.

1.6. Study objectives

Given the above, the goal of this study was to use the cunner to answer two important questions about metabolic depression in fishes: 1) do the extent (degree) of cold-induced metabolic depression, and the temperature at which metabolic depression is initiated, vary with body size (ontogeny)?; and 2) is the stress response (a phenomenon that leads to substantial increases in energy demands) of cunner modified during metabolic depression and what mechanisms might be involved? This latter question was addressed by measuring resting and post-stress plasma cortisol and catecholamine levels in 10°C and 0°C acclimated fish, and examining how the expression of genes encoding rate limiting proteins in cortisol synthesis and the glucocorticoid receptor was affected by cold acclimation.

Chapter Two: Ontogenetic Effects on Metabolic Depression in Cunner (*Tautogolabrus adspersus*)

2.1. Introduction

Body size is an important variable affecting metabolism as nearly all biological rates show a proportional dependence on this parameter (Charnov and Gillooly, 2004). The most widely used equation to describe the dependence of metabolic rate on body mass is:

$$Y = aM^{b}$$

where Y can be basal or maximal metabolic rate, a is the species - specific constant, M is body mass and b is the scaling exponent (b_b and b_m, for basal and maximal metabolic rates, respectively). According to this equation the absolute metabolic rate of an organism increases with body mass, whereas mass-specific metabolic rate (metabolic rate per unit body mass) decreases (i.e. small size fish have a higher metabolic rate per gram than larger fish). This is a widely accepted principle in biology and observed for various homeothermic as well as poikilothermic animals (Kleiber, 1932; Brody, 1945; Hemmingsen, 1960; Kleiber, 1961; McMahon and Bonner, 1983; Peters, 1983; Calder, 1984; Schmidt-Nielsen, 1984; Blaxter, 1989; Brown and West, 2000; Savage et al., 2004). However, the precise value of b_b is still controversial (e.g. is b_b = 0.75 or 0.67 for mammals?) (Dodds et al., 2001; White and Seymour, 2003 and 2005; Savage et al., 2004), and whether or not

b_b = 0.75 is universal across all taxa (Bokma, 2004; Glazier, 2005; Killen et al., 2007 and 2010).

Despite the fundamental importance of fish to aquatic ecosystems, relatively little is known about the effect of body size on the metabolic rate of fish, when compared with mammals and birds. In many species of fish, standard metabolism (b_s) has been considered to be proportional to $M^{0.8}$ (Winberg, 1956) or $M^{0.85}$ (Ricker, 1973; Brett and Groves, 1979), and this has been supported by more recent multi-species studies. Clarke and Johnston (1999) performed an inter-specific analysis of resting metabolic rate in post-larval teleosts and found that *b* was approximately 0.80. Bokma (2004) conducted an intra-specific analysis of resting metabolic rate in teleost fish, and rejected the hypothesis that b_s = 0.67 or 0.75, concluding instead that there is no single (universal) value for the *b*. White et al. (2006) surveyed 82 fish species and found an overall b_s value of approximately 0.88. Finally, the intra-specific scaling of standard metabolic rate (b_s) in three teleost fishes (ocean pout, *Macrozoarces americanus*; lumpfish, *Cyclopterus lumpus*; and shorthorn sculpin, *Myoxocephalus scorpius*) was shown to be between 0.82 and 0.84 when calculated across these species' entire life history (Killen et al., 2007). Interestingly, this mass-specific exponent can be larger, even > 1, when only the fish's larval stage is considered (Kamler, 1972; Rombough, 1988). For example, Kaufmann (1990) performed a study on two cyprinid species (*Chalcalburnus chalcoides* and *Rutilus rutilus*) and showed that the mass dependence of maximal activity was very high in the smallest larvae and decreased to a constant level in larger larvae and juveniles. Further, Killen et

al. (2007) showed that the values of b_s for *C. lumpus* and *M. scorpius* were 1.31 and 0.92, respectively, when only the larval (pre-metamorphic) life stages were considered.

Given the negative relationship between body size and mass-specific metabolic rate in post-larval fishes, it might be hypothesized that during unfavourable environmental conditions (e.g. low temperatures where food is also scarce) smaller fish would depress their metabolic rate earlier than large fish in order to conserve energy. However, a positive relationship has also been observed between body size and energy stores in some fish species (e.g. bluegill, *Lepomis macrochirus*; Cargnelli and Gross, 1997), and this might suggest that smaller cunner would become dormant later than large individuals in order to feed as much as possible before metabolic depression is initiated. This latter hypothesis is in agreement with the observations of Keast (1968 and 1977) who report that while feeding of bluegills is limited in winter, it is usually smaller not larger individuals that feed during this season. Further, it in agreement with the anecdotal observations of Dew (1976) who suggested that large cunner become dormant from 1 to 3 weeks before small fish, and remain dormant for 1 to 3 weeks longer in the spring.

Unfortunately, no study has directly examined at what temperature cunner initiate metabolic depression, or whether small fish depress their metabolism to a greater extent than larger individuals. The latter is another strategy that could be used to overcome the problems of higher mass-specific metabolic rates and potentially lower energy reserves in young/small cunner. Thus, the goal of this study was to examine the following question: Does the

temperature at which cunner enter dormancy and the degree of metabolic depression vary with body size (age)?

2.2. Materials and Methods

2.2.1. Experimental animals

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 # 07-10-KG).

Wild cunner (*Tautogolabrus adspersus*) of varying sizes (~200 mg - 100 g) were collected between July and August 2008 using seines or hand nets in Portugal Cove, Newfoundland and divided into three size ranges [< 0.50 g (YOY), 2 to 5 g (small) and ~100 g (adult)]. These fish were initially held at the Ocean Sciences Centre (Memorial University of Newfoundland) at seasonally ambient temperature and exposed to a natural photoperiod in three different size tanks (the two smaller size classes in 20 l aquaria and adult cunner in a 0.80 m diameter x 0.50 m deep tank) for several months. Thereafter, the fish to be used in this experiment were acclimated to 9 ± 0.5°C and exposed to a natural fall/winter photoperiod (9 hours light: 15 hours dark) for at least 6 months. The fish were fed natural feeds (*Daphnia*, mysid shrimp, brine shrimp, and chopped herring) and/or Gemma 0.50 mm pellets (Skretting Inc. Bayside, NB, Canada) depending on size, three times per week. Food was removed after 1 day if not consumed.

2.2.2. Respirometers and oxygen measurements

Oxygen consumption (MO_2; in mg O_2 kg^{-1} h^{-1}) measurements were made on 8 individuals per size class. MO_2 measurements on adult cunner were

made using a 22 x 10 x 13.50 cm rectangular, water jacketed, respirometer (3.00 l in volume), which included a very small internal mixing pump (Zoo Med Laboratories Inc. San Luis Obispo, CA, USA). The oxygen consumption of the two smaller size classes was made using Blazka-style swim-tunnel respirometers with flow velocity set to the minimal that would allow for effective mixing of the water (i.e., a velocity where the fish would rest calmly). A custom designed glass respirometer (60 ml volume) was used for MO_2 measurements of fish less than 1 g, while a 200 ml respirometer (Loligo Systems, ApS; Tjele, Denmark) was used for 2 - 5 g fish. To minimize visual disturbance, black plastic was draped over the respirometers and the behaviour of the fish was monitored during measurements via a mirror placed behind the respirometers.

Oxygen consumption (MO_2) was measured by turning off the inflow of water to each respirometer, and measuring the drop in water oxygen content using a Fiber-Optic oxygen meter (model FIBOX 3 LCD, PreSens, Germany) and a pre-calibrated dipping probe that was inserted into the respirometer. Data from the O_2 meter (i.e. water oxygen content in mg l^{-1}) was directly recorded to a computer using OxyView® software (PreSens; PST3 V5.32 for the 60 ml respirometer; and LCDPST3 V1.16 for the 200 ml and 3 l respirometers). The data from OxyView® was then downloaded into Logger Pro (Version 3.4; Vernier Software and Technology; Beaverton, OR, USA), and the rate of decline in water O_2 content (in mg O_2 l^{-1} min^{-1}) was determined by fitting a linear regression to the O_2-time data. Oxygen consumption (MO_2) was then calculated using the formula:

$$MO_2 \text{ (mg } O_2 \text{ kg}^{-1} \text{ h}^{-1}) = \frac{\text{(rate of decline in } [O_2] \text{ x } (V - V_m) \text{ x 60 min)}}{M \text{ x } h}$$

where V is respirometer volume in l; V_m = the mass (volume) of the fish, assuming that 1 g is equal to 1 ml of seawater; M = mass of the fish in kg; and h = hour.

The respirometers were cleaned weekly with 70% ethanol to minimize background bacterial contamination. In addition, blank measurements were performed after each MO_2 measurement to quantify any possible bacterial oxygen consumption. In the majority of cases there was negligible background oxygen consumption. However, in cases where a background rate of oxygen consumption was observed, this value was subtracted from the data for individual fish. Oxygen consumption measurements were assumed to approximate the fish's resting metabolic rate (RMR) because of the inactive nature of cunner.

2.2.3. Experimental protocol

All fish used for measurements of oxygen consumption were removed from their holding tanks approximately 36 - 48 hours after their last feeding, and placed into one of three respirometers at 9°C. After the fish were allowed to recover from transfer/netting for 48 hours, resting oxygen consumption (MO_2) was recorded for approximately 40 minutes. Then, temperature was decreased by 1°C, a second measurement of MO_2 was made approximately 6

hours later, and the temperature lowered by 1°C again. In this way, MO_2 was measured at 9 temperatures (range 9°C to 1°C) over 5 days (measurements taken at approximately 10:00 AM and 4:00 PM each day). The 6 hour interval between measurements on a particular day was chosen because Costa (2007) showed that this period was sufficient for the MO_2 of cunner to stabilize following a drop in temperature.

2.2.4. Data and statistical analyses

The temperature at which metabolic depression was initiated and the extent (degree) of metabolic depression were determined for each group from the MO_2 - temperature data. The temperature at which metabolic depression was initiated was taken as the temperature at which there was a noticeable change in the slope of the MO_2 - temperature relationship for each group. Note: although Arrhenius plots were also made of the data for all groups, this analysis did not substantially improve the interpretation of the data.

Q_{10} values for changes in MO_2 with decreasing temperature were calculated over several temperature ranges using the mean MO_2 data for all groups: 1) from 9°C to 1°C (Overall Q_{10}); 2) from 9°C until MO_2 began decreasing at a much faster rate (Q_{10} before), this point taken as the temperature where metabolic depression was initiated; and 3) from the temperature at which metabolic depression was initiated to the temperature at which MO_2 stopped decreasing (Q_{10} after) (see Table 1.1 and Figure 2.1). Q_{10} values over each temperature range were calculated using the formula:

$$Q_{10} = (R_2 / R_1)^{[10 / (T2 - T1)]}$$

where R_1 is the metabolic rate at the initial temperature (T_1), and R_2 is the metabolic rate at the final temperature (T_2) (Rome et al., 1992).

The $\log_{10}$ of mass-specific oxygen consumption was also plotted against $\log_{10}$ wet mass at each temperature so that scaling exponents (slope + 1) could be determined using linear regression analysis. Initially, repeated measures two-way ANOVA was used to examine the effects of size class and temperature on the MO_2 of cunner. However, this analysis revealed a significant interaction between the two main effects. Thus, further statistical analyses were restricted to: 1) one-way ANOVAs at 9°C and 1°C to test whether MO_2 was different between size classes at these temperatures; and 2) repeated measures one-way ANOVAs followed by Dunnett's post-hoc tests to identify significant differences in MO_2 between the acclimation temperature (9°C) and the other temperatures for each size class. In all cases, $P < 0.05$ was used as the level of statistical significance. All values are expressed as mean ± standard error of the mean (S.E.).

2.3. Results

At the acclimation temperature (9°C), the MO_2 values of the three size classes of cunner were significantly ($P < 0.05$) different (Table 2.1), with the MO_2 value of YOY cunner 1.33 and 2.44 fold greater compared with small and adult fish, respectively. This difference in MO_2 values between the three size classes resulted in a mass exponent (*b*) of approximately 0.84 at this temperature. For all three size classes, a decrease in temperature from 9°C to 1°C resulted in a dramatic drop in metabolic rate to a level where there was no significant difference in MO_2 between the three size classes (Figure 2.1). The Q_{10} values for the overall drop in metabolic rate between 9°C and 1°C were 2.76, 3.68 and 6.16 for adult, small and YOY cunner, respectively (Table 2.1).

However, the relationship between decreasing temperature and MO_2 was not linear, and could be divided into three distinct phases: 1) a gradual decrease in MO_2 where Q_{10} values ranged between 2.29 and 2.54 for all the three size classes; 2) a phase characterized by a more rapid decrease in MO_2 until each size class reached a minimal MO_2 value; and 3) a final phase (at least for adult and small cunner) where RMR was temperature insensitive. Importantly, although the rapid drop in RMR occurred over a 4 degree range for all size classes, it was initiated at different temperatures (7°C, 6°C and 5°C for adult, small and YOY cunner, respectively), and the drop in MO_2 over this temperature range was much greater for YOY cunner (Q_{10} = 14.96) compared with small (Q_{10} = 8.05) and large (Q_{10} = 4.92) fish (Table 2.1).

As temperature was initially lowered, there was little change in the mass exponent for MO_2 and it ranged from 0.82 to 0.86. However, because the metabolic rate of adult and small fish did not decrease further after 3°C and 2°C respectively, this resulted in b_s values of 0.90 and 0.94 at 2°C and 1°C, respectively (Figure 2.2).

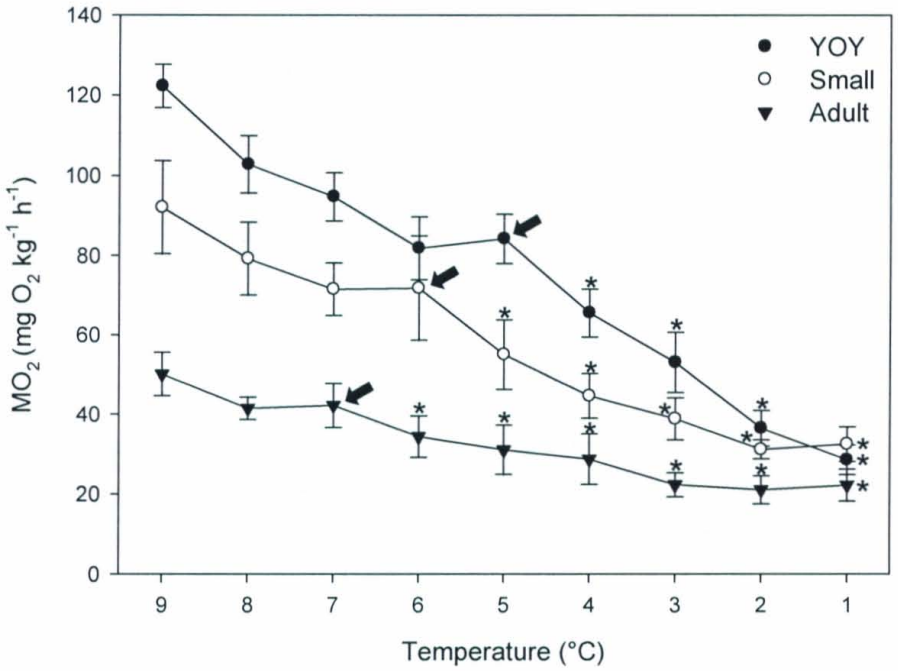

Figure 2.1 – The effect of decreasing temperature at 2°C day^{-1} on oxygen consumption (MO_2) of young-of-the-year (YOY), small and adult cunner. Values are mean ± S.E. (n = 7-8 for each size class). A repeated measures one-way ANOVA, followed by Dunnett's post-hoc tests ($P < 0.05$), was used to identify significant differences (*) in MO_2 between the acclimation temperature (9°C) and the other temperatures within each size class. The arrows indicate the temperature at which a rapid decrease in MO_2 was initiated for each size class.

Table 2.1 – Morphometric and metabolic parameters for 9°C acclimated cunner of various size classes (YOY, young-of-the-year; small and adult) when exposed to a temperature decrease from 9°C to 1°C at 2°C day^{-1}. Values are mean ± S.E. Within each row, values without a letter in common are statistically different ($P < 0.05$). Values in brackets indicate the temperature ranges over which the presented Q_{10} values were calculated. For further information see methods section and Figure 2.1.

	Size (Age) Class		
	YOY	**Small**	**Adult**
n	8	8	7
Mass (g)	0.30 ± 0.04^{a}	4.24 ± 0.31^{b}	113.49 ± 5.76^{c}
MO_2 at 9°C	122.25 ± 5.40^{a}	92.07 ± 11.64^{b}	50.13 ± 5.46^{c}
MO_2 at 1°C	28.54 ± 3.71^{a}	32.50 ± 4.36^{a}	22.24 ± 4.01^{a}
Overall Q_{10} (9 -1°C)	6.16	3.68	2.76
Q_{10} Before	2.54 (9 - 5°C)	2.29 (9 - 6°C)	2.35 (9 - 7°C)
Q_{10} After	14.96 (5 - 1°C)	8.05 (6 - 2°C)	4.92 (7 - 3°C)

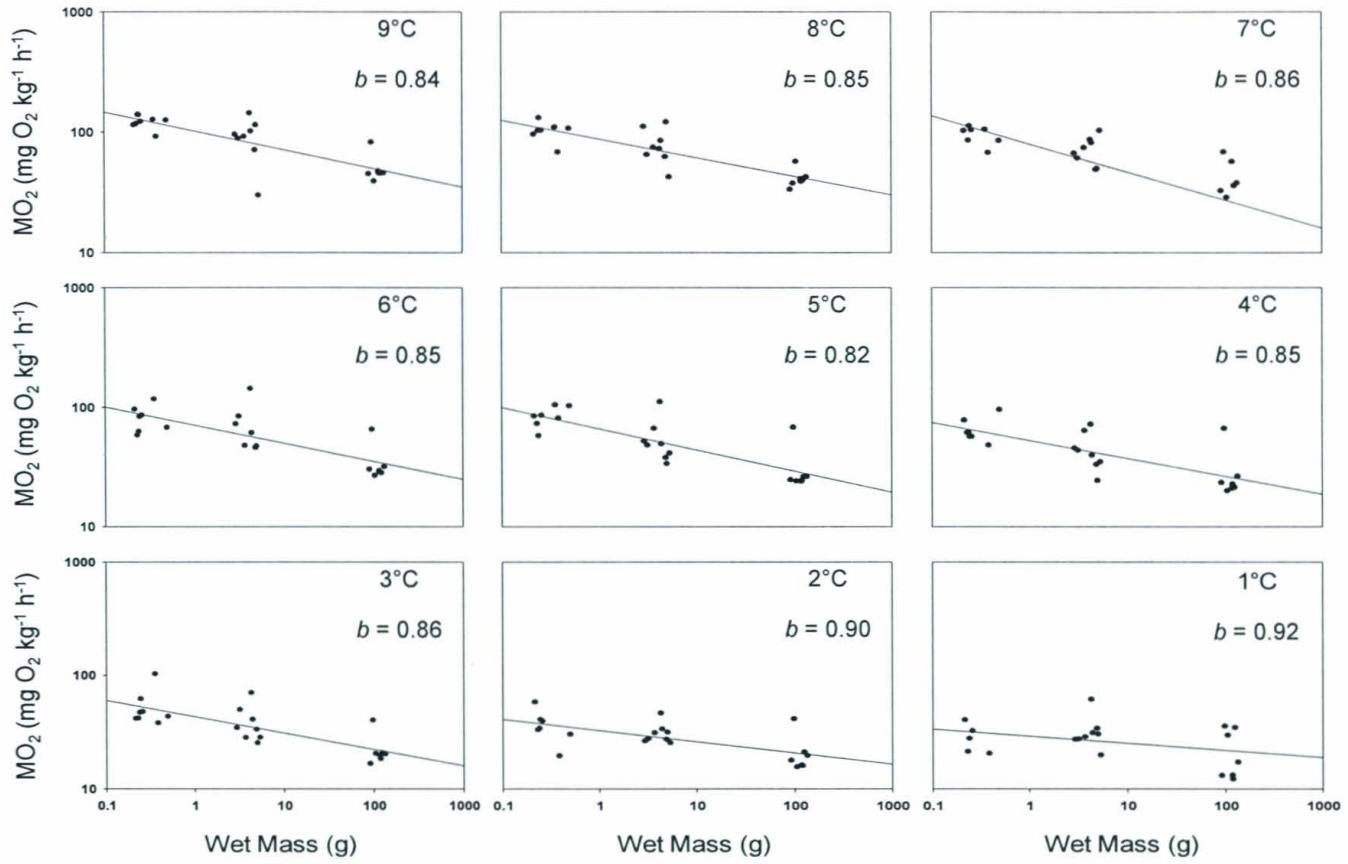

Figure 2.2 – The relationship between body mass and oxygen consumption (MO_2) for young-of-the-year (YOY), small and adult cunner during an 8°C temperature decrease over 4 days. The mass scaling exponent (*b*) at each temperature was calculated as the slope of the relationship + 1.

2.4. Discussion

2.4.1. Resting metabolic rate

In the current study, the RMR of three size classes [young-of-the-year (YOY, < 0.50 g), small (2 to 5 g) and adult (~100 g)] of 9 ± 0.5°C acclimated cunner was measured over a temperature range of 9°C - 1°C (Figure 2.1. and Table 2.1). The RMR of adult cunner at 9°C was 50.13 ± 5.46 mg O_2 kg^{-1} h^{-1}, a value similar to that reported by Corkum and Gamperl (2009) at 8°C (44.42 mg O_2 kg^{-1} h^{-1}), by Costa (2007) at 9°C (41.01 mg O_2 kg^{-1} h^{-1}), as well as by Kelly (2010) at 10°C (42.30 mg O_2 kg^{-1} h^{-1}). Similarly, the RMR of small cunner in this study (92.07 ± 11.64 mg O_2 kg^{-1} h^{-1}) is comparable with the value reported by Kelly (2010) for ~3 - 6 g cunner at 10°C (78.90 mg O_2 kg^{-1} h^{-1}). Whereas the RMR of YOY cunner at 9°C was 122.25 ± 5.40 mg O_2 kg^{-1} h^{-1}. The scaling exponent (*b*) for the RMR of cunner at the acclimation temperature (9°C) was 0.84, a value that differs substantially from either the 0.67 or the 0.75 scaling exponents that are commonly reported for endotherms. However, this value is consistent with previous fish studies where RMR was measured, and scaling exponents of 0.80 - 0.85 are reported (Post and Lee, 1996; Clarke and Johnston, 1999; Bokma, 2004; White et al., 2006; Killen et al., 2007 and 2010). This result indicates that fish capable of metabolic depression do not have an atypical scaling exponent for RMR at temperatures above those at which metabolic depression is initiated.

RMR decreased in all the three size classes of cunner as temperature was lowered. However, the magnitude of the decrease was dependent on the temperature range over which the change in RMR was measured (see Table

2.1). In the higher temperature range (between 9°C to 7°C for adult, 9°C to 6°C for small, and 9°C to 5°C for YOY fish), RMR gradually decreased (Q_{10} = 2.35, 2.29, and 2.54 for adult, small and YOY fish, respectively) with water temperature (Figure 2.1 and Table 2.1). These data are in agreement with the Q_{10} values reported by Curran (1992) for cunner from Woods Hole in the higher temperature range of her study (Q_{10} = 2.40; temperature 12°C - 22°C), the data of Costa (2007) (Q_{10} = 2.50) for adult cunner from Newfoundland when exposed to a temperature drop from 14°C to 5°C, as well as with Sayer and Davenport (1996) who reported a Q_{10} of 2.71 for the goldsinny wrasse between 10°C and 6°C. These Q_{10} values are also within the general range (1.8 - 4) reported for the direct effect of temperature on chemical and biological processes (Rome et al., 1992) and on the resting/routine metabolic rate of other teleost fishes (Rome et al., 1992; Guppy and Withers, 1999; Clarke and Johnston, 1999; Kemp, 2009; Killen et al., 2010). Thus, the drop in MO_2 at the higher temperatures in this study can be considered a normal effect of temperature on metabolism.

Further reductions in temperature resulted in a more rapid decrease in the RMR for all the three size classes of cunner. Adult cunner showed a 55% decrease in RMR (Q_{10} = ~5) between 7°C and 3°C, the RMR of small cunner decreased by 65% (Q_{10} = ~8) between 6°C and 2°C, and YOY reduced RMR by 80% (Q_{10} = ~15) between 5°C and 1°C (Figure 2.1 and Table 2.1). This abrupt drop in RMR over these 4°C temperature ranges was associated with very high Q_{10} values, and clearly indicates that the decrease in cunner RMR was related to an active downregulation of metabolism, and was not a normal

temperature-dependent change. Cold-induced metabolic depression, and its associated high Q_{10} values for metabolism, have been documented in several previous studies. For example, Costa (2007) showed that a seasonal or acute temperature decrease from 5°C to 0°C resulted in a 60 - 70% decrease in the RMR of adult Newfoundland cunner (Q_{10} = ~10). Cunner from Woods Hole were reported by Curran (1992) to have high Q_{10} values for RMR when seawater temperature dropped from 12°C to 6.4°C (Q_{10} = 5.50 - 8.90); the higher temperature (12°C) at which Woods Hole cunner initiated metabolic depression is likely explained by the fact that this population is exposed to a warmer seasonal thermal range (2.5°C to 22°C) than in Newfoundland (-1°C to 14°C). Finally, the goldsinny wrasse, also a member of the family Labridae, exhibits winter dormancy and dramatically decreases mean oxygen uptake rates (Q_{10} = 542.01) when temperature is lowered from 6°C to 4°C (Sayer and Davenport, 1996). Further evidence of the capacity of cunner to enter metabolic depression at similar temperatures to those reported in the present study comes from studies that have examined the cellular basis for this phenomenon in this species. For example, Lewis and Driedzic (2007) measured the rate of protein synthesis in several tissues of cunner from Newfoundland, and reported that this species showed a large reduction (55 - 66%) in protein synthesis when the seawater temperature dropped from 8°C to 4°C (Q_{10} values ranging from 6 to 21). Further, MacSween et al., (unpublished) report that indices of mitochondrial function (e.g. State 3 respiration) are much lower at 0°C than at 10°C (Q_{10} = ~7 - 10).

Although RMR decreased across most of the temperature range to which the fish were exposed, it did not decrease at the lowest two temperatures for both large (adult) and small cunner (Figure 2.2). This suggests that when the metabolism of this species is fully depressed there is no further scope for temperature-dependent decreases in RMR. Although this hypothesis needs to be examined further, our results are consistent with Curran (1992). This author showed that although cunner from Woods Hole decreased their metabolic rate significantly from 12°C – 6°C (Q_{10} = 8.50), no further decreases in RMR were recorded as temperature was lowered from 6°C to 3°C.

2.4.2. Effects of ontogeny

While the evidence for cold-induced metabolic depression in cunner is convincing (Curran, 1992; Lewis and Driedzic, 2007; Costa, 2007; Corkum and Gamperl, 2009), it was not known prior to this study whether ontogeny affected aspects of metabolic depression in this species. The data reveal that the temperature at which metabolic depression is initiated in cunner increases with body mass [from 5°C for YOY cunner to 6°C and 7°C in small and large (adult) fish, respectively (Figure 2.1)]. This result agrees with the anecdotal observations of Dew (1976) who suggested that large cunner become dormant from 1 to 3 weeks before smaller fish. This may be because YOY cunner do not have sufficient energy stores to sustain themselves over the winter and must feed as long as possible in the fall before metabolic depression is initiated. This hypothesis is supported by research on the bluegill which showed that larger fish emerge from the winter starvation period

in better energetic condition (amount of stored lipids) than smaller individuals (Cargnelli and Gross, 1997). A result the author's attributed to the positive relationship between body size and energy stores and the negative relationship between body size and mass-specific metabolic rate.

It is also clear from the RMR and Q_{10} data, where the latter were ~15, 8 and 5 for YOY, small and adult cunner, respectively, that YOY cunner do indeed depress their metabolism to a greater extent than larger/older cunner when faced with cold temperatures. The physiological mechanisms mediating the greater reduction in RMR in YOY cunner are not known. However, mitochondrial function (State 3 and State 4 respiration, and proton leak) appears to play a key role with regards to the low routine metabolic rate of cunner at temperatures > 5°C, and this species' capacity to enter metabolic depression (MacSween et al., unpublished). Thus, it is possible that variations in the extent of metabolic depression between cunner of varying sizes are due to differences in mitochondrial function/regulation. Other potential mechanisms that may be involved include changes in the rate of protein synthesis and the activity of Na^+ K^+ ATPase. Lewis and Driedzic (2007) showed that acclimation to 0°C is concomitant with large reductions in protein synthesis (between 65% and 85% for most tissues). MacSween et al. (unpublished) showed that the proportion of hepatocyte metabolism attributed to Na^+ K^+ ATPase fell from approximately 20% to 30% when these cells were acutely exposed to a drop in temperature from 10°C to 0°C.

The later initiation of metabolic depression, the greater decrease in RMR in YOY fish, and the temperature-insensitivity of RMR in small and large

cunner at cold temperatures had an unexpected consequence. These 3 effects caused the scaling exponent (*b*) for RMR to increase from the 0.82 - 0.86 measured at 9 - 3°C to 0.90 at 2°C and 0.94 at 1°C (Figure 2.1). This result provides another example against the use of a single, universal, value of *b* with regards to fish metabolism. Further, it shows that the scaling of metabolic rate with body size (age) in cunner is dependent on temperature. This finding is in agreement with the inverse relationship found between temperature and scaling exponents when the combined data of 89 species of teleost were examined (Killen et al., 2010).

Chapter Three: Cold-Induced Changes in Stress Hormone Levels and Steroidogenic Transcript Levels in Adult Cunner (*Tautogolabrus adspersus*), a Fish Capable of Metabolic Depression

3.1. Introduction

The stress response in teleosts is characterized by the release of primary (predominant) stress hormones, catecholamines (epinephrine and norepinephrine) and corticosteroids (cortisol), from the chromaffin cells and the interrenal cells of the head kidney, respectively (Nilsson et al., 1976; Mazeaud and Mazeaud, 1981; Donaldson, 1981). The circulating levels of stress-induced catecholamines and cortisol vary greatly depending on several factors including the type of stressor, and the rate of hormone synthesis, release and degradation (Randall and Perry, 1992; Gamperl et al., 1994a). For example, in fishes, post-stress levels of plasma catecholamines increase from resting levels of less than 10 nM to post-stress values of over 300 nM, while levels of plasma cortisol generally vary from 2 - 42 ng ml^{-1} in resting fish to 20 - 500 ng ml^{-1} post- stress (Gamperl et al., 1994a; Wendelaar Bonga, 1997). This endocrine stress response promotes the mobilization of energy stores to support the increase in energy demands associated with stress. Moreover, it mediates respiratory, circulatory, osmotic and metabolic adjustments (Mazeaud et al., 1977; Chan and Woo, 1978; Lidman et al., 1979; Perry and Wood, 1989; Barton and Iwama, 1991; Vijayan et al., 1991; Randall

and Perry, 1992; Wendelaar Bonga, 1997), the latter including effects on the metabolism of carbohydrates, proteins and lipids (Mommsen et al., 1999).

Regardless of the typical characteristics of the stress response in fishes, variability has been reported in several species of fish. For example, plasma cortisol levels in the chub (*Leuciscus cephalus*) at rest (50 - 100 ng ml^{-1}) and during/following 5 minutes of handling and disturbance (1500 ng ml^{-1}) exceed those reported for most fish species (Pottinger et al., 2000). The sea raven (*Hemitripterus americanus*) has a delayed post-stress cortisol response compared with salmonids (Vijayan and Moon, 1994), and it has been suggested that given its low metabolic activity, this altered response is an adaptation to prevent excess energy mobilisation in this species. Finally, in polar fishes (both Arctic and Antarctic), research suggests that cortisol is not a primary stress hormone and that with the exception of heat stress or extremely stressful situations, these fishes have a diminished adrenergic response compared to temperate teleosts (Egginton, 1994; Davison et al., 1995; Forster et al., 1998; Whiteley and Egginton, 1999; Whiteley et al., 2006). However, there is a lack of data on temperate fish species at temperatures of approximately 0°C to support this latter conclusion, or the hypothesis that an attenuated stress response is a specialization for life in sub-zero waters. With regards to cunner, a North Atlantic wrasse species capable of metabolic depression, Dew (1976) found that dormant individuals swam away in a dazed and spasmodic fashion when disturbed. In contrast, Green and Farwell (1971) found that cunners were so lethargic at temperatures < 5°C that when rocks were disturbed they did not always move

away, and suggested that cunners at these temperatures are highly vulnerable to environmental changes because they are unable to avoid them.

Based on the above data, and that the release of stress hormones increases an organism's metabolic demands, one would predict that cunner downregulate their stress response concomitant with metabolic depression at cold temperatures. However, this hypothesis has never been tested. Thus, resting and post-stress plasma cortisol and catecholamine concentrations, and the transcript levels of key genes involved in the synthesis of cortisol (StAR and P450*scc*; see Figure 3.1), were measured in the head kidney of 10°C and 0°C acclimated cunner. Further, as the sensitivity of cells and tissues to a given hormone is dependent on the number (density) of receptors (Clark and Peck, 1977; Danielson and Stallcup, 1984; Vanderbilt et al., 1987), and studies on glucocorticoid receptor (GR) regulation in fish demonstrate an inverse correlation between plasma cortisol levels and the density of tissue GR (Pottinger, 1990; Maule and Schreck, 1991; Lee et al., 1992; Pottinger et al., 1994; Shrimpton and Randall, 1994; Shrimpton and McCormick, 1999), GR transcript levels for cunner acclimated to both temperatures were also measured in the head kidney and liver.

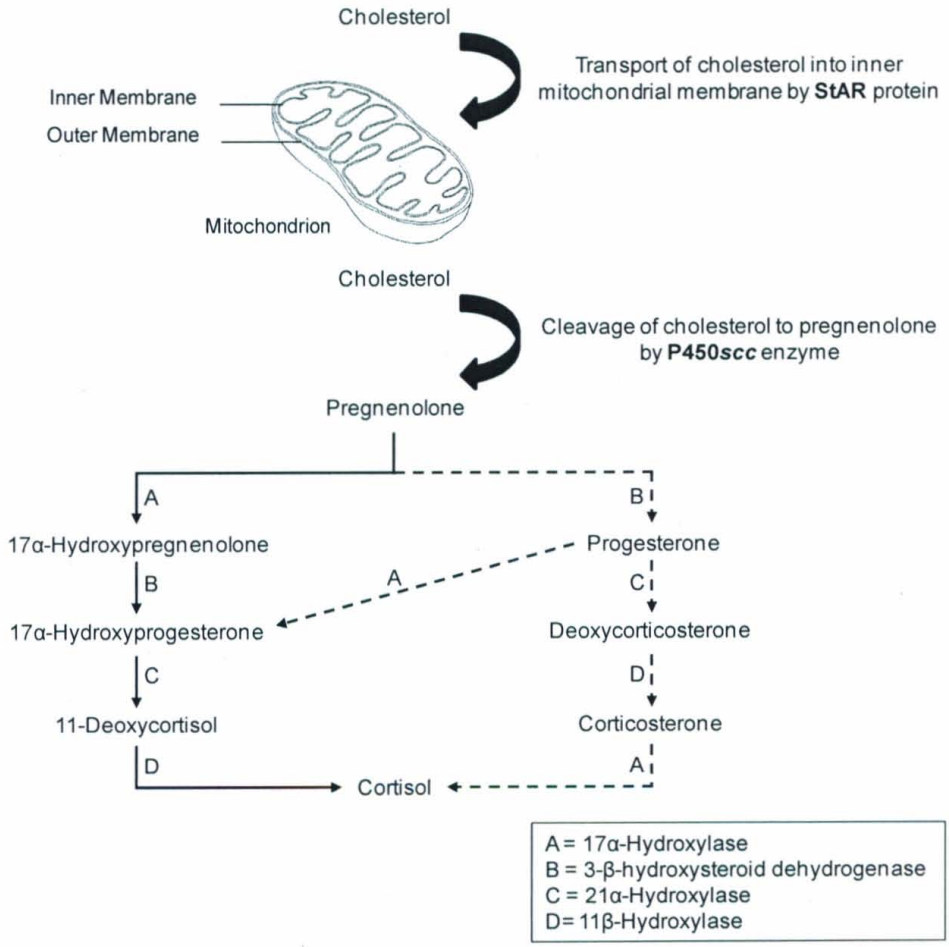

Figure 3.1 – Generalized diagram illustrating the rate limiting steps in cortisol biosynthesis (Mommsen et al., 1999; Stocco, 2000; Sierra, 2004; Gelsin and Auperin, 2004; Arukwe, 2008). The dashed-lines indicate the less efficient pathway (Sangalang et al., 1972).

3.2. Materials and Methods

3.2.1. Experimental animals

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 # 07-10-KG).

Wild cunner (*Tautogolabrus adspersus*) were collected between July and August 2008 by hand nets in Portugal Cove, Newfoundland and initially held in tanks at the Ocean Sciences Centre (Memorial University of Newfoundland) supplied with flow-through aerated seawater at seasonally ambient temperature and photoperiod. Later, the fish to be used in this experiment were acclimated to circulating aerated seawater of 0 $\pm$ 0.4°C (photoperiod 9 hours light: 15 hours dark) or 10 $\pm$ 0.5°C (photoperiod 16 hours light: 8 hours dark) for 2 - 3 months prior to sampling. The 10°C cunner were fed chopped herring (*Clupea harengus*) three times a week to apparent satiation, whereas, the 0°C fish were dormant and generally did not feed. However, some chopped herring was offered to the 0°C fish once a week, and removed after 1 day if not consumed.

3.2.2. Sampling regime

The two tanks (0°C and 10°C) were lined with netting 4 - 5 days prior to sampling to allow the fish to become accustomed to the presence of the netting. Three samplings were then performed on fish from each temperature.

In the first sampling, blood and tissue samples were collected to examine differences in resting cortisol levels and in the expression of genes involved cortisol synthesis and tissue responsiveness between 0°C and 10°C acclimated fish. To accomplish this, individual fish (n = 8 per group) from both temperatures were carefully netted, anaesthetized in seawater containing 0.40 g l^{-1} of MS-222 (Syndel Laboratories Ltd, Vancouver, BC, Canada), given a sharp blow to the head (cerebral percussion), and quickly had their blood collected. Blood samples were collected by caudal puncture within 2 minutes of netting using previously heparinised (100 U ml^{-1} heparin, Sigma Aldrich, St. Louis, MO, USA) syringes (Becton Dickinson, Franklin Lakes, NJ, USA), and the majority of the blood was transferred to 1.5 ml centrifuge tubes and stored on ice. These blood samples were subsequently used to obtain plasma (by centrifugation at 10,000 x g for 1 minute) for the measurement of resting (0 h; pre-stress) plasma cortisol levels. Head kidney (for the measurement of StAR, P450*scc* and GR mRNA expression) and liver (for the measurement of GR mRNA expression) were collected in 2.5 ml cryovials. All of these samples were quickly frozen in liquid nitrogen and stored at -80°C until further processing and analyses.

In the second sampling, to study the effects of stress on plasma catecholamine levels, individual fish (n = 8 per group) from both temperatures (0°C and 10°C) were exposed to a net stress (1 minute air exposure in a hand net), given a sharp blow to the head, and blood was immediately collected as described above. However, prior to storage, the plasma (200 µl) was transferred to Eppendorf tubes containing 15 µl of 0.20 M EDTA and 15 µl of

0.20 M glutathione. These samples were later analysed for maximum post-stress catecholamine levels. The transcript levels of genes encoding proteins involved in catecholamine synthesis were not measured. This is because catecholamines are stored prior to release (Randall and Perry, 1992), and thus, the transcript levels of genes involved in catecholamine synthesis may not be directly related to the capacity of cold-acclimated fish to elevate plasma levels following an acute stress. Further, catecholamines are rapidly released into the circulation (i.e. in < 2 min post-stress) and have a very short biological half-life (< 10 min) due to the combined effects of tissue accumulation and metabolic degradation (Nekvasil and Olson, 1986; Randall and Perry, 1992; Gamperl and Boutilier, 1994; Vijayan and Moon, 1994; Wendelaar Bonga, 1997; Rothwell et al., 2005). Therefore, the effect of stress on plasma catecholamine levels was assessed at one time point (immediately) post-stress.

Finally, the remaining fish from both groups were exposed to a net stress by lifting the tank netting in the air for 1 minute, and had their blood sampled at 1, 2, 4, 12 and 24 hours post-stress following the same procedures described above (n = 8 per time point). The plasma obtained from these blood samples was used to establish the time-course of post-stress plasma cortisol levels.

At all three sampling points, the inactive nature of cunner (at 0°C and 10°C) made it possible to carefully net a particular individual without chasing. This minimized the physical stress (i.e. repeated netting) imposed on the remaining fish within the tank.

In addition to the above described three experiments, a final sampling was performed on an additional group of fish (n = 3) acclimated to 10°C. Various tissues (brain, gill, head kidney, heart, liver, muscle, ovary, posterior kidney, and spleen.) were collected, quickly frozen in liquid nitrogen and stored at -80°C until further processing and analyses. These samples were subsequently used to assess the tissue distribution of StAR, P450*scc* and GR in order to compare expression profiles with other fish species.

3.2.3. Measurement of plasma catecholamine and cortisol levels

The plasma catecholamines epinephrine (E) and norepinephrine (NE) were measured using high performance liquid chromatography (HPLC, Bioanalytical Systems Inc. Lafayette, IN, USA) with electrochemical detection (+650 mV) after extraction with alumina (Bioanalytical Systems Inc. LCEC Application Note 14). Separation of the catecholamines was performed using a reverse phase column (ODS, 3.00 mm i.d. x 10 cm long, 3 µm pore size; model MF 8954), and an aqueous mobile phase (containing per litre: 7.088 g of monochloroacetic acid, 186.1 mg $Na_2EDTA\text{-}2H_20$, 15 ml acetonitrite and 32.30 mg sodium octyl sulphate, pH 3.00 - 3.05) pumped at a flow rate of 1 ml min^{-1}. E and NE plasma concentrations were calculated relative to NE/E synthetic standards (75 ng ml^{-1} NE, 75 ng ml^{-1} E), and DHBA (3, 4 dihydroxybenzylamine) was used as an internal standard.

Plasma cortisol levels were determined in duplicate using an enzyme-linked immunosorbant assay (ELISA) kit (NEOGEN Corp. Lexington, KY, USA), and a SpectraMax M5e microplate reader (Molecular Devices,

Sunnyvale, CA, USA) at an absorbance of 650 nm. Intra- and inter-assay variation did not exceed 10%.

3.2.4. Preparation of RNA from tissues

Total RNA was extracted from flash frozen tissues using TRIzol Reagent (Invitrogen, Burlington, ON) following the manufacturer's protocol. With the exception of liver, 45 µg of total RNA was incubated in a 100 µl reaction containing Buffer RDD DNA digest buffer (1x final concentration) and 6.8 Kunitz U DNase I (RNase-Free DNase Set, QIAGEN, Mississauga, ON) at room temperature for 10 minutes to degrade any residual genomic DNA. The entire reaction was then column purified using the RNeasy MinElute Cleanup Kit (QIAGEN) following the manufacturer's protocol. Total RNA extracted from the liver did not bind to the QIAGEN column during purification. Therefore, any contaminating genomic DNA was removed from liver total RNA using the TURBO DNA-free kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Briefly, 10 µg of total RNA was incubated in a 50 µl final volume containing TURBO DNase buffer (1X final concentration) and 2 U of TURBO DNase. The reaction was incubated for 30 minutes, another 2 U of TURBO DNase was added, then incubated for another 30 minutes. Ten µl of DNase Inactivation Reagent was added and the samples incubated for 5 minutes at room temperature with occasional mixing. The reaction was then centrifuged at 10,000 x g for 1.5 minutes at room temperature and 45 µl of the supernatant was transferred to a fresh tube to avoid the DNase Inactivation Reagent. RNA quantity and purity were measured using A260/280 and

A260/230 NanoDrop UV spectrophotometry, and integrity was assessed using 1% agarose gel electrophoresis with visual comparison to a DNA size marker (1 kb ladder, Invitrogen). The running buffer was 1X TAE; ethidium bromide (1 μg ml^{-1} final concentration) was added to the gel to visualize the RNA upon exposure to UV light.

3.2.5. First-strand cDNA synthesis

First-strand cDNA was synthesized from 1 μg of DNase I-treated total RNA. Briefly, the total RNA was reverse-transcribed in a Techne TC - 412 thermal cycler (VWR, Mississauga, ON) with heated lid at 37°C for 50 minutes in a 20 μl reaction using random primers (250 ng, Invitrogen) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (200 U, Invitrogen), with the manufacturer's first strand buffer (1X final concentration) and DTT (10 mM final concentration).

3.2.6. cDNA cloning and sequence analyses

Partial cDNAs for cunner StAR and P450*scc* were amplified using nested reverse transcription - polymerase chain reaction (RT-PCR). Degenerate primers were designed based upon consensus sequences from conserved areas of aligned fish cDNAs. The sequences of all primers used in cDNA cloning are presented in Table 3.1. First-strand cDNA (corresponding to 50 ng of input total RNA) that had been synthesized from ovarian total RNA was the template in the first PCR reaction. One-fiftieth of the first PCR reaction was then used as template in the nested PCR reaction. PCR reaction conditions

and cycling parameters were the same for both the initial and nested PCRs. PCR amplification was performed using TopTaq DNA polymerase (QIAGEN). Briefly, 50 µl reactions were prepared containing the applicable template, 1.25 U of TopTaq DNA polymerase, the TopTaq PCR buffer (1X final concentration), 0.2 mM dNTPs and 0.2 µM each of forward and reverse primer. Touchdown PCR was used with 40 cycles of [94°C for 30 sec, 65°C decreasing by 0.5°C per cycle (to 45.5°C at cycle 40) for 30 sec, and 72°C for 1 min]. PCR products were electrophoresed on a 1.5% agarose gel with ethidium bromide staining and compared to a DNA size marker (100 bp ladder, Invitrogen), excised and purified using the QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocol. StAR was subcloned into a TOPO TA cloning vector, pCR II-TOPO (Invitrogen) following the manufacturer's protocol. Briefly, 6 µl ligation reactions were prepared containing 4 µl of gel purified PCR product, 1 µl of salt solution and 1 µl (10 ng) of TOPO vector. P450*scc* was subcloned into pGEM-T Easy vector (Promega, Madison, WI) following the manufacturer's protocol. Briefly, 10 µl ligation reactions were prepared containing Rapid Ligation Buffer, T 4 DNA Ligase (1x final concentration), pGEM-T Easy Vector (50 ng), T4 DNA Ligase (3 Weiss U) and 3 µl of gel purified PCR product. Transformations were then performed using Subcloning Efficiency DHSα Chemically Competent Cells (Invitrogen). Individual clones were screened for the presence of inserts by purifying the plasmid DNA using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's protocol. Plasmid DNA (1 µg) was digested in a 10 µl reaction with 10 U of *Eco*RI and React 3 buffer (1X final concentration)

(Invitrogen) for 1 h at 37°C, and then subjected to 1% agarose gel electrophoresis with visual comparison to a DNA size marker (1 kb ladder, Invitrogen). Positive clones were sequenced on both strands at the Genomics and Proteomics (GaP) laboratory (Memorial University of Newfoundland). Briefly, DNA was amplified from approximately 100 ng of plasmid DNA using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the unincorporated BigDye terminators removed using the BigDye XTerminator Purification Kit (Applied Biosystems). The purified sequencing reactions were processed by capillary electrophoresis using the 3730xl DNA analyzer (Applied Biosystems).

The partial cDNA for cunner GR was amplified using RT-PCR by Tiago Hori. The degenerate primers used were for the gilthead seabream (*Sparus aurata*; Acerete et al., 2007) (Table 3.1).

Sequence data was compiled and analyzed using Vector NTI Advance 10 (Invitrogen). The identity of the StAR, P450*scc* and GR partial cDNAs was confirmed by a BLASTx 2.2.20 search of the NCBI GenBank non-redundant (nr) protein sequences database using a translated nucleotide query.

The partial cDNA sequences for StAR and P450*scc* were then used to design primers for RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE). For GR, a second partial cDNA was cloned to walk closer to the 5' end of the coding sequence (CDS) prior to 5' RACE. Genome walking was used to clone the remaining sequence of the 3' end of the CDS and a portion of the 3' UTR prior to 3' RACE to break up the long 3' UTR into more reasonable fragments based upon the presence of restriction enzymes within.

Methods to obtain this additional sequence information were performed by Jennifer Hall. Full length sequences were deposited into GenBank under the following accession numbers: StAR (GU596481), P450*scc* (GU596480) and GR (GU596482).

3.2.7. *Quantitative reverse transcription - polymerase chain reaction* (QPCR)

Messenger RNA levels of genes encoding StAR, P450*ssc* and GR were quantified by QPCR using SYBR Green I dye chemistry with normalization to 18S ribosomal RNA via a commercially available TaqMan assay and the 7500 Fast Real-Time PCR system (Applied Biosystems). 18S ribosomal RNA was chosen as the normalizer gene due to the limited sequence data available for cunner, and that it was stably expressed between the 0°C and 10°C fish (see below). The QPCR primers were designed based upon the full length cDNAs for StAR, P450*scc* and GR. The sequences of all primers used in QPCR are presented in Table 3.1. The quality of these primers was tested to ensure that a single product was amplified (dissociation curve analysis) and that there was no primer-dimer present in the no-template control. Amplification efficiencies (Pfaffl, 2001) were also calculated using cDNA synthesized from head kidney (StAR and P450*scc*) and liver (GR) as template. cDNA from 5 fish was combined to generate two pools (0°C and 10°C), with each fish contributing equally to the pool. A 5-point 1:5 dilution series starting with cDNA (corresponding to 10 ng of input total RNA) was analyzed for each pool. This dilution series was also performed for the 18S ribosomal RNA TaqMan assay using cDNA synthesized from both head kidney and liver as template to

ensure that 18S levels were consistent between the 0°C and 10°C pools, and hence that it was an acceptable normalizer. The amplification efficiencies were: StAR (99.6%), P450scc (93.7%), GR (80.3%) and 18S (liver, 80.5%; head kidney, 71.4%). These efficiencies represent the average of that obtained for the 0°C and 10°C pools. Amplicon sizes were: StAR (126 bp), P450scc (116 bp) and GR (114 bp).

PCR amplification of the target genes was performed in a 25 µl reaction using 1X Power SYBR Green PCR Master Mix (Applied Biosystems), 50 nM each of forward and reverse primer and cDNA (corresponding to 10 ng of input total RNA). Expression levels of the target genes were normalized to 18S ribosomal RNA, using the Eukaryotic 18S rRNA Endogenous Control (VIC / MGB Probe, Primer Limited) (Applied Biosystems). PCR amplification of 18S was performed in a separate 25 µl reaction using 1X TaqMan Universal PCR Master Mix, with AmpErase UNG (Applied Biosystems), 1X probe/primer mix and cDNA (corresponding to 0.5 ng of input total RNA). The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of (95°C for 15 sec and 60°C for 1 min), followed by dissociation analysis (+1% increases every 30 seconds from 60°C to 95°C). On each plate, for every sample, the target gene and endogenous control were tested in triplicate. The fluorescence threshold cycle (C_T) was determined automatically using the 7500 Software Relative Quantification Study Application (Version 2.0) (Applied Biosystems). When the standard deviation of C_T values within a triplicate was higher than 0.50, the outlier was discarded and the average C_T was calculated using the remaining two C_T

values. If there was no clear outlier, all three C_T values were discarded and a value of 'ND' (not detected) was assigned. The $2^{-\Delta\Delta CT}$ relative quantification method (Livak and Schmittgen, 2001), with incorporation of the amplification efficiencies for the gene of interest and normalizer assays was used to calculate the relative starting quantity (RQ) of each transcript. For each target gene, the individual with the lowest normalized expression (mRNA) level was set as the calibrator sample (assigned RQ value = 1). Gene expression data are presented as mean (± standard error) RQ relative to the calibrator.

3.2.8. Statistical analyses

Initially, two-way ANOVA was used to examine the effects of time and temperature on cortisol levels in cunner. However, this analysis revealed a significant interaction between the two main effects. Thus, further statistical analyses were restricted to: 1) one-way ANOVAs followed by Dunnett's post-hoc tests to identify significant differences in cortisol levels between the control value (measurement at 0 hour) and the other values (measurements at other time points) within each acclimation group; and 2) a two-tailed *t*-test to identify significant differences in resting cortisol levels (measurement at 0 hour) between acclimation groups. A two-tailed *t*-test was also used to identify significant differences in transcript levels and post-stress catecholamine levels between the 0°C and 10°C acclimation groups. In all cases, $P < 0.05$ was used as the level of statistical significance. All values are expressed as mean ± standard error of the mean (S.E.).

Table 3.1 – Sequences of oligonucleotides used in cDNA cloning and QPCR.

cDNA	Nucleotide Sequence (5'-3')*	Direction	Application	Position of 5' end in cDNA
StAR	CTGCAGRAGKCYATCAGYATYC	Forward	Degenerate	405
	TCCACCTGBGTYTGMGAWAG	Reverse	Degenerate	936
	ATGGARCARATGGGRGANTGG	Forward	Degenerate (nested)	579
	GGRATCCARCCCTTKTRRTCT	Reverse	Degenerate (nested)	913
	TGCACTGCAGAAAGCCATCA	Forward	QPCR	401
	TCCAGCTTGAATACCTTCCCG	Reverse	QPCR	526
P450*scc*	CCGCCTACAGRGAYTACAGGAAC	Forward	Degenerate	411
	CYTGGTTRAAGATGCCATCCCA	Reverse	Degenerate	860
	GASGTGGGWCAGGAYTTTRTGGC	Forward	Degenerate (nested)	548
	GWGAYGCAGTCAATGAARTG	Reverse	Degenerate (nested)	744
	GAAGTGATTTCCCCGAAGGT	Forward	QPCR	500
	GTCCATTTGTTCTGGCCACT	Reverse	QPCR	615
GR	AGTGCTCCTGGCTGTTYCTNATG**	Forward	Degenerate	2030
	TTTCGGTAATTGGTTGCTGATGAT**	Reverse	Degenerate	2529
	CATCTATCGTTCTTCCATCCAG	Forward	QPCR	395
	ATGTACCAACAGTGGCAGTGAC	Reverse	QPCR	508

* Degenerate base symbols: B: C+G+T, K: G+T, M: A+C, N: A+C+G+T, R: A+G, S: C+G, W: A+T, Y: C+T.

** Primers obtained from Acerete et al. (2007).

3.3. Results

3.3.1. Plasma catecholamine and cortisol levels

Post-stress levels of plasma epinephrine and norepinephrine were 9.09 fold and 4.77 fold higher, respectively, ($P < 0.05$, $n = 8$) in the 10°C acclimated cunner compared to 0°C acclimated fish (Figure 3.2). Epinephrine and norepinephrine reached ~220 nM and ~67 nM, respectively, in the 10°C group. Resting (0 h) plasma cortisol levels were also different between the two groups, with relatively low values measured in 0°C acclimated fish (~15 ng ml^{-1}) and elevated levels of ~65 ng ml^{-1} in 10°C acclimated fish (Figure 3.3). Cortisol levels in the 0°C acclimated fish increased ($P < 0.05$, $n = 8$) to ~107 ng ml^{-1} by 2 hours post-stress and returned to basal levels by 24 hours post-stress (Figure 3.3). In contrast, cortisol levels in the 10°C acclimated fish showed a tendency to decrease post-stress; although this trend was not significant ($P = 0.06$ at 2 and 4 h post-stress, $n = 8$). Nevertheless, it is clear that plasma cortisol did not increase in these fish when exposed to net stress (Figure 3.3).

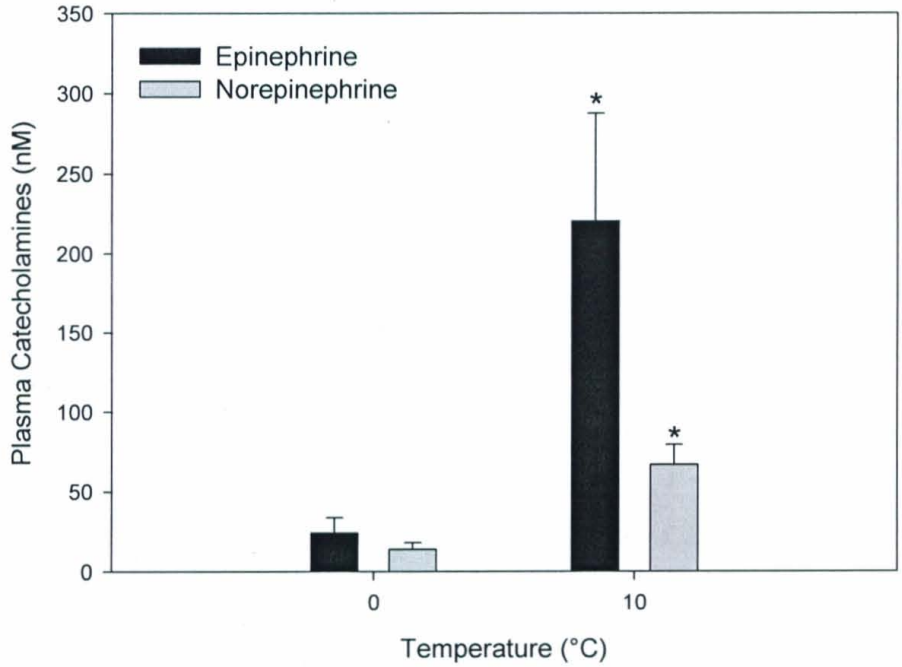

Figure 3.2 – Post-stress (~2 min) plasma catecholamines levels (nM) in 0°C and 10°C acclimated cunner subjected to an acute handling stress (1 minute out of water). Values are mean ± S.E. (n = 8 for each group). A two-tailed *t*-test ($P < 0.05$) was used to test for significant differences (*) between acclimation groups.

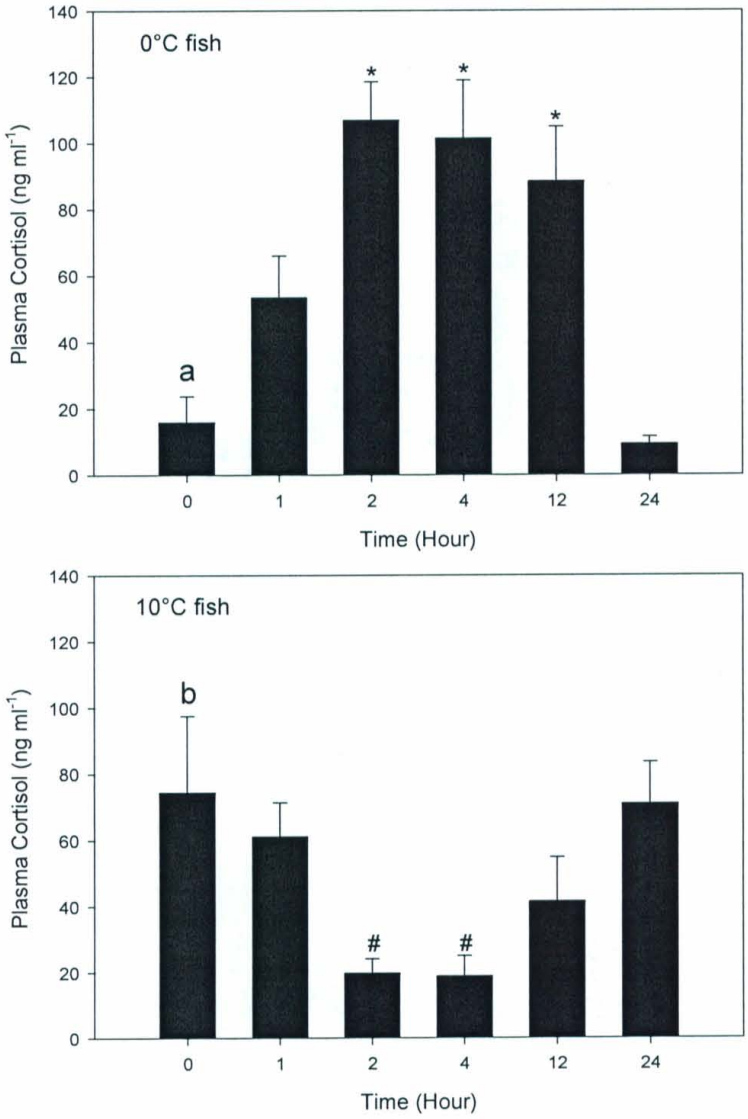

Figure 3.3 – Plasma cortisol levels (ng ml^{-1}) in 0°C and 10°C acclimated cunner subjected to an acute handling stress (1 minute out of water). Fish were sampled at time 0 (pre-stress) and at 1, 2, 4, 12 and 24 hours post-stress. Values are mean ± S.E. (n = 8 per time point for each group). One-way ANOVA, followed by Dunnett's post-hoc test ($P < 0.05$), was used to test for significant differences (*) from the control value (measurement at time 0 hour) within each acclimation group. # indicates a near significant difference ($P = 0.06$) from the control value. Dissimilar letters indicate a significant ($P < 0.05$) difference between acclimation groups prior to the stressor (at 0 hour).

3.3.2. The effect of temperature on StAR, P450scc and GR transcript levels

Transcript levels of P450*scc* were significantly higher (by 3.25 fold; $P < 0.05$) in the head kidney of 10°C acclimated cunner compared to 0°C acclimated fish (Figure 3.4). However, there was no significant difference ($P = 0.2$) in StAR transcript levels in the head kidney of 10°C acclimated cunner compared to 0°C acclimated fish, although the fold increase in transcript levels was very similar to that seen for P450scc. GR transcript levels were 1.70 fold and 1.29 fold ($P < 0.05$) lower in the head kidney and liver, respectively, of 10°C acclimated fish compared to 0°C acclimated fish (Figure 3.5).

3.3.3. Tissue distribution of StAR, P450scc and GR transcripts

Transcript levels of StAR, P450*scc* (Figure 3.6) and GR (Figure 3.7) in various tissues from adult cunner ($n = 3$) were measured using QPCR. StAR transcript levels were highest in the head kidney and ovary, whereas lower levels were detected in brain, heart, liver and posterior kidney. StAR transcripts were not determined in the other tissues (Figure 3.6). The highest transcript levels for P450*scc* were detected in head kidney and ovary, whereas lower levels were detected in brain, posterior kidney and spleen. P450*scc* transcripts were not determined in the other tissues (Figure 3.6). GR transcripts were expressed amongst the tissues analysed. However, transcript levels were highest in the gill, heart, posterior kidney and spleen (Figure 3.7).

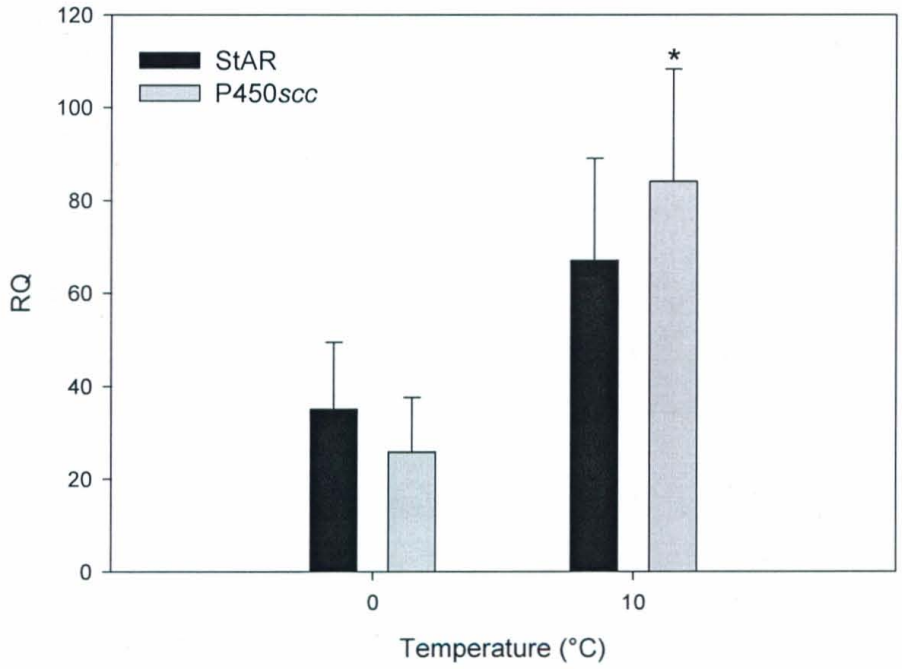

Figure 3.4 – QPCR analysis of the effect of acclimation temperature (0°C vs. 10°C) on transcript levels of StAR and P450*ssc* in the head kidney of cunner. RQ (relative quantity) values, normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest normalized expression of each gene of interest (see methods section), are presented as mean ± S.E. (n = 8 for each group). A two-tailed *t*-test ($P < 0.05$) was used to test for significant differences (*) between acclimation groups.

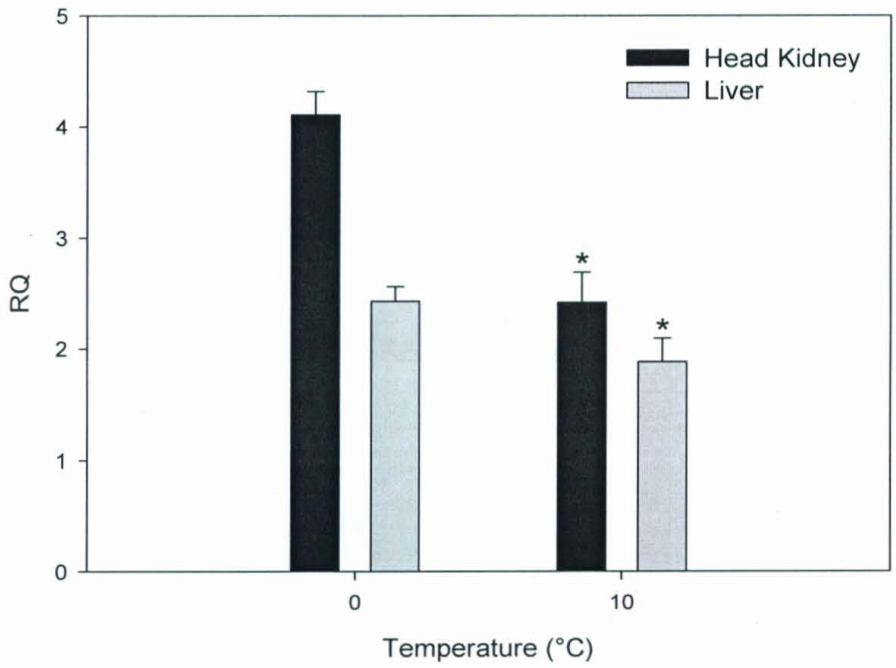

Figure 3.5 – QPCR analysis of the effect of acclimation temperature (0°C vs. 10°C) on transcript levels of GR in the head kidney and liver of cunner. RQ (relative quantity) values, normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest normalized GR expression (see methods section), are presented as mean ± S.E. (n = 8 for each group). A two-tailed *t*-test ($P < 0.05$) was used to test for significant differences (*) between acclimation groups.

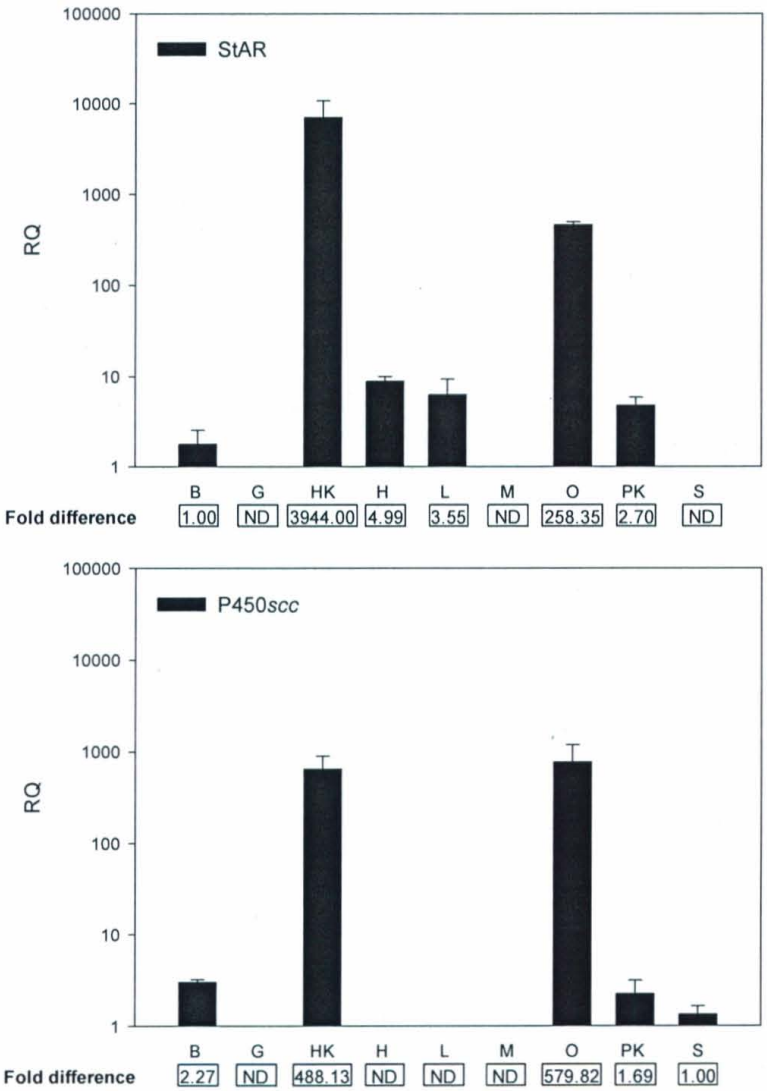

Figure 3.6 – QPCR analysis of the tissue distribution of StAR and P450*ssc* transcripts in cunner. RQ (relative quantity) values, normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest normalized expression for each gene of interest, are presented as mean ± S.E. (n = 3). The calibrator samples were: brain individual # 3 (StAR) and spleen individual # 3 (P450*scc*). B: brain, G: gill, HK: head kidney, H: heart, L: liver, M: muscle, O: ovary, PK: posterior kidney, S: spleen. Fold differences, calculated as [(average RQ of tissue of interest) / (average RQ of tissue with lowest normalized expression for each gene of interest)], are presented in the boxes. ND = not detected (see methods section).

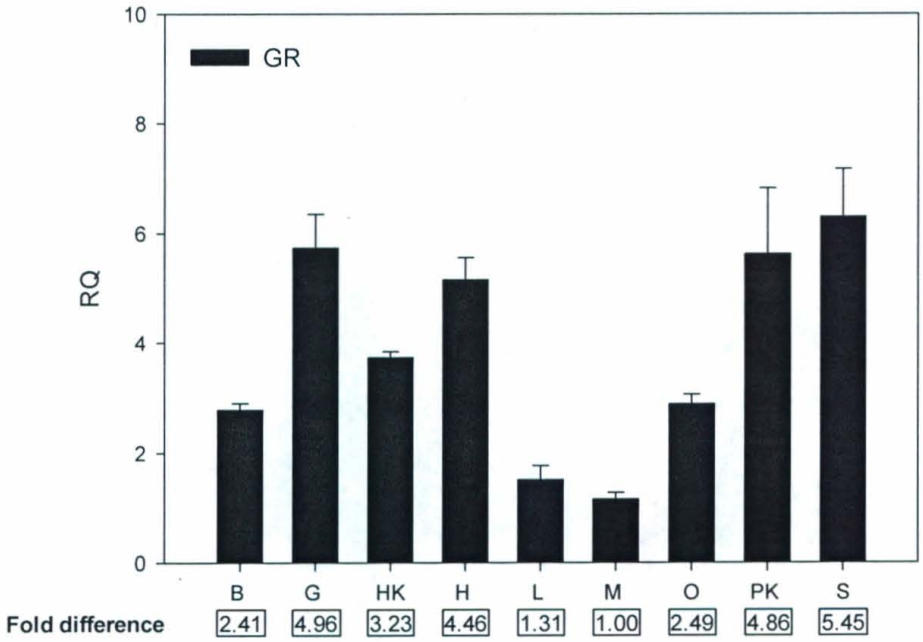

Figure 3.7 – QPCR analysis of the tissue distribution of GR transcript in cunner. RQ (relative quantity) values, normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest normalized GR expression, are presented as mean ± S.E. (n = 3). The calibrator sample was liver individual # 1. B: brain, G: gill, HK: head kidney, H: heart, L: liver, M: muscle, O: ovary, PK: posterior kidney, S: spleen. Fold differences, calculated as [(average RQ of tissue of interest) / (average RQ of tissue with lowest normalized GR expression)], are presented in the boxes.

3.4. Discussion

3.4.1. Plasma catecholamine and cortisol levels

The present study shows that acclimation to 0°C vs. 10°C has a considerable effect on the hypothalamic - sympathetic - chromaffin cell (HSC) axis and the hypothalamic - pituitary – interrenal (HPI) axis of cunner. The 10°C acclimated cunner had post-stress epinephrine and norepinephrine levels that were 9.09 fold and 4.77 fold, respectively, higher at 2 minutes post-stress compared with fish acclimated to 0°C (Figure 3.2). Catecholamines mobilize energy stores to support the increased energy demands associated with stress (Perry and Wood, 1989; Randall and Perry, 1992; Reid et al., 1998), increase oxygen uptake at the gills through increases in ventilation, branchial blood flow and oxygen diffusing capacity, and elevate cardiac output and the oxygen transport capacity of the blood (Randall and Perry, 1992; Wendelaar Bonga, 1997). These responses significantly increase energy demand, and thus, the blunted response in 0°C acclimated cunner in a state of metabolic depression may be a strategy to conserve energy.

However, there are other possible explanations for the decreased humoral adrenergic response in this species. These include the direct effects of cold temperature, and that these fish are so lethargic, and have such limited locomotory capacity, that the 'flight-or-flight' response would not be of much benefit. Support for the idea that prolonged exposure to cold resulted in the low post-stress catecholamine levels measured in 0°C cunner comes from studies on Antarctic and Arctic (e.g. *Boreogadus sadai*) fish species.

This research shows that, with the exception of heat stress or extremely stressful situations, these fishes have a diminished adrenergic response compared to temperate teleosts (Egginton, 1994; Davison et al., 1995; Egginton and Davison, 1998; Forster et al., 1998; Whiteley and Egginton, 1999; Whiteley et al., 2006). This lack of any adrenergic humoral response has largely been attributed to the pre-dominance of cholinergic over adrenergic control of the cardiovascular system (Nilsson et al., 1996; Egginton and Davison, 1998; Davison et al., 1997). However, recent research (Skov et al., 2009) shows that while adrenaline is not an important mediator of heart rate in Antarctic fishes, it significantly enhances myocardial contractile force. This data argues against the hypothesis that post-stress catecholamine levels are low in cold-water adapted species because they are not important in cardiovascular regulation. Alternatively, it is possible that cold water (cold-adapted) teleosts have a limited capacity to elevate epinephrine and norepinephrine post-stress because they do not require catecholamine modulation of blood oxygen carrying capacity due to their low metabolic rate and the high solubility of oxygen in the environment (Whiteley et al., 2006). Finally, the release of catecholamines from chromaffin cells is mediated primarily by preganglionic cholinergic fibres of sympathetic nerves (Nilsson et al., 1976; Mazeaud and Mazeaud, 1981). Thus, the possibility that the release of catecholamines from the chromaffin cells was delayed in 0°C cunner, and that the peak was missed by only sampling at 2 minutes post-stress cannot be ruled out. However, this is unlikely. Studies on low temperature compensation in the peripheral nerves of Antarctic fish species

demonstrate that the conduction of the nervous impulses is maintained down to temperatures well below 0°C (for a review, see MacDonald and Montgomery, 2005). Further, plasma epinephrine levels in 3°C - 6°C acclimated rainbow trout reach maximal levels by 2 min following epinephrine injection, and decrease to 50% of peak epinephrine levels by 5 min post injection (Gamperl et al., 1994b). This time-course is similar to that observed for 10°C acclimated fish (Tang and Boutilier, 1988).

Cunner acclimated to 0°C had relatively low resting plasma cortisol levels (~ 15 ng ml^{-1}) and displayed a robust cortisol response to stress (Figure 3.3). The values for resting cortisol suggest that prolonged exposure to 0°C does not represent a significant environmental stress for this species. This finding is in contrast to data for Atlantic cod where cortisol levels were still approximately 120 ng ml^{-1} after 18 days of exposure to 0°C (Staurnes et al., 1994), but not surprising given the cunner's ecology in Newfoundland. In this region of Canada, the cunner lives in near-shore areas year round, and water temperature of the ocean surface remains below 5°C for nearly 6 months of the year, 3 of which are around 0°C. The abrupt, and large, increase in plasma cortisol concentrations in response to acute handling stress in 0°C acclimated cunner strongly suggests that metabolic depression does not preclude a robust HPI axis response in fishes. Further, it contrasts with data for Antarctic (Whiteley and Egginton, 1999) and Arctic (Whiteley et al., 2006) fish species, and provides clear evidence that not all cold-adapted fish have a diminished cortisol response to acute stressors. What benefits the elevated post-stress cortisol concentrations would have for these

metabolically depressed fish, and why the HPI and HSC axes exhibit divergent responses to a standardized stressor, is not immediately apparent. However, it has been reported that behavioural coping strategy (reactive vs. proactive; Koolhaas et al., 1999) has a distinct effect on trout (*Oncorhynchus mykiss*) post-stress cortisol levels, and that fish that are high responders with respect to cortisol have diminished stress-induced catecholamine levels (Pottinger and Carrick, 1999; Øverli et al., 2005; Schjolden et al., 2005; Pottinger, 2006). These data show that the divergent stress response of cunner acclimated to 0°C is not unique amongst fishes, and raise the possibility that the cunner's primary stress responses may be partially influenced by their cryptic behaviour.

In contrast to the low resting plasma cortisol levels in 0°C cunner, those for 10°C acclimated fish were atypically high (~65 ng ml^{-1}: Gamperl et al., 1994a; Wendelaar Bonga, 1997) and did not respond to the imposed stressor (Figure 3.3). This unusual post-stress pattern of plasma cortisol concentrations in the 10°C acclimated fish raised concerns about the experimental methods used in this study. Cunner are much more active at water temperatures of 10°C compared to 0°C, and thus, it was possible that introduction of the netting 4 - 5 days prior to the experiment may have stressed the fish, and consequently caused the observed elevated resting cortisol levels. For this reason, resting and 2 hour post-stress plasma cortisol measurements were repeated on two additional groups of fish (n = 8 for each group) acclimated to 10°C: 1) one group held in a tank without netting; and 2) a group in an identical tank in which the netting was introduced 4 - 5 days

prior to sampling. The data (not shown), confirmed that resting plasma cortisol concentrations were elevated in 10°C cunner (~40 ng ml^{-1}) and that there was no increase in cortisol levels post-stress. These results strongly suggest that the elevated resting plasma cortisol levels, as well as the lack of a post-stress increase in cortisol in 10°C fish, was not related to the experimental design.

Although cunner are evolutionarily of tropical origin (i.e. a member of the Labridae), the elevated resting plasma cortisol levels in 10°C acclimated fish suggests that they have adapted physiologically and biochemically to the cold waters of Newfoundland, and are stressed by chronic exposure to water temperatures of 10°C and above. At present, there are no data on the upper thermal tolerance of cunner that can be used to further evaluate this hypothesis. However, results from studies on the stress physiology and thermal tolerance of Atlantic cod (*Gadus morhua*) support this conclusion. For example, several studies indicate that 16°C is the upper critical temperature for cod (Pörtner, 2002; Sartoris et al., 2003; Lannig et al., 2004; Gollock et al., 2006; Pérez-Casanova et al., 2008a and 2008b), and King et al. (2006) showed that the cortisol response to net stress in 14°C vs. 4°C acclimated cod is very similar to that shown by the 10°C and 0°C acclimated cunner in the present study. Specifically: 1) 14°C acclimated Atlantic cod had cortisol levels (~30 ng ml^{-1}) that were much higher than measured in 4°C acclimated fish (~5 ng ml^{-1}); 2) the fold increase in cortisol levels following the stressor was much lower in 14°C vs. 4°C acclimated cod (2 and 12 fold, respectively); and 3) post-stress cortisol levels in 14°C acclimated cod fell

significantly below those measured pre-stress for an extended period. In addition, this conclusion is supported by the work of Barton et al. (1987) on rainbow trout. These authors showed that rainbow trout fed cortisol-treated feed for 10 weeks (i.e. a model for chronic stress) did not show a post-stress (30 seconds handling stress) increase in cortisol levels, and had plasma cortisol levels that were significantly lower than pre-stress values from 3 hours post-stress onward. There are at least two possible explanations for why cortisol levels did not increase, or fell, in the 10°C acclimated cunner post-stress. First, Barton et al. (1987) showed that chronic cortisol elevation for 10 weeks prevented juvenile rainbow trout from increasing plasma cortisol levels following handling stress. Thus, it is possible that continuous negative feedback on the HPI axis, resulting from high resting cortisol concentrations, compromised the ability of 10°C acclimated cunner to elicit an interrenal response to a subsequent stimulus (Barton et al., 1987; Wendelaar Bonga, 1997; Mommsen et. al., 1999; Miller and O´Callaghan, 2002; Swaab et al., 2005). Second, both elevated temperatures and chronic stress have been shown to increase the rate of cortisol clearance from the plasma (Redding et al., 1984; Barton and Schreck, 1987; Wendelaar Bonga, 1997; Mommsen et. al., 1999).

3.4.2. The effect of temperature on StAR, P450scc and GR transcript levels

Transcript levels of P450*scc* were significantly higher (3.25 fold) in the head kidney of 10°C cunner compared to 0°C fish, and this magnitude of difference in transcript levels was also shown for StAR (Figure 3.4). The

positive relationship between StAR and P450*scc* transcript levels, and resting cortisol levels, was not surprising given the crucial role that these two genes play in steroidogenesis (Mommsen et al., 1999; Stocco, 2000; Sierra, 2004; Gelsin and Auperin, 2004; Arukwe, 2008), and that studies conducted on mammals and several fish species show that mRNA levels of StAR and P450*scc* tend to mirror those of plasma cortisol. For example, hypophysectomized rats have markedly lower levels of StAR and P450*scc* mRNA and adrenal steroids, whereas ACTH treatment results in significant increases in StAR mRNA levels and corticosterone secretion (Ariyoshi et al., 1998). Castillo et al. (2008) showed that sea bream (*Sparus aurata*) exposed to a crowding stress for 15 days had circulating cortisol concentrations of ~130 ng ml^{-1} and 2.5 fold higher levels of StAR transcripts compared to the control group. Kusakabe et al. (2002) reported that the increased plasma cortisol levels in rainbow trout following an acute stressor were accompanied by higher (2 fold) head kidney StAR transcript levels. Finally, Gelsin and Auperin (2004) showed that large post-stress increases in plasma cortisol were associated with an increase in StAR and P450*scc* mRNA levels in rainbow trout, and that the expression levels of these two genes were positively correlated. This led these authors to conclude that the two genes are subjected to a similar mechanism of transcriptional regulation.

In contrast to the higher levels of plasma cortisol and StAR and P450*scc* mRNA expression in 10°C acclimated fish, transcript levels of GR were significantly lower (1.70 fold and 1.29 fold) in the head kidney and liver of this group (Figure 3.5). Based on mammalian studies that show a positive

correlation between GR mRNA and protein expression (Vedeckis et al., 1989; Breslin et al., 2001), and on the numerous studies that have reported lower glucocorticoid receptor levels concomitant with elevated plasma cortisol (Maule and Schreck, 1991; Pottinger et al., 1994; Shrimpton and Randall, 1994; Mommsen et al., 1999; Shrimpton and McCormick, 1999; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003), this result suggests that the tissues of 10°C acclimated cunner had a decreased responsiveness to circulating cortisol levels, and that this was at least, in part, transcriptionally regulated. However, there is some uncertainty as to whether the lower GR mRNA levels reported for 10°C acclimated cunner were directly related to the elevated cortisol levels measured in these fish. For example, while Terova et al. (2005) reported that sea bass (*Dicentrarchus labrax*, L.) exposed to crowding stress (100 kg per m^3) for 3 months had cortisol concentrations of 280 ng ml^{-1} and significantly (4 fold) reduced GR mRNA levels, *in vivo* and *in vitro* studies on rainbow trout show that GR mRNA expression increases when hepatocytes are exposed to elevated cortisol levels (Satthiyaa and Vijayan, 2003; Vijayan et al., 2003). Further, several studies show that whether GR transcript levels increase or decrease post-stress is stressor- and time-dependent (Bears et al., 2006; Takahashi et al., 2006; Stolte et al., 2008). Clearly, additional experiments must be performed to determine to what extent temperature influences GR mRNA levels, and to define the relationships between circulating cortisol levels, GR mRNA expression and GR protein expression in this species.

3.4.3. Tissue distribution of StAR, P450scc and GR transcripts

QPCR analysis showed that high StAR and P450*scc* transcript levels were found in head kidney and ovary, but that several other tissues expressed these transcripts at much lower levels [StAR: brain, heart, liver and posterior kidney; P450*scc*: brain, posterior kidney and spleen (Figure 3.6)]. Although studies on several fish species (e.g. gilthead seabream and Atlantic cod) have shown that these two transcripts were expressed exclusively in the head kidney and gonads (Goetz et al., 2004; Kazeto et al., 2006; Castillo et al., 2008), other have shown that StAR and P450*scc* transcripts were detected in non-steroidogenic tissues, such as brain, heart, intestine, pyloric caecae, spleen and posterior kidney (Hsu et al., 2002; Kusakabe et al., 2002; Li et al., 2003; Nunez et al., 2005; Arukwe, 2008). These contrasting results suggest that StAR and P450*scc* tissue distribution may be species-specific. However, the physiological role played by the two transcripts in these other tissues has yet to be clarified. Transcript levels of StAR were much higher (15.27 fold) in head kidney than in ovary, whereas transcript levels for P450*scc* were similar in both tissues. The result for StAR is consistent with data for the gilthead seabream (Castillo et al., 2008). However, it is opposite to that observed for cod (Goetz et al., 2004), and Kazeto et al. (2006) showed that P450*scc* is more highly expressed in the kidney of Japanese eel than in the ovary. These inter-specific differences in transcript expression were not unexpected as there is significant variation in the stress responsiveness of fishes (Gamperl et al., 1994a; Wendelaar Bonga, 1997; Mommsen et al., 1999) and both Goetz et al. (2004) and Kazeto et al.

(2006) show that steroidogenic gene expression in the fish ovary is dependent on the state of maturation.

Glucocorticoid receptor-encoding transcript was ubiquitously expressed in all of the tissues examined (Figure 3.7). This finding is in agreement with previous studies on several fish species (e.g. gilthead seabream, rainbow trout and fathead minnow; *Pimephales promelas*) where GR transcripts were detected in adipose, brain, gill, gonad, heart, intestine, kidney, liver, muscle, pituitary, skin and spleen (Ducouret et al., 1995; Bury et al., 2003; Filby and Tyler, 2007; Acerete et al., 2007).

Chapter Four

Summary

There were two principal findings from this research. First, this study demonstrated that ontogeny has a pronounced impact on aspects of metabolic depression in cunner. This conclusion is based on data showing that: 1) the extent of metabolic depression was ~80% (Q_{10} = ~15) for young-of-the-year (YOY) fish, ~65 (Q_{10} = ~8) for small fish, but only ~55% (Q_{10} = ~5) for adults; and 2) the temperature at which metabolic depression was initiated in cunner increased with body size (age), i.e. 5°C in (YOY) cunner vs. 6°C and 7°C in small and adult fish, respectively. However, several research questions readily come to mind. The first question arising from this data is why do YOY cunner initiate metabolic depression at a lower temperature (later) than large fish? It is possible that, in contrast to older fish, YOY cunner do not have sufficient energy stores to sustain themselves over the winter and must feed as long as possible in the fall before metabolic depression is initiated. This hypothesis is supported by a study on a northern population of bluegill (*Lepomis macrochirus*) which showed that larger fish emerge from the winter starvation period in better energetic condition (amount of stored lipids) than smaller individuals (Cargnelli and Gross, 1997). There are two possible ways to examine if energy stores influence the capacity of timing of metabolic depression in cunner of different sizes (ages): 1) determine the total body energy content for cunner of varying sizes in the fall using proximate analysis; and 2) perform feeding trials where cunner feeding frequency/dietary energy

content are varied, and examine whether nutritional state has an effect on the temperature at which varying sizes of cunner enter metabolic depression. The second question is whether differences in the sensitivity of metabolism to temperature between cunner of varying sizes translates into variations in their upper thermal tolerance. This has recently been examined by Kelly (2010), and the results show that the Critical Thermal Maximum (CTM) of cunner increases with body mass. The CTM of large cunner was significantly higher than the CTM measured for both YOY and small fish (maximum difference over the size range measured ~2°C).

Second, this study demonstrates that acclimation temperature has a considerable effect on the hypothalamic-sympathetic-chromaffin-cell (HSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis of cunner. For example, 10°C acclimated cunner had higher (9.09 fold and 4.77 fold) post-stress epinephrine and norepinephrine levels, respectively, compared with 0°C acclimated fish. A robust HSC response would significantly increase the cunner's energy demand, and thus, the blunted response in 0°C acclimated cunner in a state of metabolic depression may be a strategy to conserve energy. However, more studies are needed to determine whether the low post-stress catecholamine levels of 0°C acclimated cunner are 'normal' for life in very cold waters (as suggested by work on Antarctic species) or directly related to being in a state of metabolic depression. Further, cunner acclimated to 0°C had relatively low resting plasma cortisol levels (~15 ng ml^{-1}) and displayed a robust cortisol response to stress, whereas 10°C acclimated fish had elevated resting plasma cortisol levels (~65 ng ml^{-1}) and did not respond

to the imposed stressor. The elevated resting cortical levels in 10°C acclimated cunner suggest that these members of the Labridae (predominantly a tropical fish family) are so adapted to the cold waters of Newfoundland that they are stressed by chronic exposure to water temperatures of 10°C and above. This is an interesting hypothesis that should be tested using cunner from various latitudes and acclimated to a wide range of temperatures. Finally, transcript levels of GR were 1.70 fold and 1.29 fold lower in the head kidney and liver, respectively, of 10°C acclimated cunner compared with 0°C acclimated fish. This result suggests that the cells of 10°C acclimated cunner had a decreased responsiveness to circulating cortisol levels. However, additional experiments are needed to determine whether the lower levels of GR mRNA reported for 10°C acclimated cunner were related to the elevated cortisol levels measured in these fish or acclimation temperature, and to define the relationships between circulating cortisol levels, GR mRNA expression and GR protein expression in this species.

In conclusion, there are various aspects of cold-induced metabolic depression in fish that could potentially be investigated using the cunner, not only due to their unique metabolic characteristics, but also because of their capacity to tolerate various environmental stressors.

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