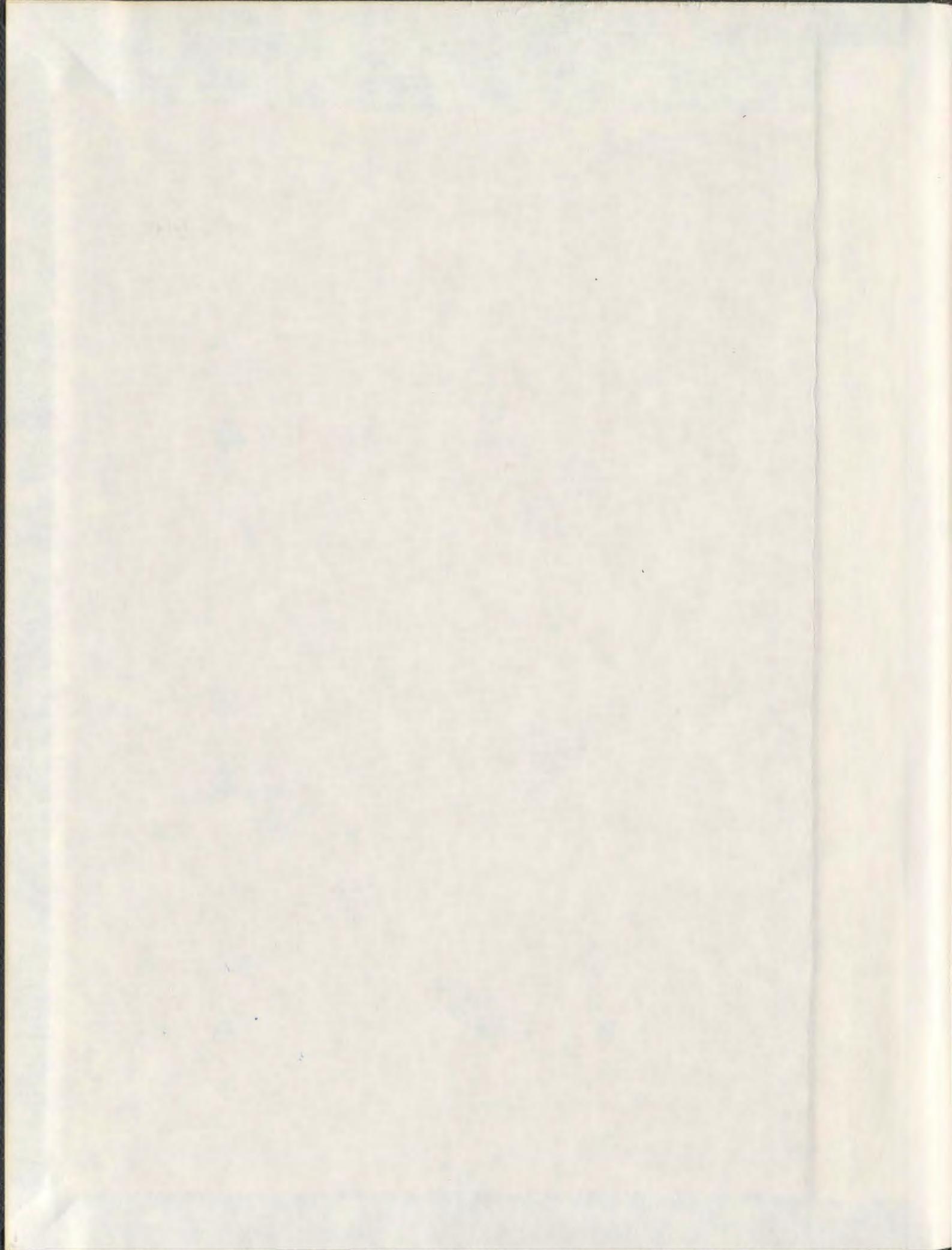


PROTEIN SYNTHESIS IN HYPOMETABOLIC FISHES

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**PROTEIN SYNTHESIS IN HYPOMETABOLIC FISHES**

by

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- ABSTRACT -

The objective of this doctoral thesis was to investigate protein synthesis in two species of hypometabolic teleosts: the north temperate labrid, *Tautoglabrus adspersus* (cunner) and the Amazonian cichlid, *Astronotus ocellatus* (oscar). The flooding dose methodology, which measures *in vivo* rates of tissue protein synthesis following the injection of a large dose of radiolabelled phenylalanine, was used to measure rates of amino acid incorporation at three key time points with respect to metabolic depression: 1) the entrance into metabolic depression; 2) while in a metabolically depressed state; and 3) during the return to regular activity levels. Additionally, rates of phenylalanine incorporation in the subcellular protein pool were determined in cunner in response to acute hypothermia and hypoxia. In general, an active decrease in protein synthesis (55-65%), in both the whole tissue and subcellular protein pools, accompanied the metabolic depression observed at the whole animal level. However, tissue specific responses in protein synthesis were evident in both species and appeared to play an adaptive role in extending survival time while in an energetically compromised state. Only a modest decrease in brain protein synthesis was observed in hypoxic oscar (30%), which may be linked to the maintenance low levels of activity for predator avoidance. Protein synthesis was defended in the mitochondrial protein pool of the cunner gills during both acute hypoxia and hypothermia challenges, suggesting its importance to the maintenance of ion and gas exchange in this tissue. In addition, a significant hyperactivation of liver protein synthesis occurs in metabolically depressed cunner, which may be associated with the production of antifreeze proteins at extreme low temperatures. During the post-dormancy recovery period a significant hyperactivation of protein synthesis occurred in white

muscle, heart and liver in eunner. In contrast, post-hypoxic oscars do not experience a hyperactivation in protein synthesis despite a significant increase in oxygen consumption during recovery. This latter result suggesting that the accumulation of a 'protein debt' is either stress specific or an artifact of the length of time spent in a hypometabolic state.

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### **- CO-AUTHORSHIP STATEMENT -**

In the following thesis work, I was responsible for intellectual design, carrying out of the experiments, the analysis of data (both biochemical and statistical), and the preparation of the manuscripts. The only exception to this is the work presented in Chapter Four. This experiment was part of a collaborative project with Isabel Costa, who was responsible for the measurement of routine metabolic rate in the oscar and for statistical analysis of this data. Isabel also assisted with the analysis of plasma lactate. Ms. Costa has graciously given permission to have her portion of the work included in this thesis as it completes the story of how the oscar has adapted to low oxygen stress at various levels of physiological organization.

**- PUBLICATION STATEMENT -**

The work presented in Chapter 3 has been published as the following paper: J.M. Lewis and W.R. Driedzic. (2007). Tissue specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperature labrid, *Tautoglabrus adspersus*. American Journal of Physiology, Regulatory and Integrative Physiology, 293: 474-481.

The work presented in Chapter 4 has been published as the following paper: J.M. Lewis, I. Costa, A.L. Val, V.M.F. Almeida-Val, A.K. Gamperl and W.R. Driedzic. (2007). Responses to hypoxia and recovery: repayment of oxygen debt is not associated with compensatory protein synthesis in the Amazonian cichlid, *Astronotus ocellatus*. Journal of Experimental Biology. 210: 1935-1943.

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**- ABBREVIATIONS AND SYMBOLS -**

<b>ATP</b>	adenosine tri-phosphate
<b>Ca<sup>2+</sup></b>	calcium
<b>CaCl<sub>2</sub></b>	calcium chloride
<b>CPM</b>	counts per minute
<b>CS</b>	citrate synthase
<b>DHAP</b>	dihydroxy-acetone phosphate
<b>DO</b>	dissolved oxygen
<b>DPM</b>	disintegrations per minute
<b>DTNB</b>	5,5'-dithiobis (2-nitrobenzoic acid)
<b>EDTA</b>	ethylenediamine tetraacetic acid
<b>eIFs</b>	eukaryotic initiation factors
<b>GPDH</b>	glycerol 3 phosphate dehydrogenase
<b>HEPES</b>	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
<b>KCl</b>	potassium chloride
<b>KCN</b>	potassium cyanide
<b>K<sub>2</sub>HPO<sub>4</sub></b>	dipotassium phosphate
<b>KH<sub>2</sub>PO<sub>4</sub></b>	potassium phosphate
<b>LDH</b>	lactate dehydrogenase
<b>NaCl</b>	sodium chloride
<b>NAD<sup>+</sup></b>	nicotinamide adenine dinucleotide
<b>NADH</b>	nicotinamide adenine dinucleotide reduced form
<b>NaHCO<sub>3</sub></b>	sodium bicarbonate

**Na<sub>2</sub>HPO<sub>4</sub>** sodium phosphate dibasic

**Na<sup>+</sup>/K<sup>+</sup> ATPase** sodium/potassium adenosine triphosphatase

**NaOH** sodium hydroxide

**MgSO<sub>4</sub>** magnesium sulphate

**PCA** perchloric acid

**Phe** phenylalanine

**Q<sub>10</sub>** temperature coefficient

**RNA** ribonucleic acid

**RoMR** routine metabolic rate

**SCCR** succinate cytochrome c reductase

**TCA cycle** tricarboxylic acid/citric acid cycle

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## - CHAPTER ONE -

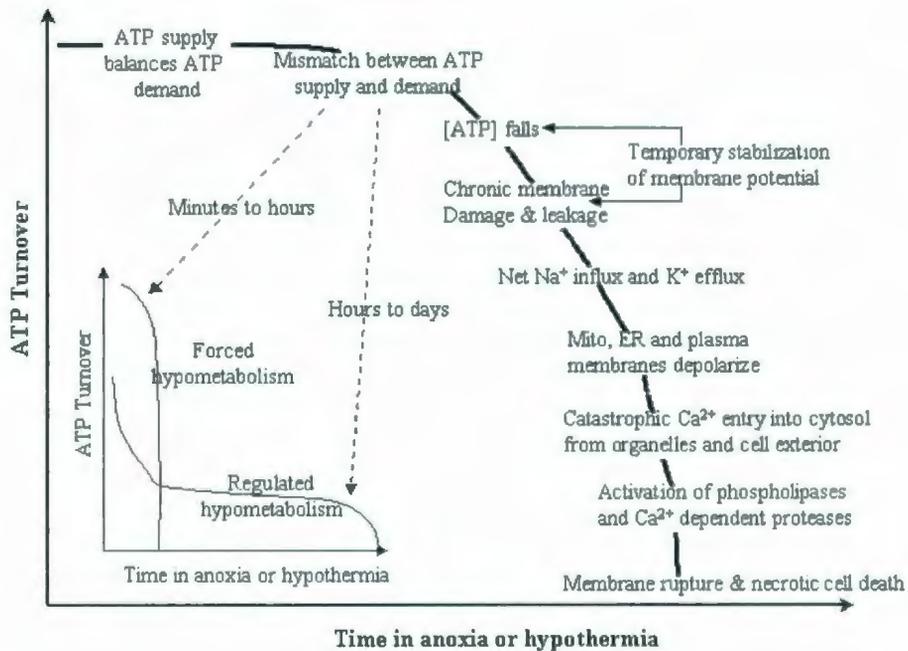
### INTRODUCTION

#### 1.1 Cellular Physiology of Metabolic Depression

The first observation of metabolic depression in animals dates back to 1702 when Antony van Leeuwenhoek documented that desiccated creatures named “animalcules” that appeared lifeless resumed regular activity when rehydrated (van Leeuwenhoek *in* Guppy 2004). Our understanding of metabolic depression has advanced greatly since this discovery of anhydrobiosis in invertebrates, and there is now a copious amount of literature available on the behavioural adaptations associated with mammalian hibernation, as well as on the physiological mechanisms and adaptive changes that allow for hypoxia/anoxia tolerance. Metabolic depression is now known to be a strategy that is widely used and conserved across animal phyla, and occurs in various organisms when exposed to short-term stress (diving response in mammals and reptiles and daily torpor exhibited by small mammals and birds) or long-term stressors ranging in duration from months (mammalian hibernation) to years (dormant eggs/cysts of invertebrates). As well, it can occur on a seasonal, opportunistic or preventative basis (Guppy et al. 1994; Guppy and Withers 1999; Guppy 2004; Storey 2007). Current research indicates the mechanisms utilized to cope with environmental challenges become more complex as the amount of time an organism is required to remain hypometabolic increases. The extent of whole animal metabolic depression ranges from 60-95% and is accompanied by reduced activity levels and feeding (Storey 2007). Often, animals will sequester themselves into sheltered areas whilst in a dormant state to protect themselves from the

threat of predation. Although mechanisms that allow organisms to cope with environmental stressors can be found at all levels of biological organization, it is now accepted that the mechanisms responsible for metabolic depression are found at the cellular level (Hochachka and Guppy 1987).

Metabolic depression is achieved through a coordinated net suppression of all cellular processes, and allows the organism to achieve a new, lower and balanced rate of ATP turnover when exposed to adverse conditions. If cells are unable to adjust physiologically to maintain the balance between ATP supply and demand, the cell will suffer irreversible damage that ultimately will lead to the organism's death (Boutilier 2001; Storey 2001; Storey and Storey 2004) (Figure 1.1).



**Figure 1.1** Schematic diagram of ATP turnover as a function of the duration of environmental stress. Main figure details the cascade of events that lead to cell necrosis. The inset demonstrates that the regulated suppression of ATP turnover (metabolic depression) extends survival time (recreated from Boutilier 2001).

In addition to the global suppression in metabolic rate there are several criteria that are necessary for long-term survival in a hypometabolic state: 1) storage of endogenous fuels such as glycogen and lipids, 2) the employment of alternative pathways for fuel usage in order to limit the accumulation of deleterious and toxic end products; 3) the coordination of metabolic responses in cells and organs through signal transduction mechanisms; 4) the initiation of defense mechanisms that stabilize macromolecules and promote long term survival, such as stress and antifreeze proteins; 5) changes in gene expression; and 6) differential regulation and prioritization of ATP consuming processes, such as ion pumping, growth and development and protein synthesis (Hochachka 1986, 1997; Boutilier and St-Pierre 2000; St-Pierre et al. 2000a; Boutilier 2001; Carey et al. 2003; Storey and Storey 2004; Storey 2007). Although the processes behind metabolic depression appear to be conserved across animal phyla, the main energy conserving mechanism used by a particular organism, or tissues within that organism, may vary in response to the environmental stress it encounters (Storey and Storey 2004).

Terms such as torpor, hibernation, dormancy and quiescence are used to refer to metabolic depression and the choice of term depends on the study animal or the environmental stress to which it is exposed. In some scientific fields, the terms torpor and hibernation are used specifically to refer to the metabolic depression observed in mammals during the winter and great concern is raised when hibernation or torpor are used when referring to periods of hypometabolism in ectotherms. The textbook definition of torpor is "a state of inactivity often with lowered body temperature and reduced metabolism that some homeotherms enter so as to conserve energy stores" (Randall et al. 1997). Hibernation is defined as "a period of deep torpor or winter

dormancy in animals in cold climates, lasting weeks or months” and dormancy as “the general term for reduced body activities including sleep, torpor, hibernation (winter sleep) and estivation” (Randall et al. 1997). Through comparison of the textbook definitions it becomes evident that the terms are quite similar. In fact, the term hibernation, when traced back to its Latin root, simply means to spend the winter in sequestration. Limiting the use of this term to only describe the overwintering behaviour of mammals is an artifact of the early studies in the field being limited to mammalian examples (Ultsch 1989). The focus of this thesis is hypothermia or hypoxia induced metabolic depression in teleosts; therefore, to avoid confusion or conflict the only terms that will be interchanged with “metabolic depression” will be hypometabolism or dormancy.

## **1.2 Role of Protein Turnover in Metabolic Depression**

In mammals, 90% of cellular respiration is mitochondrial, 20% of which is due to proton leak and 80% of which is used for ATP coupled processes. Of the 80% of respiration that is coupled with ATP, the two major energy consuming processes are protein synthesis and ion motive ATPases (specifically  $\text{Na}^+/\text{K}^+$  ATPase) (Rolfe and Brown 1997). The downregulation of these dominant energy consuming processes is now accepted to be largely responsible for allowing the cell to depress its metabolism (Boutilier 2001).

Protein synthesis comprises 18-30% of the whole animal metabolic rate for ATP coupled processes (Hand and Hardewig 1996; Rolfe and Brown 1997; Boutilier 2001). This contribution is increased even further when the cost of protein degradation is considered (35-41%) and RNA synthesis can account for an additional 11% (Land et al.

1993). Suppression of protein synthesis is an intrinsic response that plays an integral role in the metabolic depression of the cell, and does not just occur as a reaction to ATP limitation (Storey and Storey 2004). Recent studies demonstrate that the inhibition of protein synthesis, to approximately 10-50% of pre-dormancy levels, occurs in most tissues during metabolic depression in response to hypothermia, hypoxia/anoxia and desiccation in animals from all phyla (Table 1.1). These results indicate that the suppression of protein synthesis is a highly conserved and an integral mechanism to the global metabolic depression of the animal. As shown in the previous examples, protein synthesis is substantially reduced during hypometabolism, but there is little information available on the role of protein degradation in whole animal metabolic depression. As protein degradation can account for up to 22% of ATP turnover (Land et al. 1993; Land and Hochachka 1994) it must be reduced in addition to protein synthesis during metabolic depression. This reduction in protein degradation contributes towards the conservation of energy turnover at the whole animal level and limits the accumulation of nitrogenous waste (toxic by products of proteolysis). In addition, the prevention of a deficit in cellular protein facilitates a rapid recovery from extended periods of dormancy (Storey and Storey 2004).

Many researchers are focusing on identifying the mechanisms that control the reduction in protein turnover during metabolic depression; however, considerably more effort is needed before definite answers can be elucidated. Early evidence that intracellular  $Ca^{2+}$  levels, pH effects and changes in redox state play roles as signaling events for translational control is beginning to emerge (Hofmann and Hand 1994; Dorovkov et al. 2002). Also, studies on mammalian cell lines are pointing towards the

**Table 1.1** Comparison of the degree of protein synthesis inhibition during metabolic depression in various tissues and organisms. The rate of protein synthesis is expressed as a percentage of the control rate (normal level of activity) (Replicated from Storey and Storey 2004).

Species	Condition	Tissue	Protein Synthesis (% of Control)	Reference
<i>Spermophilus tridecemlineatus</i> (13-lined ground squirrel)	Hibernation	Brain	34	Frerichs et al. (1998) Hittel & Storey (2002) Hittel & Storey (2002)
		Kidney	15	
		Brown adipose tissue	104	
<i>Trachemys scripta</i> (red-eared slider turtle)	Anoxia	Hepatocytes	8	Land et al. (1993) Fraser et al. (2001)
		Heart, liver, brain, muscle & others	~0	
<i>Neobatrachus centralis</i> (Australian desert frog)	Aestivation	Liver	33	Fuery et al. (1998)
<i>Carassius carassius</i> (crucian carp)	Anoxia	Liver	5	Smith et al. (1996)
		Heart	52	
		Red muscle	52	
		White muscle	56	
<i>Helix aspersa</i> (land snail)	Aestivation	Hepatopancreas	23/30	Pakay et al. (2002)/Guppy et al. (2000)
		Foot Muscle	53	
<i>Littorina littorea</i> (common periwinkle)	Anoxia	Heart, liver, brain, muscle	~ 0	Larade & Storey (2002)
<i>Austrofundulus limnaeus</i> (killifish)	Diapause	Whole embryo	7	Podrabsky & Hand (1999, 2000)
<i>Artemia franciscana</i> (brine-shrimp)	Anoxia	Pre-emergent embryos	11	Hofmann & Hand (1994)

reversible phosphorylation of translational components (eukaryotic initiation factors, eIFs) and the state of the ribosome assembly as the regulating sites of protein synthesis in reversible bouts of metabolic depression (van Breukelen and Martin 2001; van Breukelen and Martin 2002; Storey and Storey 2004; van Breukelen et al. 2004). Recently, the idea that the regulation of transition into and out of the hypometabolic state is accompanied by specific changes in gene expression and protein turnover has become of great interest (Storey 1996).

In contrast to the global suppression in protein turnover in cells during metabolic depression, examples of the up-regulation of genes and the synthesis of *de novo* proteins have appeared through gene expression studies (Storey and Storey 2004). The upregulation of these genes, despite the global metabolic depression of cellular processes, has been linked to the protection of cellular metabolism and macromolecules, as well as the synthesis of *de novo* products that have stress-specific functions (such as heat shock or antifreeze proteins) (Storey 2007). An anticipatory up-regulation of genes has also been shown, where transcript levels of specific genes are elevated without a corresponding elevation in their protein product (Storey and Storey 2004; Storey 2007). The advantage of storing translationally ready transcripts for essential cellular processes may be linked to the facilitation of rapid post-dormancy recovery, which is equally as important to the survival of the animal as metabolic depression itself. Animals are required to emerge from dormancy in a fully functional state in order to avoid predation, forage for food to replenish fuel supplies and prepare for reproduction. With respect to protein synthesis this entails the breakdown of accumulated denatured proteins, the replacement of atrophied muscle protein from extended periods of inactivity, the

synthesis of new proteins to support growth and the resumption of regular activity levels. Research into the response of protein synthesis in the post-dormancy recovery period has been limited to only a few studies, but these studies indicate that a hyperactivation of protein synthesis rates occurs during arousal periods in some hypometabolic animals. For example, increases of 160% and 120% were measured during recovery in anoxic turtles, *Chrysemys picta* and hibernating ground squirrels, *Spermophilus lateralis*, respectively (Land and Hochachka 1994; van Breukelen and Martin 2001). Similar results were obtained from long tailed gophers (*Citellus undulatus*), in which a 1.5-2 fold increase in amino acid incorporation was measured during arousal from hibernation (Zhegunov et al. 1988). Where possible, the response of protein synthesis during the post-dormancy recovery period has been included in this thesis work to investigate if this mechanism, like the depression of protein synthesis during the onset of metabolic depression, is conserved across vertebrate classes.

### **1.3 Metabolic Depression in Fish**

In fish, examples of metabolic depression have been described in response to hypothermia, anaerobiosis and anhydrobiosis, but research is often limited to whole animal physiological responses. In periods of anhydrobiosis, African and South American lungfish (*Protopterus aethiopicus* and *Lepidosiren paradoxa*) burrow into the mud and wait out the dry season in a self-made cocoon. In both species of lungfish a reduction in metabolic rate, of approximately 50%, is accompanied by a suite of biochemical adaptations that include the accumulation of muscle glycogen for fuel storage and cessation of ammonia production due to a shift in metabolic processes

(Delaney et al. 1974; Mesquita-Saad et al. 2002). The crucian carp (*Carassius carassius*) and its close relative the goldfish (*Carassius auratus*) are frequently used as model organisms in the investigation of hypoxia/anoxia induced metabolic depression. The crucian carp is one of the most anoxia-tolerant vertebrates and is capable of surviving for several months in almost anoxic environments while overwintering in ice covered lakes (water temperatures approaching 0°C) (Nilsson and Renshaw 2004). In addition to the 70% reduction in whole animal metabolic rate (Smith et al. 1996), goldfish and crucian carp gills undergo morphological changes, which involve controlled apoptosis of intralamellar cells, that increase the surface area for gas exchange by approximately 7.5 fold (Sollid et al. 2003, 2005). As well, similar to other anoxia tolerant species, these fish accumulate substantial glycogen stores for energy supply via anaerobic metabolism. However, unlike other anoxia tolerant species some cyprinids possess the unique ability to prevent acidosis by converting pyruvate to ethanol as opposed to lactic acid (Shoubridge and Hochachka 1980).

Examples of hypothermia-induced metabolic depression in fish have not been as thoroughly investigated at the biochemical level. The existence of low temperature induced metabolic depression has been suggested to occur in the brown bullhead (*Ictalurus nebulosus*), the large mouth bass (*Micropterus salmoides*) (Crawshaw 1984), toadfish (*Opsanus tau*) (Matthews and Haschemeyer 1978), the American eel (*Anguilla anguilla*) (Walsh et al. 1983), goldsinny wrasse (*Ctenolabrus rupestris*) (Sayer and Davenport 1996) and the cunner (*Tautoglabrus adspersus*) (Green and Farwell 1971, Curan 1992). These studies have based their conclusions on visual observations of fish reducing their activity level, sequestering themselves into shelters/burrowing into

substrate and decreasing oxygen consumption to a greater extent than can be explained by temperature effects alone (i.e.  $Q_{10} > 2$ ). Unlike hypometabolic amphibians, reptiles and cyprinids, these fish all enter what appears to be a low temperature induced hypometabolic state without the confounding factor of low oxygen. To date there has been little to no investigation into the biochemical and regulatory mechanisms underlying the whole animal metabolic depression in these species, despite their aptness as model organisms for research in the field of metabolic depression.

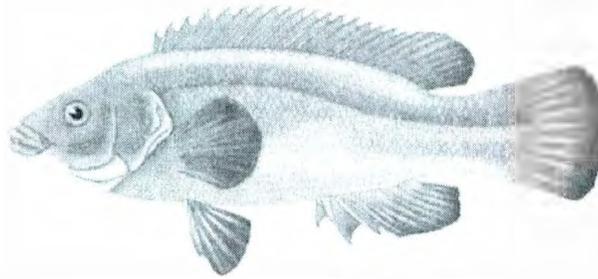
#### **1.4 Objective of Thesis**

The objective of this thesis was to further advance the study of metabolic depression in teleost fish beyond the behavioural and whole animal physiological level. To achieve this goal, a description of the contribution of protein synthesis at the whole tissue and subcellular level to the depression of whole animal metabolism was carried out. The role of protein synthesis was examined in two teleost species from the sub-order Labroidei which experience metabolic depression in response to varying types of environmental stress. The cunner (*Tautoglabrus adspersus*) a north temperate member of the Labridae family (Figure 1.2) was the main study species. The cunner utilizes metabolic depression as an adaptation to allow this northern member of a typically tropical family of teleosts to survive seasonal exposure to subzero water temperatures in its habitat during the winter. Changes in rates of protein synthesis and translational capacity (total RNA content) in response to the entrance into and recovery from winter dormancy (Chapter Three) were measured in the cunner. As well, the cunner was used to investigate if tissue specific protein synthesis at the sub-cellular level is reduced in response to varying environmental

stressors, one it is naturally exposed to (hypothermia) and one it is not (hypoxia) (Chapter Five). Ultimately, the aim of this chapter was to determine if metabolic depression is an intrinsic response that can be used to extend survival in the face of any unfavourable environmental condition, as well as to determine if adaptations to stress are conserved across the various levels of cellular organization.

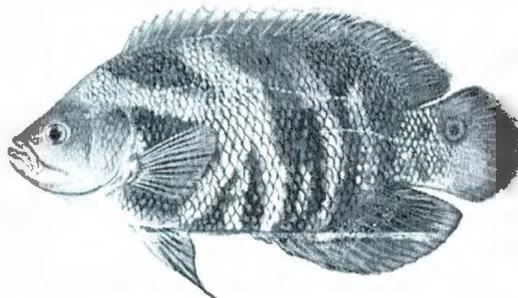
A comparative approach was also undertaken in this thesis through the inclusion of a second study species, the oscar (*Astronotus ocellatus*). The oscar, or acará-açu as it is known locally in Brazil, is a hypoxia tolerant Amazonian cichlid that is quickly emerging as suitable alternative study species to the cyprinids for studying hypoxia induced metabolic depression in teleosts. The oscar resides in the floodplains of the Amazon basin, and is capable of surviving hypoxia at elevated water temperatures (28°C) on a diurnal and seasonal basis (in contrast to the low temperatures which accompany hypoxia stress in the cyprinids). Chapter Four describes the response of protein synthesis in the oscar in relation to whole animal metabolic depression (oxygen consumption rate), during the entrance into and recovery from acute hypoxia exposure.

Through the comparison of results between these two teleost species, and with hypometabolic animals in general, this thesis investigates if there is a tissue specific response in protein synthesis during the metabolic depression and recovery. As well, it will provide initial insight into the contribution of protein synthesis to the metabolic depression observed at the whole animal level and if this response is conserved across environmental stresses and levels of subcellular organization.



**Figure 1.2** Illustration of the north temperate Labrid, *Tautoglabrus adspersus*. Common name: cunner.

([www.fishbase.org](http://www.fishbase.org)).



**Figure 1.3** Illustration of the Amazonian cichlid, *Astronotus ocellatus*. Common names: oscar, acará-açu.

([www.myfwc.com](http://www.myfwc.com)).

## -CHAPTER TWO-

### **Preliminary assessment of protein synthesis in the cunner, *Tautoglabrus adspersus* using flooding dose methodology.**

#### **2.1 Introduction**

The majority of studies that investigate *in vivo* rates of protein synthesis measure the flux of an amino acid or nitrogen that has been labeled with a tracer substance, such as a  $^3\text{H}$ ,  $^{14}\text{C}$  or  $^{15}\text{N}$  (Houlihan et al. 1995a). Several methods have been developed that use amino acid administration to measure protein synthesis, but the most reliable and frequently used is the "flooding method" which was first described in 1950's by Loftfield and colleagues (Keller et al. 1954; Loftfield and Eigner 1958). The principle behind this approach is that the injection of labeled amino acid contained in a solution of unlabelled amino acid, in amount greater than the endogenous free pool (i.e. a flooding dose), will allow the specific activities in all the free amino acid pools (plasma and intracellular) to be equal. This enables the specific activities of all the free pools to be elevated to the same extent as well as remaining constant for an extended period after injection (Garlick et al. 1994). The incorporation of the labeled amino acid into protein can be measured over a period of time giving an average rate of protein synthesis (Houlihan et al. 1995a).

The flooding dose methodology utilized in recent studies is commonly based upon the approach developed by P.J. Garlick et al. (1980) using [ $^3\text{H}$ ] phenylalanine as the tracer substance. Phenylalanine has become the preferred amino acid as it has a small tissue free pool and is highly soluble; therefore, making it easier to deliver a flooding dose in a smaller injection volume (Houlihan et al. 1995a). As well, simple and reliable

assays exist for the analysis of phenylalanine content in tissues (McCaman and Robins 1962; Gerasimova et al. 1989), and it has been shown in mammals that the injection of a flooding dose of phenylalanine neither stimulates nor inhibits protein synthesis (Garlick et al. 1994). Accurate interpretation of rates of protein synthesis through use of the flooding dose methodology is contingent upon the fulfillment of several validation criteria: i) the injection dosage enables the tracer amino acid to fully flood and rapidly equilibrate with the intracellular free pool; ii) the specific radioactivity of the free pool remains elevated and stable over the period of time which protein synthesis is measured; iii) the labeling of tissue protein begins immediately post-injection and is linear over the incorporation time (Garlick et al. 1980; Houlihan et al. 1995a).

The time scale chosen to measure protein synthesis has ranged from minutes up to 24 hours depending on the study species and environmental condition (Houlihan et al. 1995a and references within). Protein synthesis, like many other physiological processes, is highly temperature dependent (Houlihan et al. 1995a), and rates of protein synthesis are typically higher in endotherms than ectotherms (Sayegh and Lajtha 1989). In fish, a wide range of incorporation times have been reported, which is not surprising given the ability of teleosts to adapt to varying environmental conditions and the sensitivity of protein synthesis to nutritional state, developmental stage/body size, environmental oxygen levels and temperature (nutritional state: Smith 1981; Houlihan et al. 1989; Lied et al. 1983; Lyndon et al. 1992; McMillan and Houlihan 1992; Lyndon et al. 1993; developmental state/body size: Millward et al. 1981; Houlihan et al. 1986; Houlihan et al. 1995b; Carter et al. 1998; Peragón et al. 1998; environmental oxygen levels: Buc-Calderon et al. 1993; Smith et al. 1996; Smith et al. 1999; temperature: Das and Prosser

1967; Haschemeyer 1968; Lajtha and Sershen 1975; Matthews and Haschemeyer 1978; Haschemeyer et al. 1979; Smith and Haschemeyer 1980; Haschemeyer 1983; Fauconneau and Arnal 1985; Loughna and Goldspink 1985; Watt et al. 1988; Foster et al. 1992; Sephton and Driedzie 1995; McCarthy et al. 1999; West and Driedzie 1999; Storeh et al. 2005; Treberg et al. 2005). Due to the high variability in rates of protein synthesis it is prudent that the validation criteria are demonstrated to be successfully fulfilled for each new species and environmental condition.

## **2.2 Use of flooding dose methodology for *Tautogolabrus adspersus***

As this was the first attempt to measure protein synthesis in the cunner (*Tautogolabrus adspersus*), preliminary analysis of protein synthesis via the flooding dose method was conducted at 14°C (August) in order to determine an appropriate incorporation time before beginning the seasonal experiment. This was especially important given the seasonal experiment would measure protein synthesis in fish from 8°C in the fall of the year (November) until water temperatures returned to 8°C in the summer (July), which would include measurement at subzero temperatures at the lower end of the fish's temperature range. Measurements of protein synthesis in Atlantic cod (*Gadus morhua*) were shown to be successful over a similar range of water temperatures using incubation times of up to nine hours (Treberg et al. 2005). Compared to the Atlantic cod, the cunner is a relatively sluggish species that has a substantially lower resting metabolic rate (40% lower based on mass independent oxygen consumption measurements) (Costa 2003). In addition, cunner are observed to enter a torpid-like state at low water temperatures (Green and Farwell 1971), which would potentially lower rates of protein synthesis even further.

Therefore, the incubation time was extended and protein synthesis was measured from two to twenty hours post-injection to accommodate the lower metabolic rate of this species.

### *2.2.1 Animal Collection and Sampling*

Cunner were collected in Portugal Cove (Newfoundland, Canada) in August 2003 using a hoop net and transported to the Ocean Sciences Centre where they were held in 1 m<sup>2</sup> tanks supplied with flow through seawater from an ambient water source and a natural photoperiod. Fish were fed chopped herring ad libitum one to two times a week, with uneaten food removed the following day. After a two week acclimation period, sixteen cunner were randomly selected from the tanks and tagged for individual recognition, by attaching coloured beads to the bottom of the dorsal fin where it meets the dorsal musculature. Fish were then placed in a separate 1 m<sup>2</sup> experimental tank for one week prior to sampling, during this time food was withheld to ensure complete clearance of the gut. On the day of sampling, fish were injected intraperitoneally with 1.0 ml 100 g<sup>-1</sup> of [2,3-<sup>3</sup>H] phenylalanine (Amersham International) solution and returned to their tanks. The injection solution consisted of 135 mM phenylalanine in addition to sufficient [2,3-<sup>3</sup>H] phenylalanine to ensure a dosage of 100 µCi ml<sup>-1</sup> in a saline solution containing: 150 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 10 mM NaHCO<sub>3</sub>, 5 mM D-glucose, 5 mM HEPES and 2 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8 at 20°C (as described in Treberg et al. 2005). After an incubation time of 2, 4, 6 or 20 hours, four fish at each time were killed by a blow to the head followed by a severing of the spine. Brain, liver and white muscle were excised, in that order, blotted dry and frozen in liquid nitrogen. Tissues were also excised

from un-injected cunner (n = 4) to obtain endogenous levels of free phenylalanine to determine if the concentration of phenylalanine in the injection bolus was sufficient to fully flood the free amino acid pool. All samples were stored at  $-70^{\circ}\text{C}$  until analysis.

### *2.2.2 Sample Preparation and Scintillation counting*

Approximately 100 mg of each tissue was homogenized with a Polytron in 9 volumes of 6% perchloric acid (PCA) except for liver, which was homogenized in 4 volumes of PCA. Homogenized samples were left on ice for 10-15 minutes, after thoroughly mixing a 1 ml aliquot was transferred to a microcentrifuge tube and centrifuged for 5 minutes at  $15,600 \times g$  to separate the free pool (supernatant) from the protein bound phenylalanine (pellet). The supernatant was removed and frozen at  $-20^{\circ}\text{C}$  until analysis for the free pool phenylalanine content and specific radioactivity. The protein pellet was re-suspended, and washed in 1.0 ml of 6% PCA, by vortexing and then centrifuging as described above. The supernatant was then discarded, and the wash step was repeated until the radioactivity in the discarded supernatant was at background levels. This ensured that only protein bound  $^3\text{H}$  phenylalanine was being measured in the protein pellet. After sufficient washing, 1.0 ml of 0.3 M NaOH was added to the tube containing the protein pellet, the protein pellet was incubated in a water bath held at  $37^{\circ}\text{C}$  until fully dissolved, and the dissolved protein was stored at  $-20^{\circ}\text{C}$  until analysis for protein content and protein bound radioactivity. Aliquots of the original supernatant from the homogenized tissue and the dissolved protein were added to 10 ml of Ecolume scintillation cocktail and counted on a Packard Tri-carb 2100TR liquid scintillation counter to obtain the [2,3- $^3\text{H}$ ] phenylalanine content of the free and protein bound phenylalanine pools, respectively.

### *2.2.3 Biochemical Assays*

Free pool phenylalanine content was measured from the PCA extraction supernatant and phenylalanine standards in 6% PCA by a fluorometric assay following the protocol described in McCaman and Robins (1962). Protein content of the tissue was determined from the NaOH solubilized protein pellet using the BioRad D<sub>c</sub> kit (Bio-Rad Laboratories, California), based on the Lowry assay, using standards made from bovine serum albumin.

### *2.2.4 Statistical Analysis*

Mean tissue phenylalanine content and specific activity were compared between sample times using a one-way ANOVA with Tukey's post-hoc tests for multiple comparisons. The incorporation of radioactivity into protein was determined by linear regression. Data were log-transformed when necessary. In all cases  $p < 0.05$  was considered significant and data is expressed as mean  $\pm$  SEM.

## **2.3 Results**

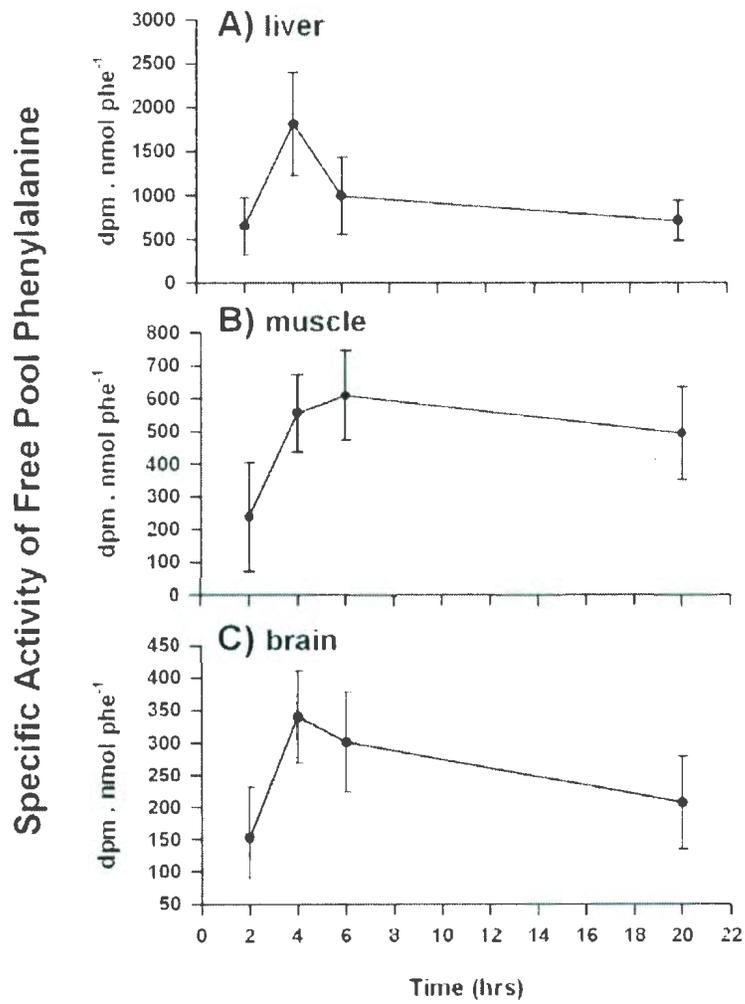
### *2.3.1 Flooding of Free Amino Acid Pool*

The injection dosage, which contained 135 mM per 100 g<sup>-1</sup> body mass of unlabeled phenylalanine, should theoretically increase the free-pool phenylalanine content to the same extent. Baseline levels of phenylalanine obtained from non-injected cunner were 0.12  $\pm$  0.02, 0.14  $\pm$  0.04, 0.09  $\pm$  0.01 nmol  $\cdot$  mg<sup>-1</sup> fresh tissue for liver, white muscle and brain, respectively. Results for the post injection phenylalanine content were pooled for all time points (n=16) as values were not significantly different over time, within a tissue. The free phenylalanine content was approximately 8-fold higher in all tissues post

injection,  $1.00 \pm 0.09$ ,  $1.24 \pm 0.25$ ,  $0.71 \pm 0.08$  nmol · mg<sup>-1</sup> in liver, white muscle and brain tissue, respectively. The significant elevation of free pool phenylalanine post-injection indicates the injection bolus was successful in flooding the free pool over the full twenty hours.

### *2.3.2 Specific Activity of Free Phenylalanine Pool*

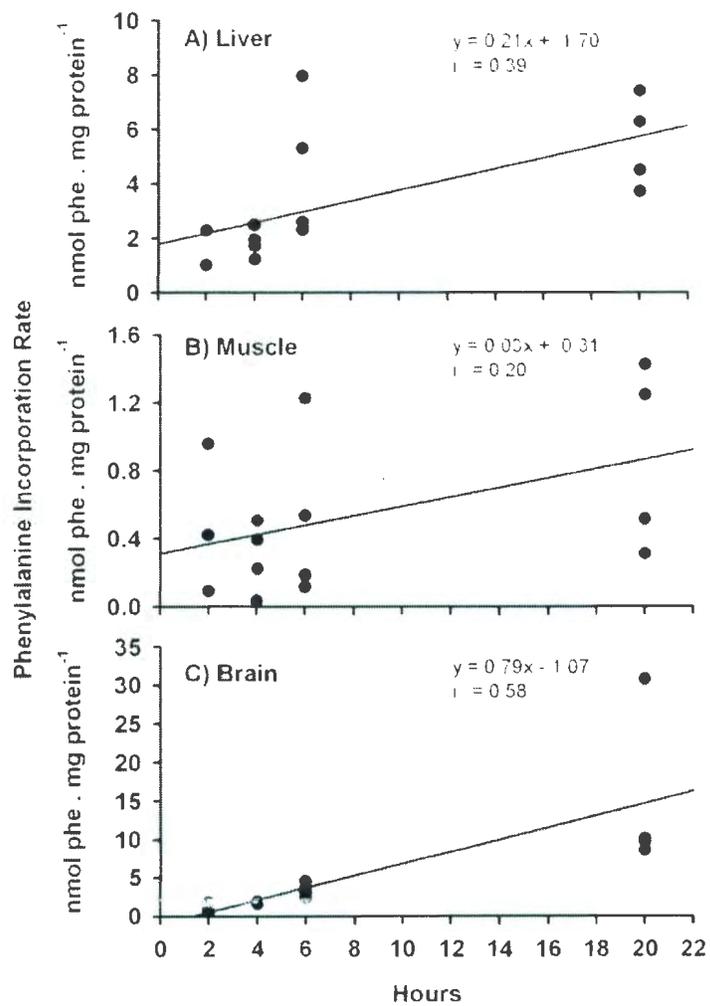
The second validation criterion dictates that intracellular free pool radioactivity (dpm · nmol phe<sup>-1</sup>) should increase rapidly post-injection and remain elevated and stable for the time period over which it is being measured. The specific activity of free phenylalanine was calculated from the radioactivity in the protein-free supernatant and the concentration of free phenylalanine. In the cunner, there were no significant differences in the specific activities of the intracellular free-pool over the twenty hours (Figure 2.1). However, the data suggests that in future experiments (described in Chapters Three and Five), which are to be conducted at lower temperatures (between 8°C and 0°C) the specific activity at the two hour time point may not be fully elevated.



**Figure 2.1** Post-injection time course for specific radioactivity of the free pool phenylalanine in cunner at 14°C for liver (A), muscle (B) and brain (C). Values are means  $\pm$  SEM,  $p < 0.05$  is significant.

### *2.3.3 Linear Incorporation of Phenylalanine into Protein*

To determine the incorporation of phenylalanine into the whole tissue protein pool incubation times between 2 and 20 hours were used. Regression analysis showed that the incorporation of phenylalanine was linear with time (2-20 hours) for liver ( $p = 0.017$ ) and brain tissue ( $p = 0.001$ ). Due to high individual variation a significant linear incorporation was not achieved in white muscle from 2 to 20 hours, but this relationship approached significance when incorporation from 4 to 20 hours was analysed ( $p = 0.09$ ) (Figure 2.2). Further, in all three tissues, the y-intercepts of the fitted lines were not significantly different from zero indicating that incorporation began immediately post-injection. Whole tissue incorporation rates were  $0.56 \pm 0.09$ ,  $0.07 \pm 0.02$  and  $0.58 \pm 0.08$  nmol phe  $\cdot$  mg  $\text{pr}^{-1} \cdot$  hr $^{-1}$  for liver, white muscle and brain tissue, respectively.



**Figure 2.2** Post-injection time course for the incorporation of radiolabeled phenylalanine into the protein pool in cunner at 14 °C for (A) liver, (B) white muscle and (C) brain. Individual values are plotted, n = 4 fish at each sample time.

## 2.4 Discussion

Successful analysis of *in vivo* protein synthesis rates in an organism via the flooding dose methodology is contingent upon fulfillment of the validation criteria. Demonstrating the fulfillment of these criteria becomes particularly important when measuring protein synthesis in a new species or under varying experimental conditions. The purpose of this preliminary work was to determine a time period over which protein synthesis could be measured for a hypometabolic teleost species with the expectation that future experiments would be carried out at the extreme low temperatures at which this fish is exposed to in its environment. As predicted, a much longer incubation time than is the norm for this procedure in mammals and other ectothermic animals (between 40 minutes and 6 hours) (Houlihan et al. 1995a) was necessary to accurately measure protein synthesis in the cunner. The dosage injected was successful in flooding the phenylalanine pool and allowing the specific activities of the free phenylalanine pool to be fully elevated and stable over the twenty-hour incubation time in the cunner in all three tissues (Figure 2.1). The determination of the specific activity of the free phenylalanine ( $\text{dpm} \cdot \text{nmol phe}^{-1}$ ) is contingent upon the assumption that phenylalanine was not being catabolised and that the radioactivity being measured is incorporated into phenylalanine. It has been shown that the flooding dose of phenylalanine does not impact rates of protein synthesis (Garlick et al. 1980) and a recent study has shown through HPLC analysis that there was no significant conversion of phenylalanine to other compounds, specifically tyrosine up to eight hours post-injection (Pakay et al. 2002). If phenylalanine was being catabolised, the label would be ending up in lipid intermediates, gluconeogenic precursors or oxidized by the mitochondria and would be exhibited as a

marked decrease in phenylalanine content and specific activity. Our results, which show stability of the free phenylalanine content ( $\text{nmol} \cdot \text{mg tissue}^{-1}$ ) and specific radioactivity in cunner over the full incubation time, suggest that the radiolabel is retained in the form of phenylalanine. The incorporation of phenylalanine into the various tissues was significant and linear between 2 and 20 hours post-injection for liver and brain, but not for white muscle (Figure 2.2). However, the incorporation of phenylalanine in the white muscle protein pool did approach linearity between 4 and 20 hours post-injection. The slower rate of phenylalanine uptake in the white muscle of the cunner is consistent with previous studies that show low protein turnover in white muscle as compared to other tissues (Poernjic et al. 1983, Sayegh and Lajtha 1989, Houlihan 1991, Storch et al. 2005). Rates of protein synthesis in the liver of the cunner were approximately 1.5 times lower than rates reported for Atlantic cod liver, despite being measured at a higher temperature ( $14^{\circ}\text{C}$  for the cunner as compared to  $11^{\circ}\text{C}$  for the cod) (Treberg et al. 2005). Nevertheless, as all the validation criteria were fulfilled, the reduced rates of protein synthesis in the tissues of the cunner are not a result of ineffective application of the “flooding method” approach to measuring protein synthesis. Rather these lower rates of phenylalanine incorporation are most likely due to the lower metabolic rate and sluggish lifestyle of the cunner compared to other North Atlantic teleosts (Costa 2003). The need for an extended incorporation time in this fish may result in a slight underestimation the rate of protein synthesis due to amino acid recycling, particularly in tissues of higher rates of protein turnover such as the liver. However, as rates of protein synthesis obtained in the liver and brain remain linear and do not plateau or decline, amino acid recycling does not seem to be a source of error up to twenty hours post injection.

In conclusion, this preliminary study demonstrated that the measurement of protein synthesis in the cunner required a longer incubation time than has been previously used for teleosts. Based on results from this study showing that the specific activity of the free phenylalanine may not have been fully elevated at two hours post-injection, it was decided to extend the incubation time to allow the measurement of protein synthesis from four to twenty-four hours in further studies. The extended incorporation time became particularly important for the seasonal study, which tracked rates of protein synthesis in cunner over water temperatures ranging from 8°C to 0°C, which included its winter dormancy and recovery periods.

## - CHAPTER THREE -

### **Tissue specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperate labrid, *Tautoglabrus adspersus*.**

#### **3.1 Introduction**

Animals utilize metabolic depression as a way to survive periods of unfavourable environmental conditions such as low temperature, hypoxia and desiccation. Behavioral studies suggest the cunner (*Tautoglabrus adspersus*), a north temperate member of the mainly tropical labridae family of teleosts that is indigenous to the North-West Atlantic Ocean, enters a period of inactivity once water temperature decreases to 4°C in the fall of the year. During this time cunner aggregate in rock crevices, remain motionless and refrain from feeding until water temperature returns to 4°C in the spring (Green and Farwell 1971). Measurements of physiological parameters during the period of behavioral depression are sparse; however, the metabolic rate of cunner in the winter was found to be approximately 75% lower than in summer (Hauggaard and Irving 1948). More recently a study which tracked oxygen consumption rates in cunner over a full year has shown an 83% depression in resting metabolism during winter, with a  $Q_{10}$  value of 9.9 between 5°C and 0°C (Costa 2007). This elevated  $Q_{10}$  value suggests the winter dormancy in cunner observed by Green and Farwell (1971) to be a result of an active depression in metabolism. In the spring of the year, a significant increase in metabolic rate was observed at 5°C (Costa 2007), which supports the observed increase in activity and feeding of these fish at 4°C in the spring (Green and Farwell 1971). A similar active depression in metabolic rate was not found in the brown bullhead (*Ictalurus nebulosus*) or the largemouth bass (*Micropterus salmoides*) between 17°C and 3°C, despite the

behavioral observations of dormancy at low temperatures (Crawshaw 1984). The occurrence of metabolic depression in the cunner provides a novel model to study the biochemical mechanisms behind hypothermic metabolic depression in an ectothermic vertebrate, without the confounding factor of low oxygen as in turtles or temperate amphibians.

The synthesis of macromolecules, such as protein and RNA is a major contributor to overall metabolic rate, with protein synthesis accounting for 18-30% and RNA synthesis accounting for approximately 10% of cellular energy expenditure (Hawkins 1991; Guppy et al. 1994; Rolfe and Brown 1997). As such, it is not surprising these two processes have been demonstrated to be sensitive to ATP supply (Buttgereit and Brand 1995; Wieser and Krumschnabel 2001). Several studies have investigated tissue specific changes in rates of protein synthesis during metabolic depression in various vertebrate classes in response to a plethora of environmental stressors, and have demonstrated levels of depression ranging from approximately 50% to a complete suppression of the synthesis of protein (Yacoe 1983; Zhegunov et al. 1998; Land et al. 1993; Bailey and Driedzic 1996; Smith et al. 1996; Frerichs et al. 1998; Fuery et al. 1998; Fraser et al. 2001). Of those vertebrate classes that have been studied, information on *in vivo* protein synthesis rates and metabolic depression in teleosts is limited with only one study (Smith et al. 1996) investigating the changes in protein synthesis during anoxia induced metabolic depression in crucian carp. This investigation of the seasonal changes in tissue specific protein synthesis rates in the cunner is the first study to investigate *in vivo* protein synthesis in a teleost in relation to hypothermia induced metabolic depression.

Hyperactivity during arousal from metabolic depression is commonly observed at the behavioral level as increased feeding and activity; however, research into the biochemical changes during the recovery period is limited. Those few studies that have investigated changes in protein synthesis during the recovery period have provided contradictory results. *In vitro* studies on various tissues from hibernating ground squirrels and on hepatocytes from anoxia tolerant turtles have shown a definite hyperactivation of protein synthesis during recovery (Zhegunov et al. 1988; Land et al. 1993). In contrast, the only *in vivo* analysis of protein synthesis, which was also on the anoxia tolerant red-eared slider turtle, did not show any hyperactivation of protein synthesis rates after acute anoxia exposure (Fraser et al. 2001). These disparate results may indicate a problem with comparing *in vitro* versus *in vivo* applications, or may be due to the varying exposure time to the environmental stress.

The objective of the present study was to utilize the cunner as a novel model to investigate changes in protein synthesis in relation to the observed metabolic depression that occurs in response to seasonal low temperatures. Distinct time periods over the annual temperature cycle were selected for measurement: periods of normal activity before metabolic depression, entrance into and during winter dormancy and during the recovery period. It was hypothesized that rates of protein synthesis in the cunner would be actively depressed to levels greater than would be predicted from temperature effects alone and this change would be supported by changes in the capacity of the tissue to synthesize proteins. During the recovery period, a hyperactivation of *in vivo* rates of protein synthesis was expected to occur in order to repay the protein debt accumulated during the lengthy winter dormancy experienced by cunner.

## **3.2 Materials and Methods**

### *3.2.1 Animal Collection*

Cunner were collected via hoop net in Portugal Cove (Newfoundland, Canada) in August 2003 and transported to the Ocean Sciences Centre where they were held in tanks with flow through seawater from an ambient water source and exposed to a natural photoperiod through room lighting that was controlled by a natural photosensor. Cunner were fed chopped herring ad libitum one or two times a week while animals were active. During winter dormancy cunner do not normally feed; however, food was offered once a week with uneaten food removed the following day. All animals in this study were held and treated in accordance with Canadian Council of Animal Care guidelines.

### *3.2.2 Validation of the Flooding Dose Methodology*

The measurement of protein synthesis was achieved by administering a flooding dose of radiolabeled phenylalanine based upon the methods first described in Garlick et al. (1980) and since used extensively for the measurement of protein synthesis in teleosts (Houlihan 1991 and references within). Preliminary work was conducted to determine an appropriate incubation time over which protein synthesis could be measured in cunner due to its low metabolism and sluggish lifestyle compared to other North Atlantic teleost species (Chapter Two). It was determined that an incubation time of four to twenty-four hours would be necessary for successful application of the “flooding method” approach in the cunner.

### *3.2.3 Seasonal Changes in Tissue Specific Rates of Protein Synthesis*

Rates of protein synthesis rates in cunner were measured at five key points relating to various states of activity, from October 2003 through August 2004: 8°C (November) when fish were fully active and feeding; 4°C (December) during the entrance into winter dormancy; 0°C (March) while fish are fully dormant; 4°C (June) when fish begin recovery from winter dormancy and 8°C (July) once fish had returned to an active state and resumed feeding. Fish were allowed to reach the above experimental temperatures by following the ambient water temperature cycle. Figure 3.1 shows a seasonal ambient water temperature profile for Logy Bay, Newfoundland which is the source of water for the Ocean Sciences Centre and is representative of the seasonal cycling of water temperatures of inshore waters along the Newfoundland coast. Once the desired experimental temperature was reached, fish (92 – 270 g, average  $174 \pm 5.1$  g) were randomly selected, tagged for individual recognition and placed in a separate experimental tank one week prior to sampling. Water temperature in this tank was maintained at the experimental temperature by mixing ambient with either heated or chilled seawater. During this time, food was withheld to ensure complete clearance of the gut. After one week of confinement, fish were injected intraperitoneally with 1.0 ml  $100 \text{ g}^{-1}$  of [2, 3- $^3\text{H}$ ] phenylalanine (Amersham International) solution. This injection solution consisted of 135 mM phenylalanine in addition to sufficient [2,3- $^3\text{H}$ ] phenylalanine to ensure a dosage of  $100 \mu\text{Ci ml}^{-1}$  in a saline solution containing 150 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , 10 mM  $\text{NaHCO}_3$ , 5 mM D-glucose, 5 mM HEPES and 2 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.8 at 20°C. Fish were immediately placed back into the experimental tank post-injection and based on behavioural observations returned

to a quiescent state within minutes. After an incubation time of 4, 8, 16 or 24 hours, four fish were killed by a blow to the head followed by a severing of the spine. Whole brain, whole liver and heart ventricular tissue were excised, blotted dry and immediately frozen in liquid nitrogen. A section of white muscle was taken from the dorsal musculature above the lateral line, care was taken to remove skin and scales and the tissue was then frozen in liquid nitrogen. Gills were excised and lamellae were scraped from the gill arches, the lamellar tissue was then flash frozen in liquid nitrogen. Samples were stored at  $-70^{\circ}\text{C}$  until analysis.

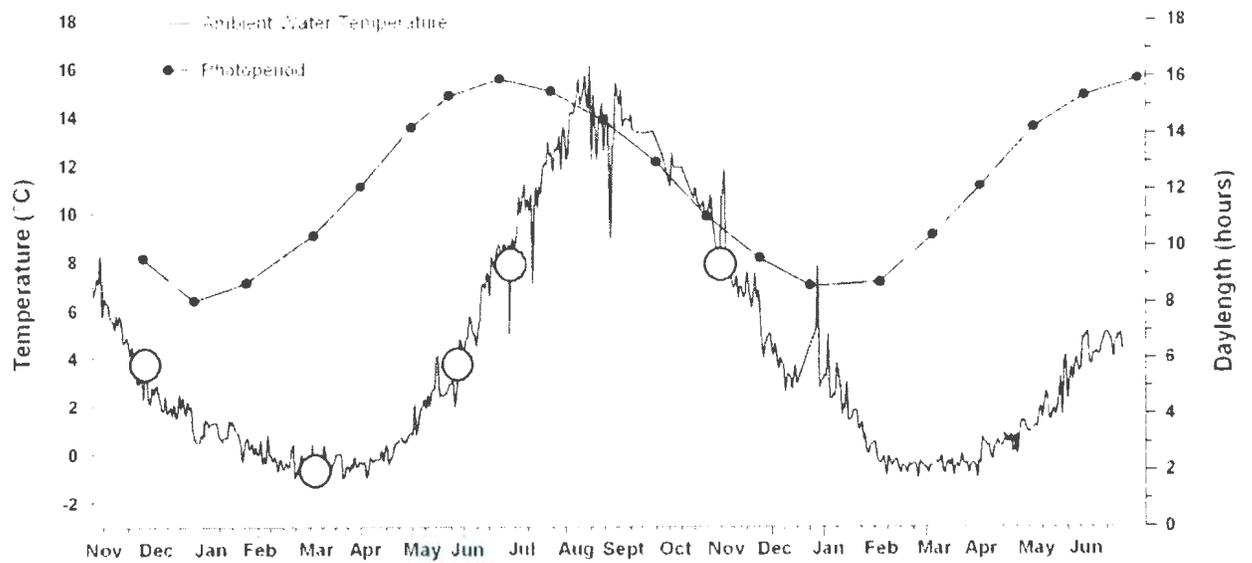
#### *3.2.4 Sample Preparation and Biochemical Analyses*

Samples were prepared and analysed for specific and protein bound radioactivity as previously described in Chapter Two.

#### *3.2.5 Determination of Total RNA Content*

A second cohort of cunner of comparable size to the fish used for the analysis of protein synthesis (92-482 g, average  $194 \pm 17.0$  g,  $p = 0.252$ ) were tracked over a full temperature cycle (March 2005 to January 2006) in order to obtain samples for analysis of the total RNA content in all tissues analyzed for protein synthesis. Six cunner were sampled at the same temperatures and corresponding time of the year as described for the protein synthesis experiment. Fish were killed and tissues were excised and frozen as described above. Total RNA was extracted from the samples using TRIzol reagent (Invitrogen), which is a commercially available modification of the single-step RNA isolation method (Chomezynski and Sacchi 1987) following the procedure outlined in

Treberg et al. (2005). Total RNA, expressed as microgram of RNA per gram tissue (wet weight) was determined spectrophotometrically by subtracting the absorbance at 320 nm from the absorbance at 260 nm.



**Figure 3.1** Seasonal temperature for the ambient water source for the Ocean Sciences Centre, Logy Bay Newfoundland and daylengths (hours between sunrise and sunset) for a Newfoundland latitude (47° North). Sample temperatures for the current study are indicated by open circles (Modified from Levesque et al. 2005).

### 3.2.6 Statistical Analyses

Mean tissue phenylalanine content, specific activity over the incubation time and RNA data were compared using a one-way ANOVA with Tukey's post-hoc test for multiple comparisons. The incorporation of radioactivity into protein was determined by linear regression. Once data were confirmed to fit the validation criteria, phenylalanine incorporation rates between temperatures for each tissue were compared using a one-way ANOVA with Tukey's post-hoc tests. Data were log-transformed when necessary and in all cases  $p < 0.05$  was considered significant.

## 3.3 Results

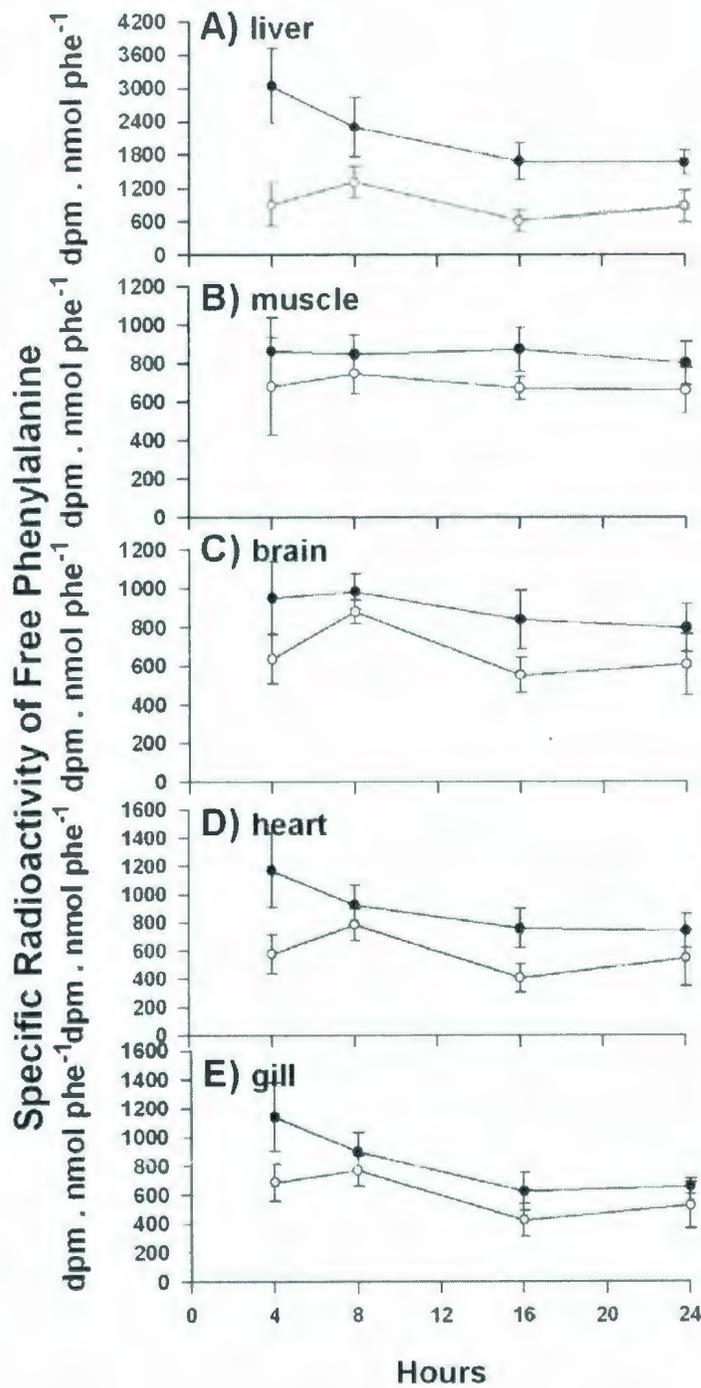
### 3.3.1 Validation of Flooding Dose Methodology

Accurate interpretation of *in vivo* protein synthesis rates via the flooding dose methodology is contingent upon fulfillment of several validation criteria. The injection dose must fully flood the phenylalanine pool and the free-pool specific radioactivity must rapidly elevate and remain stable over the incubation period. As well, the incorporation of phenylalanine into tissues must be significant and linear over the time that protein synthesis is being measured. The fulfillment of these criteria was particularly important for this study as it measured *in vivo* protein synthesis in a previously unstudied teleost species, and required that accurate measurements be made while the fish were metabolically depressed for long periods, and at extremely low temperatures.

As predicted in Chapter Two, a much longer incubation time than is the norm for this procedure was necessary to fulfill the validation criteria in the eunner. The injection dosage, which contained  $1.35 \text{ nmol} \cdot \text{mg}^{-1}$  body mass of unlabeled phenylalanine, should

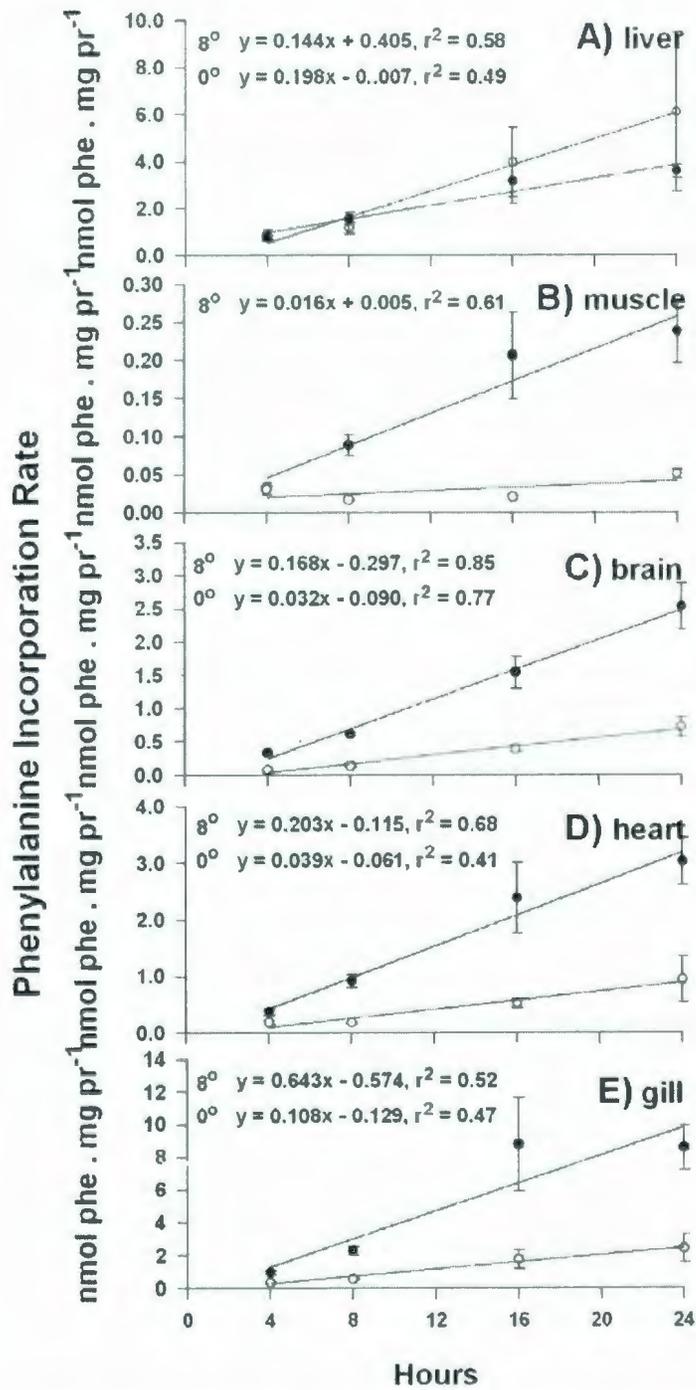
theoretically increase the free-pool phenylalanine content to the same extent. Baseline levels of phenylalanine obtained from non-injected cunner were  $0.12 \pm 0.02$ ,  $0.14 \pm 0.04$ ,  $0.09 \pm 0.01$ ,  $0.07 \pm 0.01$  and  $0.11 \pm 0.02$   $\text{nmol}\cdot\text{mg}^{-1}$  fresh tissue for liver, white muscle, brain, heart and gill, respectively. The mean post-injection phenylalanine concentration over all time points and all temperatures was  $1.38 \pm 0.01$ ,  $1.14 \pm 0.01$ ,  $1.00 \pm 0.01$ ,  $1.04 \pm 0.01$  and  $0.9 \pm 0.01$   $\text{nmol}\cdot\text{mg}^{-1}$  for liver, white muscle, brain, heart and gill respectively; indicating the free phenylalanine pool was increased by 8.1 to 14.8 fold by the injection dosage, depending on the tissue.

The specific activities of the free-pool were found to be elevated and stable over the time protein synthesis was measured as there were no significant differences in the specific activity of the free phenylalanine pool between sample times within each tissue. As well, levels of specific activity were not significantly different between experimental temperatures and as such, only data from  $8^{\circ}\text{C}$  (November) and  $0^{\circ}\text{C}$  (March) are presented in Figure 3.2 to demonstrate the successful fulfillment of the second validation criterion. For reference, figures showing the specific activity of the free phenylalanine pool for all experimental temperatures can be found in Appendix 1.



**Figure 3.2** Post-injection time course for the specific radioactivity of the free pool phenylalanine in cunner at 8°C (November) (●) and 0°C (March) (○) for liver (A), white muscle (B), brain (C), heart (D) and gill (E) tissues. Values are mean  $\pm$  SEM, n = 4 fish.

The incorporation of phenylalanine into tissues was expressed as nmol of phenylalanine incorporated per mg protein. Figure 3.3 shows regression plots for data obtained at 8°C (November) and 0°C (March) for all tissues as a representation of the range of rates of phenylalanine incorporation in cunner. Data for linear incorporation of phenylalanine into protein for all five experimental temperatures is included in Appendix I. Based on the regression equations calculated over the entire twenty hour post-injection time period, liver, brain, heart and gill tissues exhibited significant and linear incorporation rates of labeled phenylalanine at all five temperatures, and had intercepts that were not significantly different from the origin. Significant and linear incorporation of phenylalanine was only evident in white muscle at 8°C November, 4°C June and 8°C July. Protein bound radioactivity in white muscle during the winter (4°C December and 0°C March) were barely above background radioactivity levels. Therefore, rates of protein synthesis could not be calculated for white muscle at 4°C December and 0°C March. The linearity of phenylalanine incorporation into the various tissues of dormant cunner from injection time onward indicates that the disturbance of fish during injection was not substantial enough to arouse the fish and cause a return to increased or 'normal' metabolism.



**Figure 3.3** Post injection time course for the incorporation of radiolabeled phenylalanine into protein in cunner at 8 °C (November) (●) and 0°C (March) (○) for liver (A), white muscle (B), brain (C), heart (D) and gill (E) tissues. Values are mean ± SEM, n = 4 fish at each sample time.

### 3.3.2 Seasonal Changes in Tissue Specific Rates of Protein Synthesis

Protein synthesis rates obtained in cunner display the same tissue-specific hierarchy as other species with rates in gill > liver > heart > brain > white muscle across all experimental temperatures. At 8°C (in November) protein synthesis rates for liver, white muscle, brain, heart and gill were  $0.20 \pm 0.02$ ,  $0.01 \pm 0.001$ ,  $0.09 \pm 0.006$ ,  $0.12 \pm 0.01$  and  $0.36 \pm 0.05$  nmol phe · g protein<sup>-1</sup> · hr<sup>-1</sup>, respectively. Significant decreases in protein synthesis occurred in all five tissues as the water temperature decreased to 4°C (Figure 3.4). Rates in white muscle fell below detectable levels at this temperature and did not increase until water temperatures returned to 4°C in June. A decrease of approximately 55% between 8°C and 4°C was seen in liver, brain and heart, with the greatest depression occurring in gill tissue (~ 66% decrease). The only tissue that exhibited a further decrease in rates of protein synthesis when water temperature dropped to 0°C was the brain. Phenylalanine incorporation dropped to  $0.02 \pm 0.003$  nmol phe · g protein<sup>-1</sup> · hr<sup>-1</sup>, a significant decrease from rates at 8°C (78% depression), but not from 4°C. Incorporation rates in the liver underwent an unexpected significant increase to  $0.22 \pm 0.050$  nmol phe · g protein<sup>-1</sup> · hr<sup>-1</sup>, bringing protein synthesis to levels comparable to those obtained at 8°C in November. As water temperature warmed to 4°C in June, liver, heart and gill tissue maintained protein synthesis at similar rates as measured at 0°C. In contrast, brain and white muscle both increased protein synthesis significantly at this temperature, with rates in brain returning to rates similar to those recorded at 4°C in December and white muscle increasing to levels comparable to fully active fish at 8°C in November. Accompanying the return of water temperature to 8°C in July, there were significant increases in rates of protein synthesis in all tissues, except for white muscle in which the increase was non-

significant. Brain and gill protein synthesis returned to similar rates as recorded when water temperature was 8°C in the fall of the year. Interestingly, protein synthesis rates in liver and heart tissue increased to  $0.33 \pm 0.046$  and  $0.21 \pm 0.028$  nmol phe·g protein<sup>-1</sup>·hr<sup>-1</sup>, respectively, indicating a hyperactivation of protein synthesis to 165% (liver) and 175% (heart) of that measured at the equivalent temperature in the fall (8°C in November).

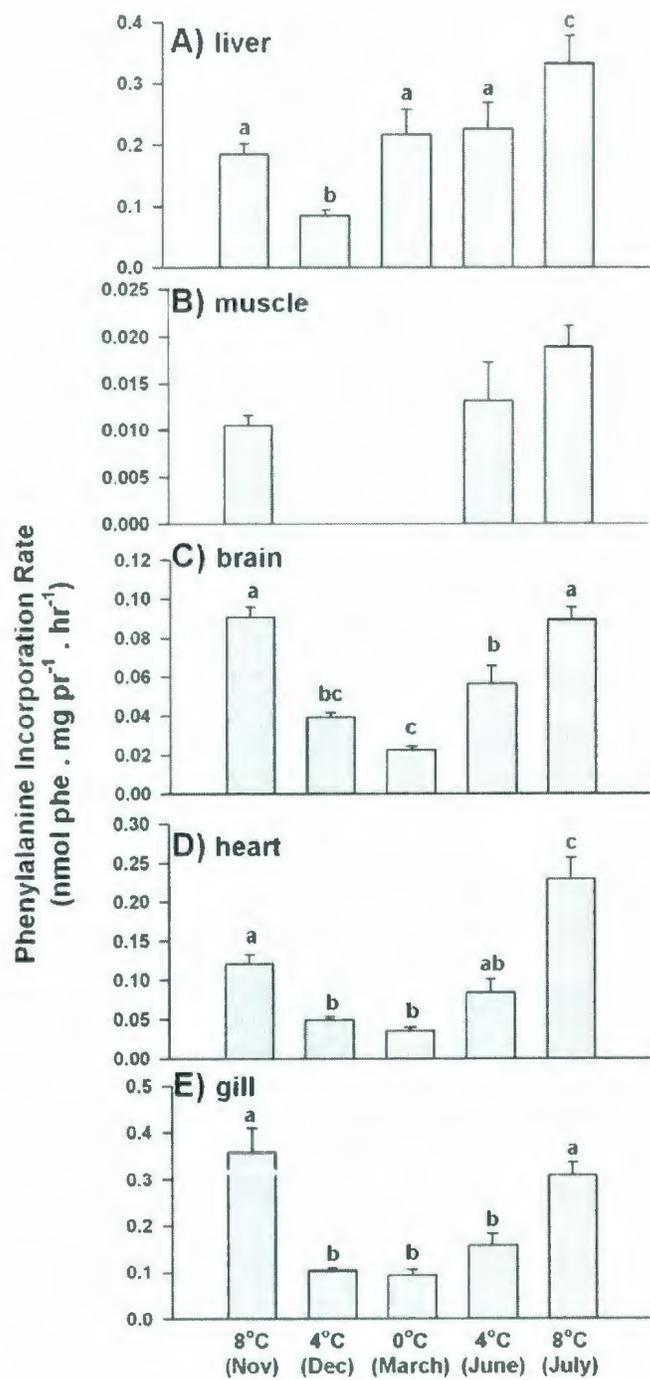
### *3.3.3 Seasonal Changes in Total RNA Content*

Total RNA content in fish sampled at 8°C (November), was the highest in liver tissue, followed by gill, brain and heart, with white muscle exhibiting the lowest levels ( $6406 \pm 627$ ,  $3214 \pm 347$ ,  $1458 \pm 488$ ,  $1459 \pm 148$ ,  $153.9 \pm 8.2$  µg · g tissue<sup>-1</sup>, respectively) (Figure 3.5). RNA content in liver, brain, heart and gill was found to decrease with water temperature, whereas levels of RNA in white muscle remained relatively low and similar despite changes in temperature. The decrease in water temperature to 0°C was accompanied by a significant reduction in total RNA content in liver, brain, heart and gill tissue relative to the 8°C sample (decreases of 74, 88, 66 and 60%, respectively). However, as water temperatures increased in the spring, several patterns were observed in total RNA content as temperature increased to 4°C, and then 8°C (July). Liver RNA content significantly increased as water temperatures reached 4°C, bringing total RNA content up to levels comparable to those obtained at 8°C in November. However, RNA levels subsequently underwent a significant decrease to  $1102 \pm 255$  µg · g tissue<sup>-1</sup> as water temperatures further increased to 8°C in July. White muscle, again maintained constant levels of RNA until water temperatures reached 8°C, when total RNA was significantly increased to  $286.5 \pm 50.8$  µg · g tissue<sup>-1</sup>. Brain and heart RNA levels

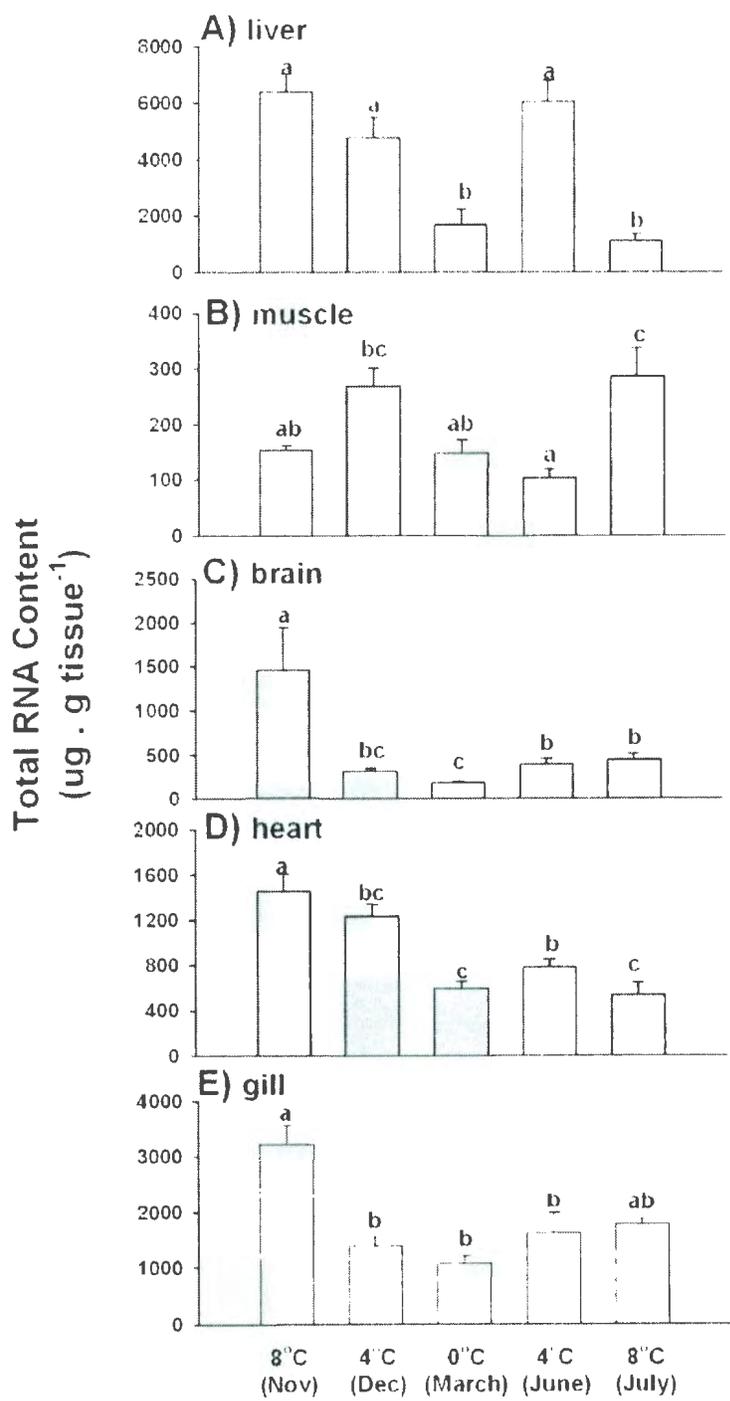
increased significantly along with the spring water temperature increase to 4°C, thereafter brain RNA was maintained at these levels whereas levels in heart returned to 0°C levels. There were no significant changes in total RNA content in gill tissue after the initial decrease that coincided with the entrance into winter dormancy.

#### *3.3.4 Comparison of Protein Synthesis and RNA content*

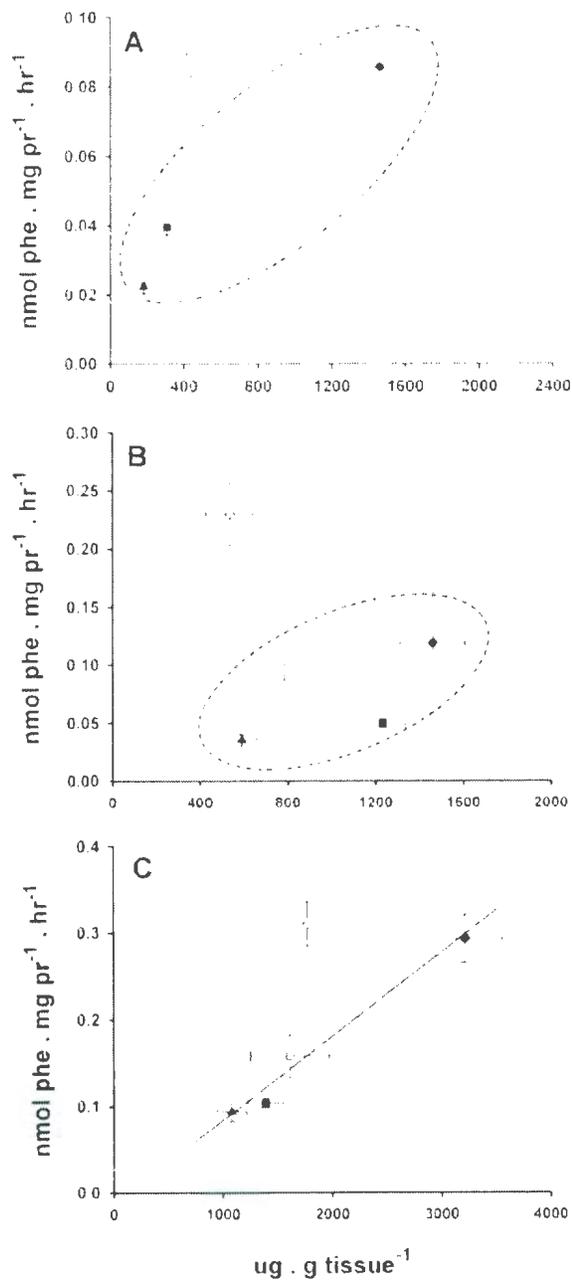
Although the rates of protein synthesis and content of RNA were obtained from different cohorts of fish, comparison of these values revealed qualitative patterns. Significant correlations were not obtained when comparing mean phenylalanine incorporation rates and mean total RNA content for any of the tissues when all experimental temperatures were included. However, a qualitative analysis of the regression plots demonstrated the 8°C (July) values were a consistent outlier in brain, heart and gill tissue (open circles; Figure 3.6). The removal of the 8°C July data points from the regressions produced a significant correlation between protein synthesis and total RNA content with temperature changes in gill tissue, but not for heart and brain; although a definite trend was observed (data grouped by ellipses; Figure 3.6).



**Figure 3.4** Rates of protein synthesis (nmol phe · mg protein<sup>-1</sup> · hr<sup>-1</sup>) in cunner at the various experimental temperatures in liver (A), white muscle (B), brain (C), heart (D) and gill (E) tissue. Values represented as mean ± SEM, n = 16 fish. Significant differences are indicated by differing letters (p < 0.05).



**Figure 3.5** Total RNA content ( $\mu\text{g RNA} \cdot \text{g fresh tissue weight}^{-1}$ ) in cunner at the various experimental temperatures in liver (A), white muscle (B), brain (C), heart (D) and gill (E) tissue. Values represented as mean  $\pm$  SEM,  $n = 6$  fish. Significant differences are indicated by differing letters ( $p < 0.05$ ).



**Figure 3.6** Comparison of protein synthesis rates (nmol phe · mg protein<sup>-1</sup> · hr<sup>-1</sup>) and total RNA content (ug RNA · g fresh tissue weight<sup>-1</sup>) in brain (A), heart (B) and gill (C) tissue at 8°C November (●), 4°C December (■), 0°C March (▲), 4°C June (□), and 8°C July (○). Regression equations calculated using mean values of protein synthesis for all temperatures except 8°C July (○). Brain:  $y = 4.22 \times 10^{-5}x + 0.02$ ,  $r^2 = 0.85$ ,  $p = 0.078$ ; Heart:  $y = 6.02 \times 10^{-5}x + 0.01$ ,  $r^2 = 0.41$ ,  $p = 0.36$ ; Gill:  $y = 9.52 \times 10^{-5}x - 0.01$ ,  $r^2 = 0.98$ ,  $p = 0.01$ .

### 3.4 Discussion

#### 3.4.1 Entrance into Metabolic Depression

Earlier evidence of metabolic depression in response to low temperature in cunner has been based on behavioral observations (Green and Farwell 1971) and oxygen consumption rates (Haugard and Irving 1948; Costa 2007). The present study shows a significant depression in the protein synthesis, providing evidence at the cellular level to complement the observations at the whole animal level. Upon the transition from 8°C to 4°C the incorporation of phenylalanine was decreased by 55% in liver, brain and heart, 66% in gill, and rates of protein synthesis in white muscle fell below detectable levels, suggesting complete suppression of protein synthesis in white muscle during the entrance into winter dormancy.

As in any study that investigates metabolic processes in ectothermic animals in relation to temperature induced metabolic depression, the question arises as to the validity of deeming cellular mechanisms an active contributor to metabolic suppression or simply a passive consequence of decreasing temperature.  $Q_{10}$  values are often calculated to determine if the changes observed in a metabolic process are due to direct temperature effects ( $Q_{10}$  value of 2-3) or active alterations in metabolism ( $Q_{10} > 3$ ). Early studies on the effects of temperature acclimation on protein synthesis established the incorporation of protein into body tissues to normally be dependent upon temperature, showing depression in rates as environmental temperatures decrease, with  $Q_{10}$  values of approximately 2-3 (Haschemeyer 1968; Haschemeyer et al. 1979; Loughna and Goldspink 1985; Watt et al. 1988). However, high  $Q_{10}$  values for protein synthesis have been reported for the toadfish, *Opsanus tau* ( $Q_{10} = 6-7$ ) (Matthews and Haschemeyer

1978; Poernjic et al. 1983) and the North Sea eelpout, *Zoarces viviparous* ( $Q_{10} = 7-10$ ) (Storeh et al. 2005). These high  $Q_{10}$  values were calculated at the lower end of the organism's temperature profile and often accompanied visual observations of sluggish behaviour in the eel pout or periods of complete inactivity in the toadfish. The high  $Q_{10}$  values in these two temperate species suggest an active depression in whole animal metabolic rate at low temperatures; however this has yet to be experimentally determined for these species.  $Q_{10}$  values calculated from the protein synthesis rates in cunner between 8°C and 4°C in the fall of the year are also higher than expected from temperature effects alone; with values of 6.7, 8.0, 9.2 and 21.0 for brain, liver, heart and gill, respectively. Interestingly, this active suppression of protein synthesis appears to make a substantial contribution towards the 69% depression in resting metabolism (RoMR) that occurs between 5°C and 0°C ( $Q_{10} = 10.5$ ), the same temperatures over which the behavioral changes are noticeable (Green and Farwell 1971; Costa 2007). The hypothesis that cunner are capable of active metabolic depression is further strengthened by results obtained in an acute temperature challenge (one degree an hour, between 5°C and 0°C). During the initial stages of the acute challenge, cunner are capable of thermal compensation, but after an hour at 0°C a rapid depression of metabolic rate occurs with a peak depression of 64% ( $Q_{10} = 8$ ) at 2-3 hours at 0°C (Costa 2007). These results suggest the depression of protein synthesis that occurs between 8°C and 4°C to be an intrinsic response, occurring in anticipation of the extended period of extreme low water temperatures experienced by the cunner during the winter months and before the decrease in whole animal metabolic rate is evident at the physiological level.

### *3.4.2 Metabolic Depression*

The only tissue to exhibit further depression in synthesis as water temperatures decreased from 4°C to 0°C was brain, bringing levels of protein synthesis to approximately 25% of rates obtained in fully active fish ( $Q_{10} = 4$ ). The negligible decrease of protein synthesis in heart and gill despite the additional decrease in environmental temperature further supports active control of protein synthesis in relation to seasonal metabolic depression. The level of depression in brain tissue falls in between the complete suppression of brain protein synthesis in anoxic turtles and hibernating ground squirrels (Frerichs et al. 1998; Fraser et al. 2001) which exist in a comatose like state during metabolic depression, and the maintenance of protein synthesis in the brain of crucian carp which remain relatively active during anoxia (Smith et al. 1996). Cunner appear to be in a quiet state during winter dormancy, but fish will become active for a short period of time if disturbed (Green and Farwell 1971). This may explain the maintenance of phenylalanine incorporation at low levels in the brain, as a representative of neuronal tissue activity, during dormancy.

The anomaly of the increase in rates of protein synthesis in liver tissue to pre-dormancy levels at 0°C (March) is perplexing as the activation of protein synthesis in this tissue, which is the major site of protein synthesis, is an energetically expensive action during a time when energy conservation is the goal. Cunner remain in shallow inshore waters where temperatures frequently decline close to the freezing point of seawater and contact with ice crystals without the protection of an antifreeze mechanism would be fatal to the cunner when they are in this supercooled state. Antifreeze proteins have been found in the skin of cunner and also circulating in the blood during the winter, but the

production site of these proteins has not been established (Valerio et al. 1990; Evans and Fletcher 2004). As circulating AFP in the blood are commonly expressed in the liver (Fletcher et al. 2001) the substantial increase in liver protein synthesis during a time of energy conservation may be linked to the production of antifreeze proteins during exposure to extreme low temperatures to enable survival.

The process of protein synthesis does not stand alone in cellular metabolism; it is closely linked with the degradation of proteins in a continuous cycle known as protein turnover. Protein degradation can account for up to 22% of ATP turnover (Land and Hochachka 1994), and thus, it is an energetically expensive process (falling just behind protein synthesis and active ion exchange) (Wieser and Krumshabel 2001). This creates a paradoxical situation in animals that undergo extended periods of metabolic depression. These animals must deal with the need to reduce protein degradation, in order to conserve ATP consumption, while avoiding the accumulation of damaged proteins in order to sustain tissue functionality. A solution to this problem has been described in anoxic turtle hepatocytes. Despite the extensive depression in ATP-dependent proteolysis (93%) during anoxic exposure, total protein degradation was only reduced by 36-41%. Thus, the remaining protein degradation is due to ATP-independent processes (Land and Hochachka 1994). Further investigation is needed in order to determine whether or not *T. adspersus* employs a similar approach with respect to protein degradation when faced with low temperature induced metabolic depression.

### *3.4.3 Recovery from Metabolic Depression*

During periods of hypometabolism, levels of protein synthesis and degradation often become mismatched (synthesis < degradation), creating a negative protein balance (Land and Hochachka 1994). In this situation there will inevitably be some accumulation of denatured or damaged proteins and unless animals can reduce energy demands and enter a maintenance mode (synthesis = degradation) there will also be some loss in protein content. Upon recovery, this relationship is reversed in anoxic turtles as levels of protein synthesis are hyperactivated (160%) while protein degradation remains below control rates (Land and Hochachka 1994). This positive protein balance is most likely necessary to allow for the replacement of damaged proteins that accumulate during dormancy. In tissues such as white muscle, this increase in synthesis and retention of protein (accompanied by decreased degradation) is manifested at the whole animal level as increased growth (Houlihan 1991).

During the initial stages of post-dormancy recovery in cunner (4°C, June samples) the synthesis of protein began to increase in a temperature dependent fashion for brain, while rates in heart, gill and liver were maintained at similar levels as measured at 0°C (March). Further, a significant increase in protein synthesis in white muscle occurs during early recovery, bringing rates of phenylalanine incorporation from undetectable levels to rates comparable to those obtained in fully active fish at 8°C in November. As the accretion of proteins in white muscle is representative of increased tissue mass, this hyperactivation of protein synthesis is most likely accompanied by a decrease in degradation and responsible for the stimulation of growth after an extended period of winter dormancy. A comparative study on two geographically different populations of

cunner shows that the Newfoundland population experiences a longer period of dormancy than more southerly populations, but the annual growth rates of the two populations is the same (Chiasson 1995). Therefore, it is possible that the hyperactivation of protein synthesis in white muscle during the recovery period allows for compensatory growth to occur in order to make up for a shorter growth season in cunner inhabiting colder waters. Although metabolism is not decreased to the same extent in Atlantic cod as in cunner, cod also exhibit compensatory protein synthesis after exposure to cold water temperatures (Treberg et al. 2005). Hyperactivation of protein synthesis was also evident in liver (165%) and heart (175%) once cunner returned to a fully active state (8°C, July). A similar level of hyperactivation of protein synthesis was documented in *in vitro* studies on ground squirrels during arousal from hibernation (Zhegunov et al. 1988) and turtles during recovery from extended exposure to anoxia (Land et al. 1993). As in these previous studies, the mechanism behind the substantial increase in rates of protein synthesis in the cunner remains elusive, but is most likely linked to the increase in overall metabolic rate, higher food consumption and activity level.

#### *3.4.4 Protein Synthesis and RNA Content*

The close relationship between rates of protein synthesis and the translational capacity of a tissue (RNA content) is well established (Houlihan 1991). Specifically, translational capacity undergoes significant reductions that parallel the depression of protein synthesis caused by decreasing temperature (Saez et al. 1982; Fraser et al. 2002), and during metabolic depression (Bailey and Driedzie 1996; Smith et al. 1996; Fraser et al. 2001).

The qualitative comparison of protein synthesis rates with total RNA content in this experiment suggests this to also be true in cunner, for brain, heart and gill tissue until cunner begin recovery from winter dormancy. In teleosts, stimulation of protein synthesis and compensatory growth has been linked to increases in translational efficiency instead of an increase in translational capacity (Houlihan 1991; Treberg et al. 2005). The absence of a significant correlation between protein synthesis rates and total RNA content in liver and white muscle throughout the experiment as well as the increase and hyperactivation observed in brain, heart and gill during recovery suggest these changes in protein synthesis are due to an increase in efficiency of translation of proteins on the ribosomes, and not a result of an increase translational capacity (number of ribosomes).

### **3.5 Conclusions**

The current study is the first to investigate the relationship between temperature and protein synthesis during both metabolic depression and recovery in a fish species that experiences winter dormancy. The depression of protein synthesis in cunner appears to be an intrinsic response, as it is an active process that contributes substantially to the initiation of the metabolic depression at the whole animal. An unexpected hyperactivation of protein synthesis occurs in the liver while the animals are in a fully dormant state that may be linked to the production of antifreeze proteins to ensure the survival in the ice laden environment. During recovery, hyperactivation of protein synthesis occurs in white muscle, heart and liver presumably to allow for compensatory growth and to repay the protein debt that is accumulated during the extended period of

winter dormancy. The biochemical mechanism(s) responsible for the stimulation of protein synthesis remains unclear, although qualitative comparisons of protein synthesis rates and total RNA content suggest an increase in translational efficiency to be a potential mechanism.

## - CHAPTER FOUR -

### **Responses to hypoxia and recovery: Repayment of oxygen debt is not associated with compensatory protein synthesis in the Amazonian cichlid, *Astronotus ocellatus*.**

#### **4.1 Introduction**

Dissolved oxygen is one of the most important environmental factors affecting the survival of animals that rely on aquatic respiration, and animals that are exposed to periods of hypoxia show adaptations at the behavioural, morphological and/or physiological level. At the physiological level, animals commonly resort to one of two strategies: 1) maintenance of low levels of activity, which is fuelled by anaerobic metabolism or 2) depression of metabolism, accomplished by decreasing ATP producing and consuming processes (Lutz and Nilsson 1997; Boutilier 2001). The latter approach allows survival for longer periods of hypoxia/anoxia due to the conservation of energy and the limited accumulation of toxic end products, such as lactate. However, there is a trade off to this approach as deep metabolic depression impairs the animal's ability to respond to external stimuli and leaves the animal vulnerable to predators.

The majority of successful oxyconformers are ectotherms that survive short bouts of hypoxia at warm temperatures, but require seasonal or behaviourally regulated decreases in environmental/body temperature to survive extended anoxia. Such animals are the crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), common frog (*Rana temporaria*) and two species of freshwater turtle (*Chrysemys picta bellii* and *Trachemys scripta elegans*) (Boutilier 2001). In these animals a depression of metabolic rate by 70-95% occurs during hypoxia/anoxia, based on oxygen consumption rates or

calorimetry (Jackson 1968; Van Waversveld et al. 1989). This depression at the whole animal level is accompanied by tissue specific decreases in protein synthesis of 50% in crucian carp (Smith et al. 1996) and 70 to 90% in fresh water turtles (Land et al. 1993; Bailey and Driedzie 1996; Fraser et al. 2001). Protein synthesis is one of the major energy consuming processes, accounting for 18-30% of cellular energy expenditure (Hawkins 1991). As such, the downregulation of protein turnover is one of the major contributing factors to the depression in ATP turnover and metabolic depression at the whole animal level (Guppy et al. 1994).

Animals that are exposed to a prolonged period of oxygen deprivation accumulate an oxygen debt that is repaid during recovery by a substantial increase in oxygen consumption. This oxygen debt has been shown to occur at both the whole animal and tissue levels in goldfish after extended hypoxia exposure (Van den Thillart and Verbeek 1991; Johansson et al. 1995). Johansson et al. (1995) predicted that a substantial increase in protein turnover would accompany the repayment of the oxygen debt, but a consistent pattern in protein synthesis during recovery from hypoxia has not been found. For example, an *in vitro* study on turtle hepatocytes exposed to 12 hours of anoxia showed a significant overshoot in protein synthesis rates to 160% of normoxic levels during recovery (Land et al. 1995). However, *in vivo* studies on crucian carp and a freshwater turtle species did not show hyperactivation of protein synthesis during post-anoxic recovery (Smith et al. 1996; Fraser et al. 2001).

The Amazonian cichlid, the oscar or acara-au (*Astronotus ocellatus*), is an ideal species to study hypoxia induced metabolic depression without the confounding variable of decreased temperature. During periods of high water, Amazon vrzas become

flooded and the surfaces of the lakes become densely covered with floating macrophytes (Val and Almeida-Val 1995). The dense surface vegetation causes extreme diurnal variation in dissolved oxygen levels, with supersaturation occurring at midday when photosynthesis is at its maximum and levels dropping close to zero during the night (MacCormack et al. 2003). *A. ocellatus* undergoes a significant decrease in RoMR (~30%) when water levels reach 20% saturation and only reverts to anaerobic metabolism once oxygen levels drop below 6% saturation, which is accompanied by a decrease in RoMR of approximately 60% (Muusze et al. 1998). These results suggest that *A. ocellatus*, like the crucian carp and freshwater turtles, is able to maintain aerobic metabolism in situations of oxygen deprivation by decreasing the rate of ATP turnover until near anoxic conditions are reached. Despite the recent studies describing the behavioural and physiological responses of *A. ocellatus* to hypoxia (Muusze et al. 1998; Sloman et al. 2006), little is known about the cellular mechanisms behind the hypoxia-induced metabolic depression in *A. ocellatus* and of its response during recovery from severe hypoxia.

The objectives of this study were to expand our knowledge of hypoxia induced metabolic depression in *A. ocellatus* to include the post-hypoxia recovery period and to investigate the tissue specific protein synthesis rates in relation to the whole animal metabolic depression. In addition, the present study is the first to obtain measurements of whole animal metabolic rate and protein synthesis under similar experimental conditions on the same population of fish.

## 4.2 Materials and Methods

Experiments were conducted at the Laboratory of Ecophysiology and Molecular Evolution, INPA, Brazil. A population of *A. ocellatus* was held in an outdoor holding tank with aerated well water (O<sub>2</sub> Saturation of 80-100%) at 28°C and fed commercial food once daily, until transferred to experimental tanks (for protein synthesis studies) or respirometers (for measurements of RoMR). In total, eight fish were used for the measurement of RoMR (156-225 g, average 186 ± 1.0 g) and 44 fish were used for the measurement of protein synthesis (70-160 g, average 95.8 ± 3.8 g). All fish were held without feeding for 48 hrs prior to beginning measurements for RoMR and protein synthesis. In fish used for analysis of RoMR and protein synthesis, measurements were taken under normoxic and hypoxic conditions as well as during the recovery from acute hypoxia. Normoxic conditions were identical to those of the holding tank (O<sub>2</sub> saturation of 80-100%), and the hypoxic challenge consisted of a step down decrease of the dissolved oxygen (DO) level. This was accomplished by bubbling nitrogen directly into the water of the experimental tank, or into the reservoir that supplied water to the respirometer. Water oxygen levels were stepped down from 100 to 70, 50, 30, 20 and 10%, with O<sub>2</sub> levels maintained at each step for one hour, and the fish held at 10% O<sub>2</sub> saturation for 3 hours prior to re-oxygenation. Re-oxygenation was achieved by bubbling air vigorously into the water, and water O<sub>2</sub> levels returned to normoxic levels within 30 to 45 minutes.

#### 4.2.1 Measurement of Routine Metabolic Rate

Individual fish were transferred to a specially designed Plexiglass<sup>®</sup> respirometer (15 x 20 x 40cm; 11.875 L) supplied with oxygen saturated water (80-100%) from a 100 L reservoir and allowed a 48 hour acclimation period before beginning the experiment. Water from the reservoir was continuously pumped through the respirometer using a submersible pump (model NK-1, Little Giant Co., USA). Water temperature and oxygen concentration were monitored through a circuit composed of tubing with extremely low gas permeability (Tygon<sup>®</sup> Food & LFL, Cole Palmer, Inc., USA) using a peristaltic pump (Masterflex L/S model 77200-12, Cole-Palmer) and flow through chambers (D201, WTW, Weilheim, Germany) containing oxygen probes (model CelloX 325, WTW) positioned in the respirometer's inflow and outflow tubing. Measurements of water oxygen levels and water flow rate (range 0.8 to 1 L · min<sup>-1</sup>) were taken at hourly intervals during the experiment (i.e. during normoxia, hypoxia and recovery from hypoxia), as well as before the placement of the fish and immediately after the removal of the fish from the respirometer in order to correct for bacterial O<sub>2</sub> consumption. Bacterial O<sub>2</sub> consumption was consistently less than 2% of the fish's RoMR and was therefore considered to be negligible. The RoMR of each fish was calculated at each measurement interval as:

$$\text{RoMR} = \frac{[(C_{O_2}(i) - C_{O_2}(o))] \times Vw \times 60}{W}$$

**Where:**

RoMR = Routine Metabolic Rate, in mg O<sub>2</sub> kg<sup>-1</sup> hr<sup>-1</sup>;

C<sub>O<sub>2</sub></sub>(i) = O<sub>2</sub> concentration in inflowing water (mg O<sub>2</sub> L<sup>-1</sup>);

C<sub>O<sub>2</sub></sub>(o) = O<sub>2</sub> concentration in outflowing water (mg O<sub>2</sub> L<sup>-1</sup>);

Vw = water flow rate through the respirometer (L min<sup>-1</sup>);

W = mass of fish (kg)

(modified from Cech 1990)

#### 4.2.2 Protein synthesis

4.2.2.1 *Normoxia*: Twelve fish were removed from the holding tank, weighed, tagged for individual recognition with coloured beads attached to the dorsal musculature at the base of the dorsal fin and transferred to a separate experimental tank under identical environmental conditions. After 48 hours, fish were injected without anaesthesia via intraperitoneal injection, with 1.0 ml 100 g<sup>-1</sup> of [2,3-<sup>3</sup>H] phenylalanine (Amersham International) solution. This injection solution consisted of 135 mM phenylalanine in a solution containing 125 mM NaCl, 3 mM KCl, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.5 mM CaCl<sub>2</sub>, 5 mM HEPES (sodium salt), 5 mM glucose, 2 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8 at 28°C, in addition to sufficient [2,3-<sup>3</sup>H] phenylalanine to ensure a dosage of 100 µCi ml<sup>-1</sup>. Following injection, fish were returned immediately to the experimental tank and after an incubation time of one, two or three hours, groups of four fish were killed by a blow to the head and immediate severing of the spine. Whole brain, whole liver and heart ventricular tissue were excised, blotted dry and immediately frozen in liquid nitrogen. A section of white muscle was taken from the dorsal musculature above the lateral line, care was taken to remove skin and scales and the tissue was then frozen in liquid nitrogen. Gills were excised and lamellae were scraped from the gill arches, the lamellar tissue was then flash frozen in liquid nitrogen. All samples were kept at -70°C until analysis.

4.2.2.2 *Hypoxia*: In this treatment, twelve fish were weighed, tagged and transferred to the experimental tank. After a 48 hour acclimation period, fish were exposed to the stepwise decrease in dissolved oxygen levels as described previously. Fish were injected immediately once water oxygen saturation reached 10%, and four fish were sampled (as

above) at one, two, and three hours after injection with DO levels maintained at 10% for the three hour hypoxia exposure.

*4.2.2.3 Recovery:* To assess changes in protein synthesis rates during recovery from hypoxia, twenty fish were exposed to an acute hypoxia challenge as described previously. After holding fish at 10% DO for three hours, air was bubbled into the experimental tank allowing the dissolved oxygen level to return to normoxic levels (80-100%). Groups of five fish were injected at hourly intervals, starting when O<sub>2</sub> saturation levels returned to normoxic levels (group 1) and ending four hours after O<sub>2</sub> returned to normoxic levels (group 4). Each group of fish was sampled one hour post-injection allowing protein synthesis to be tracked over a four hour time period during the post-hypoxic recovery. Tissues were excised and stored as previously described.

#### *4.2.3 Blood Sampling for Lactate*

Blood samples were obtained from as many fish as possible during the protein synthesis experiment, resulting in n = 4, normoxic; n = 8, hypoxic; and n = 7, recovery. Blood was drawn from the caudal vein with a heparinized syringe prior to sampling the fish for protein synthesis analysis. Blood samples were centrifuged and plasma was stored at -70°C for lactate analysis.

#### *4.2.4 Sample Preparation and Scintillation Counting*

The protocol used for the analysis of protein synthesis was modified from that described in Garlick et al. (1980). Samples were homogenized with a Polytron in nine volumes of 6% perchloric acid (PCA) except for liver, which was homogenized in four volumes of PCA. Homogenized samples were left on ice for 10-15 minutes and a 1 ml aliquot was transferred to a microcentrifuge tube. In tissues that had excess 6% PCA homogenate (liver, muscle and in some cases brain), the remaining homogenate was stored at -70°C for the analysis of lactate. The 1 ml aliquot of homogenate for protein synthesis was centrifuged for five minutes at 15,600 x g, after which the supernatant was removed and frozen at -20°C for analysis of the free pool phenylalanine content and specific radioactivity.

The protein pellet was washed by manually re-suspending the pellet in 1.0 ml of 6% PCA, vortexing, centrifuging as described above, and then discarding the supernatant. This wash step was repeated until the radioactivity in the discarded supernatant was at background levels to ensure only protein bound <sup>3</sup>H phenylalanine was being measured in the protein pellet. After sufficient washing, 1.0 ml of 0.3 M NaOH was added to the tube containing the protein pellet. The protein pellet was incubated in a water bath held at 37°C until fully dissolved. The dissolved protein was stored at -20°C until analysis for protein content and protein bound radioactivity.

Aliquots of the original supernatant from the homogenized tissue and the dissolved protein were added to 10 ml of Ultima Gold<sup>TM</sup> liquid scintillation cocktail and counted on a Beckman Coulter LS6500 liquid scintillation counter to obtain the [2,3-<sup>3</sup>H]

phenylalanine content of the free and protein bound phenylalanine pools of the tissues, respectively.

#### *4.2.5 Biochemical Assays*

Free pool phenylalanine content was measured from the PCA extraction supernatant and phenylalanine standards in 6% PCA using a fluorometric assay following the protocol described in McCaman and Robins (1962). Protein content of the tissue was determined from the NaOH solubilized protein pellet by using the BioRad D<sub>6</sub> kit (Bio-Rad Laboratories, California) using standards made from bovine serum albumin. Lactate was measured in standards in 6% PCA, plasma, liver, white muscle and brain tissue via the reduction of NAD<sup>+</sup> to NADH at 340 nm using a Sigma diagnostics kit.

#### *4.2.6 Statistical Analyses*

Comparison of oxygen consumption data was carried out by using a repeated measures ANOVA followed by a Dunnett's post-hoc test, to compare all values with the normoxic (control) value. Lactate concentrations for normoxia, hypoxia and recovery treatments were compared using a one way-ANOVA, with Tukey's post-hoc test for multiple comparisons. In the protein synthesis experiment, mean tissue phenylalanine content and specific radioactivity over the incubation time were compared using a one-way ANOVA with Tukey's post-hoc test for multiple comparisons, and the incorporation of radioactivity into protein was examined by linear regression. Once data were confirmed to fit the validation criteria, phenylalanine incorporation rates for each tissue were

compared using a one-way ANOVA followed by a Tukey's post hoc-test. In all cases  $p < 0.05$  was considered significant.

### 4.3 Results

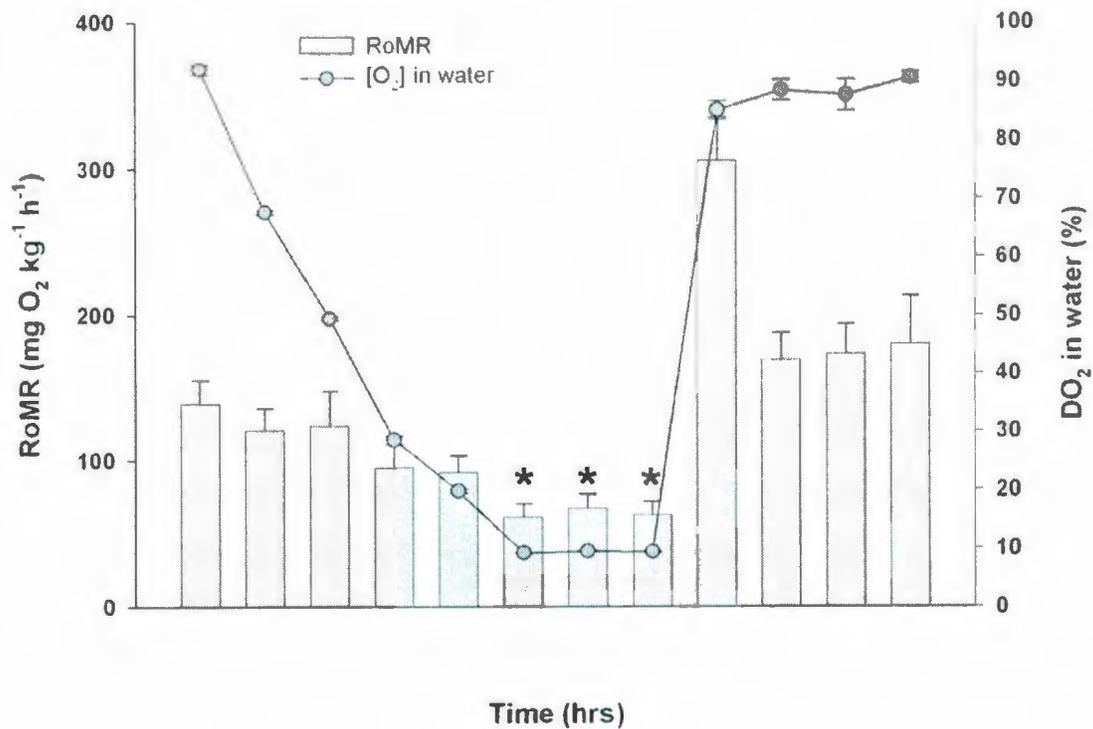
#### 4.3.1 Routine Metabolic Rate

The RoMR of *A. ocellatus* under normoxic conditions was  $138.7 \pm 16.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$  (Figure 4.1). Despite decreasing oxygen saturation of the water, there was no significant decrease in RoMR until water  $\text{O}_2$  levels reached 10% saturation ( $0.67 \pm 0.005 \text{ mg} \cdot \text{L}^{-1}$  at  $28 \pm 1^\circ\text{C}$ ). At this  $\text{O}_2$  level, oxygen consumption was decreased to  $65.1 \pm 2.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$ , a value approximately 50% of the normoxic rate. Although RoMR at one hour post-hypoxia significantly increased to  $305.5 \pm 29.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$  (a value 270% of the normoxic rate), RoMR returned to pre-hypoxic levels ( $174.0 \pm 3.1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$ ) by two hours post-hypoxia and remained at similar levels for the remainder of the experiment.

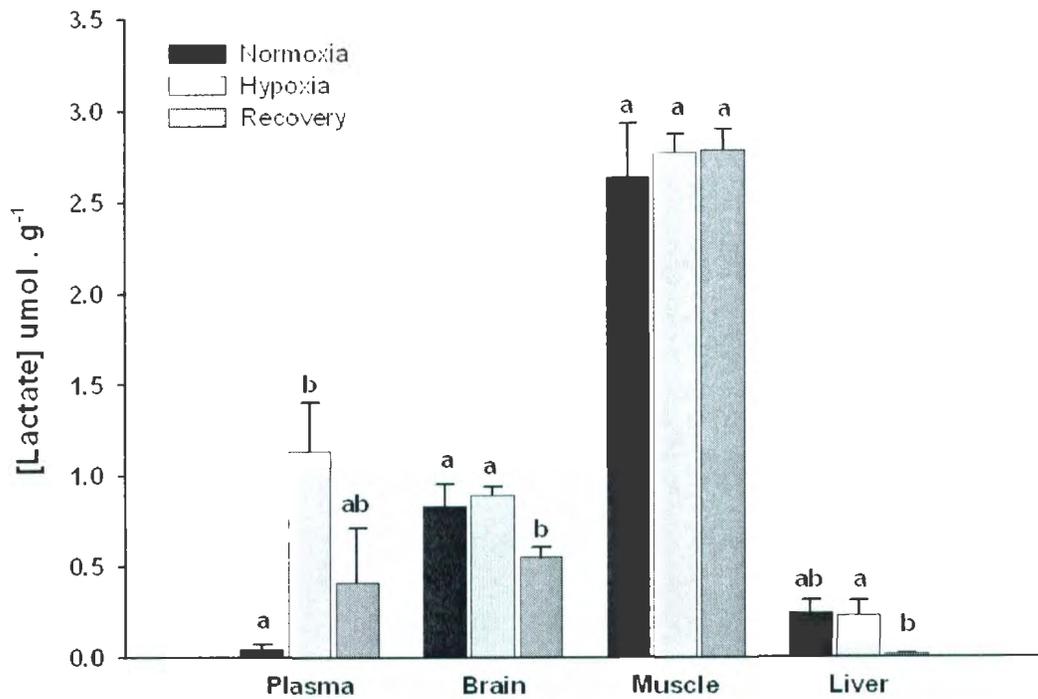
#### 4.3.2 Lactate Concentration

Lactate concentrations under normoxic conditions in the various tissues were  $0.04 \pm 0.03 \text{ umol} \cdot \text{ml}^{-1}$  for plasma ( $n = 4$ ) and  $0.25 \pm 0.06$ ,  $0.84 \pm 0.11$  and  $2.63 \pm 0.30 \text{ umol} \cdot \text{g tissue}^{-1}$  for liver ( $n = 11$ ), brain ( $n = 12$ ) and white muscle ( $n = 6$ ), respectively (Figure 4.2). As there were no significant differences in lactate concentration in any of the tissues during the three hour hypoxia or four hour recovery treatments, results were pooled within each treatment to give a mean value for hypoxic and recovery samples. During the three hour hypoxic exposure, only plasma exhibited a significant increase in lactate concentration ( $1.13 \pm 0.27 \text{ umol} \cdot \text{ml}^{-1}$ ,  $n = 8$ ). During the post-hypoxic recovery

period, plasma lactate returned to levels ( $0.42 \pm 0.30 \text{ umol} \cdot \text{ml}^{-1}$ ,  $n = 7$ ) that were not significantly different from pre-hypoxic values. Finally, significant decreases in lactate concentrations in liver ( $0.02 \pm 0.01 \text{ umol} \cdot \text{g tissue}^{-1}$ ,  $n = 20$ ) and brain ( $0.55 \pm 0.06 \text{ umol} \cdot \text{g tissue}^{-1}$ ,  $n = 20$ ) occurred during the recovery period, whereas lactate concentration in white muscle was maintained at similar concentrations over all three treatments.



**Figure 4.1** Routine metabolic rate (RoMR) in *A. ocellatus* in relation to changing levels of  $\text{O}_2$  saturation in the water. RoMR measurements are means  $\pm$  SEM,  $n = 8$  fish. Significant differences in RoMR from time 0 (normoxia) are indicated by \*,  $p < 0.05$ .



**Figure 4.2** Lactate concentration in plasma, brain, white muscle and liver tissue of *A. ocellatus* during normoxia, hypoxia and recovery from hypoxia. Values are means  $\pm$  SEM, with n values varying: Plasma normoxic (n = 4), hypoxic (n = 8), recovery (n = 7); Brain normoxic (n = 6), hypoxic (n = 10), recovery (n = 20); Muscle normoxic (n = 12), hypoxic (n = 12), recovery (n = 20); Liver normoxic (n = 11), hypoxic (n = 10), recovery (n = 9). Significant differences within each tissue are indicated by differing letters, p < 0.05.

#### *4.3.3 Validation of Protein Synthesis Methodology*

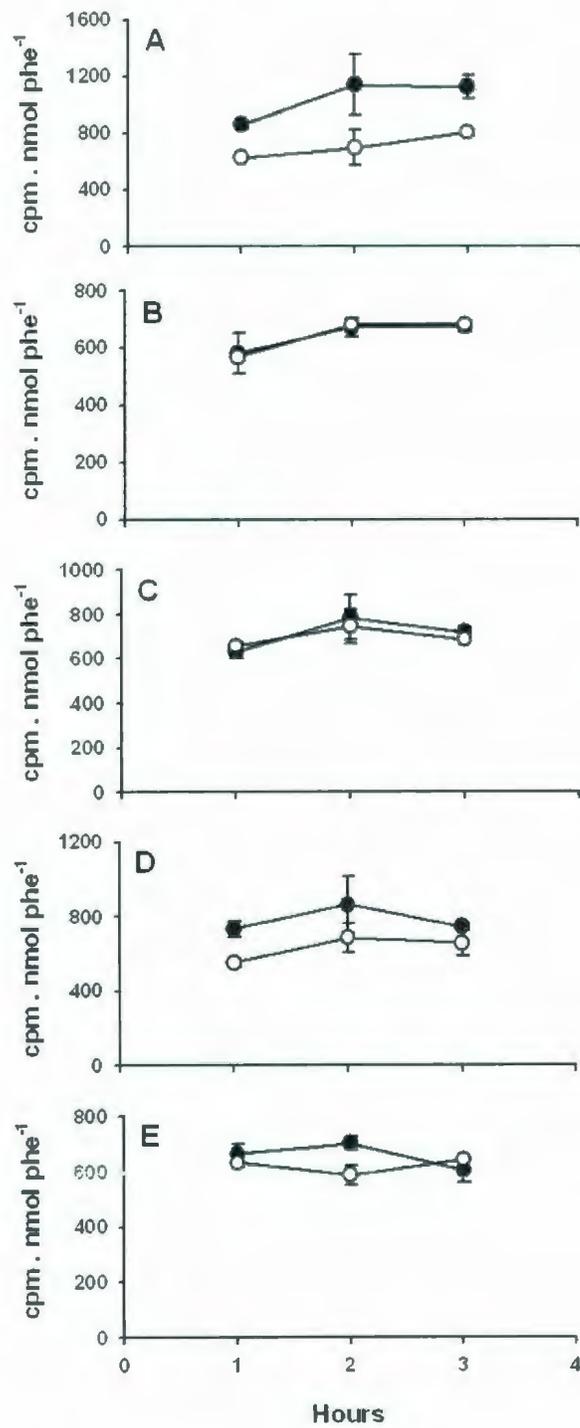
In order to accurately interpret protein synthesis rates obtained via flooding dose methodology several validation criteria must be met: 1) the injection dose must be shown to be sufficient to elevate the free phenylalanine pool of the various tissues; 2) the specific radioactivity of the free phenylalanine must increase rapidly post-injection and remain stable throughout the time protein synthesis is measured; and 3) the rate of phenylalanine incorporation must be linear and begin immediately after injection. As shown in the following sections, all three criteria were fulfilled during this experiment.

*4.3.3.1 Elevation of Free Pool Phenylalanine Content:* The concentration of free pool phenylalanine in the various tissues was not significantly different between the three treatments or between sample times within treatments. Therefore, results for normoxic, hypoxic and post-hypoxic fish were pooled and referred to as injected fish ( $n = 44$ ). Injected fish had free phenylalanine levels of  $0.55 \pm 0.04$ ,  $0.16 \pm 0.01$ ,  $0.08 \pm 0.01$ ,  $0.20 \pm 0.01$  and  $0.19 \pm 0.01$  nmol phe  $\cdot$  mg<sup>-1</sup> fresh tissue for liver, white muscle, brain, heart and gill, respectively. When compared to levels of free phenylalanine in uninjected fish (data not shown), levels were 2-fold higher in brain, white muscle and gill and five-fold higher in liver and heart tissue.

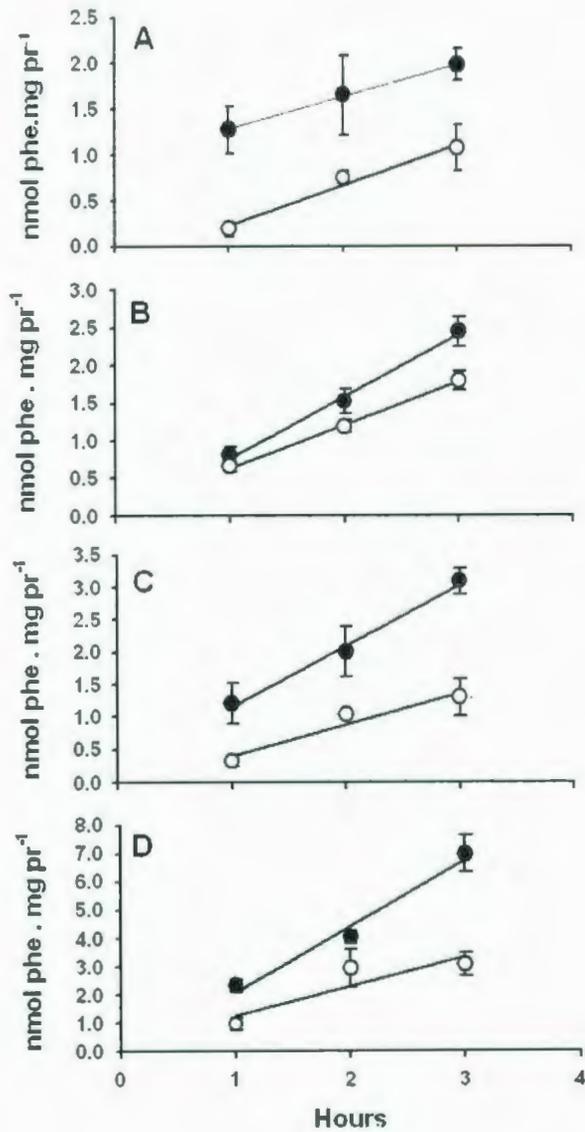
*4.3.3.2 Intracellular Free Pool Phenylalanine Specific Radioactivity:* Intracellular specific radioactivity of the free phenylalanine pool for both normoxic and hypoxic fish was elevated one hour post-injection, and remained constant over the three hours that protein synthesis was measured (Figure 4.3). On average, the specific radioactivity for

normoxic fish was  $1039 \pm 79$ ,  $641 \pm 27$ ,  $711 \pm 37$ ,  $788 \pm 61$  and  $656 \pm 22$  cpm  $\cdot$  nmol phe<sup>-1</sup> for liver, white muscle, brain, heart and gill, respectively (n = 12). For hypoxia exposed fish, the specific radioactivity for the same tissues was  $708 \pm 44$ ,  $642 \pm 17$ ,  $696 \pm 26$ ,  $622 \pm 34$  and  $620 \pm 14$  cpm  $\cdot$  nmol phe<sup>-1</sup> (n = 12). As there was no significant difference in the specific radioactivity measured in fish recovering from hypoxia, results were pooled to give mean values of  $830 \pm 23$ ,  $641 \pm 12$ ,  $702 \pm 7.0$ ,  $665 \pm 10$  and  $672 \pm 17$  cpm  $\cdot$  nmol phe<sup>-1</sup> for liver, white muscle, brain, heart and gill, respectively (n = 20).

*4.3.3.3 Phenylalanine Incorporation into Tissue Protein:* Protein synthesis rates were expressed as nmol phenylalanine incorporated per mg protein. Regression equations calculated over the three hour sampling time demonstrated significant and linear incorporation of phenylalanine into liver, brain, heart and gill tissue of both normoxic and hypoxic fish (Figure 4.4). Rates of protein synthesis for white muscle in both normoxic and hypoxic treatments were below detectable levels; therefore, rates of protein synthesis were not able to be obtained for this tissue. As linear incorporation rates were achieved for both normoxic and hypoxic fish over the three time points, rates of protein synthesis during recovery were determined using one time point only, that being one hour post-injection.



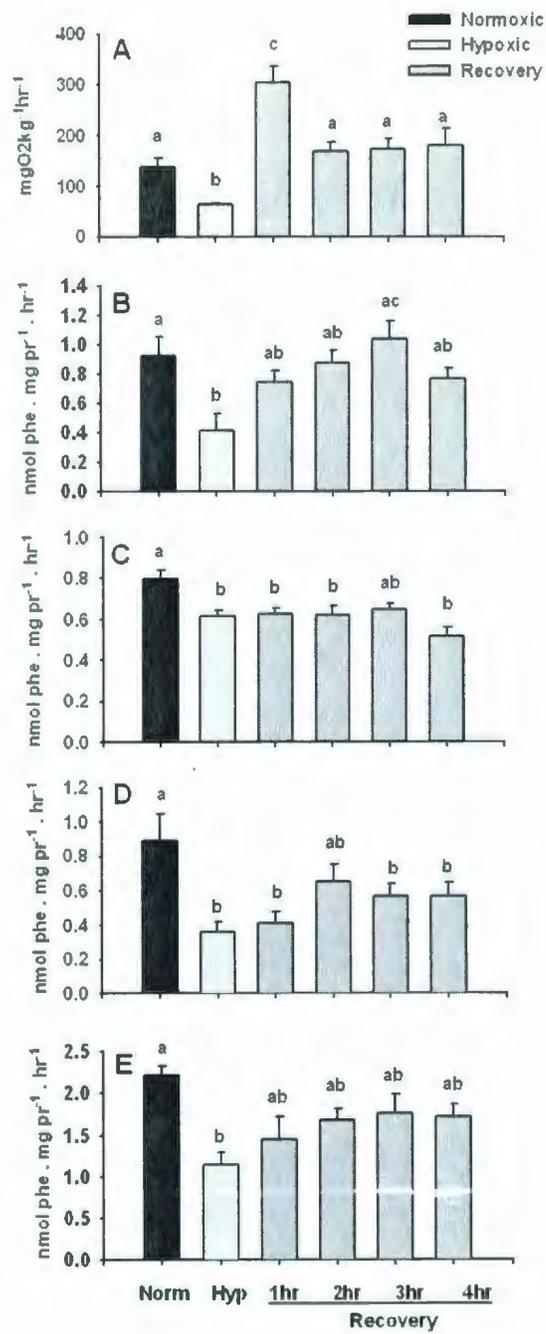
**Figure 4.3.** Post-injection changes in the intracellular free pool specific radioactivity in liver (A), white muscle (B), brain (C), heart (D) and gill (E) of *A. ocellatus* during normoxia (●) and severe hypoxia (○). Values are means  $\pm$  SEM, n = 4.



**Figure 4.4** Post-injection time course for the incorporation of radiolabeled phenylalanine into protein in *A. ocellatus* during normoxia (●) and severe hypoxia (○). (A) liver ( $y=0.47x + 0.62$ ,  $r^2=0.30$ ;  $y=0.44x - 0.22$ ,  $r^2=0.64$ ), (B) brain ( $y=0.82x - 0.05$ ,  $r^2=0.85$ ;  $y=0.57x + 0.07$ ,  $r^2=0.90$ ), (C) heart ( $y=0.94x + 0.20$ ,  $r^2=0.65$ ;  $y=0.55x - 0.157$ ,  $r^2=0.70$ ), and (D) gill ( $y=2.34x - 0.25$ ,  $r^2=0.86$ ;  $y=1.05x + 0.21$ ,  $r^2=0.46$ ). Regression equations refer to normoxic and hypoxic fish, respectively. All  $r^2$  values are significant ( $p<0.05$ ). Values are means  $\pm$  SE,  $n = 4$ , except for hypoxia exposed liver 2 hr time point, where  $n = 3$ . White muscle not shown because rates of incorporation were not detectable.

#### *4.3.4 Tissue Specific Rates of Phenylalanine Incorporation*

Rates of phenylalanine incorporation under normoxic conditions were  $0.92 \pm 0.13$ ,  $0.80 \pm 0.05$ ,  $0.90 \pm 0.16$ , and  $2.2 \pm 0.11$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup> for liver, brain, heart and gill, respectively. During exposure to hypoxia, rates of protein synthesis decreased to  $0.41 \pm 0.12$ ,  $0.62 \pm 0.03$ ,  $0.36 \pm 0.06$ , and  $1.14 \pm 0.15$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>, a depression of 56% for liver, 27% for brain, 60% for heart and 50% for gill (Figure 4.5). During recovery from acute hypoxia, no hyperactivation in protein synthesis occurred, and two different patterns in post-hypoxic phenylalanine incorporation were observed. In liver and gill, rates of phenylalanine incorporation (respectively,  $0.74 \pm 0.08$  and  $1.46 \pm 0.27$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>) were not significantly different from normoxic levels by one hour post-hypoxia, and remained at similar levels for the duration of the recovery period. In contrast, phenylalanine incorporation took longer to return to normoxic levels in brain (three hours) and heart (two hours), and in both these tissues phenylalanine incorporation was significantly less than normoxic values for the remainder of the recovery period. This latter result suggesting that full recovery of protein synthesis in brain and heart tissues takes longer than four hours to occur.



**Figure 4.5.** Routine metabolic rate (A) and tissue phenylalanine incorporation (liver (B), brain (C), heart (D) and gill (E)) in *A. ocellatus* exposed to normoxia, severe hypoxia, and during recovery from hypoxia. Values are means  $\pm$  SEM,  $n = 8$  for routine metabolic rate measurements. For phenylalanine incorporation measurements,  $n = 12$  for normoxia and hypoxia exposure, and  $n = 4$  for each time point during recovery. Significant differences are indicated by differing letters,  $p < 0.05$ .

## 4.4 Discussion

### 4.4.1 Hypoxia Induced Metabolic Depression

4.4.1.1 Routine Metabolic Rate: In the present study, RoMR was maintained at normoxic rates until dissolved oxygen levels in the water decreased to 10%. At this time, RoMR underwent a 50% depression, which was maintained for the full three hours of hypoxia-exposure. This response to hypoxia was similar to that obtained previously for oscar, in which a depression in RoMR of approximately 50% was observed once water O<sub>2</sub> levels reached 10% oxygen saturation, and a reduction of 60% was measured when water O<sub>2</sub> levels approached anoxic conditions (Muusze et al. 1998). At the lowest level of hypoxia tested in the present study (10% DO), lactate levels were only significantly increased in plasma. However, this increase only brought lactate levels to one-fifth of levels obtained in *A. ocellatus* at 6% DO (Muusze et al. 1998). These results, in combination with the absence of lactate accumulation in white muscle, indicate anaerobic metabolism is only beginning to be employed to supplement energy demands at this level of oxygen deprivation, and metabolic depression is an effective way of conserving ATP until *A. ocellatus* is faced with almost anoxic conditions.

The level of metabolic depression achieved by *A. ocellatus* is similar to that of goldfish and crucian carp, which decrease metabolic rate by approximately 70% under anoxia (Van Waversveld et al. 1989), but not as great as demonstrated by freshwater turtles (90-95% reduction; Jackson 1968). Lower levels of metabolic depression are observed in teleosts due to the maintenance of ion exchange with the environment and low levels of activity for predator avoidance. For example, in their natural environment *A. ocellatus* are susceptible to predation from air-breathing fish and aerial predators, and

laboratory experiments show that they split their time equally between unprotected normoxic environments and sheltered hypoxic environments (Sloman et al. 2006). Whereas, turtles are essentially a closed system and they retreat into their protective shell and enter a comatose-like state during periods of oxygen deprivation.

*4.4.1.2 Protein synthesis:* The use of the flooding dose methodology to measure *in vivo* protein synthesis requires that several validation criteria be fulfilled. The results from this study show that the injection dosage used successfully flooded the free phenylalanine pool during both normoxia and three hours of hypoxic exposure, causing a 2-5 fold increase in phenylalanine concentration in the various tissues. As well, the specific radioactivity of the free phenylalanine pool was elevated one hour post-injection and remained stable at this level for the three hours over which protein synthesis was measured. The final validation criterion requires the incorporation of radiolabelled phenylalanine into the tissues to be linear post-injection. This was shown for all tissues in both normoxia and hypoxia exposed fish, except for white muscle (Figure 4.4). The radioactivity of protein bound phenylalanine in white muscle was below detectable levels, indicating rates of protein synthesis in this tissue to be extremely low. Given that rates of protein synthesis in fish white muscle are extremely low as compared to mammals (Fauconneau et al. 1995), and *A. ocellatus* has a much lower mass specific oxygen uptake than other teleosts, including tropical species (Almeida-Val et al. 2006), it is not surprising that protein synthesis was unable to be detected in the white muscle of *A. ocellatus*.

The role of protein synthesis in hypoxia-induced metabolic depression in ectothermic animals has been previously described in freshwater turtles (specifically *Trachemys scripta elegans* and *Chrysemys picta bellii*) and in the crucian carp (*Carassius carassius*), and these studies show the extent to which protein synthesis is depressed to be positively linked with the degree to which activity is curtailed. For example, rates of protein synthesis were suppressed by approximately 70% in the heart of *T. scripta elegans* (Bailey and Driedzie 1996) and by >95% in various tissues in *C. picta bellii*, both species which enter a comatose like state during anoxia (Land et al. 1993; Fraser et al. 2001). Whereas, the crucian carp, which maintains low levels of activity during hypoxia/anoxia exposure, exhibits a depression in protein synthesis of approximately 50% in heart and white muscle, 95% in liver tissue, but no significant depression in the brain (Smith et al. 1996). Similar to the crucian carp, *A. ocellatus* exhibited tissue specific depression in protein synthesis when exposed to acute hypoxia exposure. Rates of protein synthesis in liver, heart and gill were depressed by 50-60%, whereas rates of protein synthesis in the brain were only depressed by 27%. Thus, our results reinforce the idea that fish need to maintain protein synthesis in the brain to prevent damage to neural tissue, and to sustain appropriate brain functions so that predators can be effectively avoided.

#### *4.4.2 Recovery from Acute Hypoxia Exposure*

A significant overshoot in oxygen consumption, to 270% of normoxic rates, was observed during the first hour of recovery, indicating that the three hour hypoxic exposure was substantial enough to cause the fish to accumulate an oxygen debt. Crucian

carp have also been shown to accumulate a substantial oxygen debt during periods of hypoxia (Van den Thillart and Verbeek 1991), and it has been suggested the hyperactivation of metabolic rate during anoxic/severe hypoxic recovery is associated with the restoration of phosphocreatine, the conversion of lactate into glycogen, and possibly an increase in protein synthesis (Johansson et al. 1995). An *in vitro* study on turtle hepatocytes exposed to 12 hours of anoxia has shown a significant increase in protein synthesis (to 160% of normoxic rates) during the first hour of recovery (Land et al. 1993). However, the present study, which measured *in vivo* protein synthesis rates, did not show any hyperactivation of protein synthesis in the various tissues during the recovery period. These results agree with other *in vivo* studies showing a hyperactivation of protein synthesis does not occur in either anoxic exposed turtles (Fraser et al. 2001) or crucian carp (Smith et al. 1996). There were two distinct patterns observed in post-hypoxic phenylalanine incorporation in *A. ocellatus*, tissues which are a main source for protein synthesis, liver and gill, phenylalanine incorporation returned to pre-hypoxic rates by one hour post-hypoxia. In contrast, protein synthesis in brain and heart took longer than four hours post-hypoxia to fully recover. The slow recovery in brain tissue is particularly interesting as its hypoxia-induced reduction in protein synthesis is half of that shown by the other tissues. The reasons for this remain elusive, however, it may be linked to the removal of a dietary source of amino acids (due to the cessation of feeding) requiring *A. ocellatus* to rely on the recycling of existing protein (ie. protein turnover) to replenish diminished supplies due to the decrease of protein synthesis during metabolic depression.

#### 4.5 Conclusions

In conclusion, the present study was successful in providing insights into the cellular mechanisms behind whole animal metabolic depression in response to hypoxia in *A. ocellatus*. The response of *A. ocellatus* to acute hypoxia and subsequent recovery, at both the physiological and biochemical level, was similar to that of the well studied anoxia-tolerant teleost, the crucian carp. However, there were tissue-specific differences in the magnitude of the hypoxia-induced depression of protein synthesis (brain 20%, other tissues 50-60%), which suggest that brain function is maintained during hypoxia to facilitate active predator avoidance. Finally, this study demonstrated that an acute (3h) exposure to severe hypoxia is substantial enough to cause *A. ocellatus* to accumulate an oxygen debt, but the repayment of this oxygen debt is not accompanied by a compensatory hyperactivation in protein synthesis. This latter finding indicates the high metabolic rate *A. ocellatus* during the first hour of recovery is attributed to an increase in cellular processes other than protein synthesis.

## - CHAPTER FIVE -

### **Tissue specific responses of mitochondrial protein synthesis during hypoxic and temperature induced metabolic depression in the north temperate labrid,**

#### ***Tautoglabrus adspersus.***

##### **5.1 Introduction**

Metabolic depression, characterized by a 60-95% reduction in basal metabolic rate, enables animals to extend survival time in the face of unfavourable environmental conditions. It has been described in all animal phyla, in every class of vertebrates and in response to various environmental stressors (anoxia, hypothermia, desiccation and restricted access to food) (reviewed in Guppy et al. 1994, Hand and Hardewig 1996, Guppy and Withers 1999). Through extensive research it has become evident that metabolic depression is reflected at all levels of biological organization and is accomplished by the establishment of a reduced rate of ATP turnover, by decreasing both energy consuming and producing processes at the cellular level (reviewed in Storey 1988, Guppy et al. 1994, Hand and Hardewig 1996, Guppy and Withers 1999, Hochachka and Lutz 2001, Storey 2002, Storey and Storey 2007). Recently, research has begun to look towards adaptations at the sub-cellular level for potential regulating mechanisms of metabolic depression (Guppy 2004). Mitochondria have been identified as a potential site for the control of metabolic depression as 90% of cellular respiration is mitochondrial, 80% of which is coupled to ATP synthesis (the remaining 20% is due to proton leak) (Rolfe and Brown 1997). The development of model systems from the following three hypometabolic animals has begun to elucidate the response of mitochondria during metabolic depression: 1) hepatocytes from hibernating mammals

(Martin et al. 1999, Barger et al. 2003, Muleme et al. 2006, Brown et al. 2007), 2) isolated skeletal muscle cells from hypoxic frogs (St. Pierre et al. 2000abc) and 3) isolated hepatopancreas cells from estivating snails (Bishop and Brand 2000, Guppy et al. 2000, Bishop et al. 2002). The reduction of active (state 3) mitochondrial respiration has emerged as a cellular adaptation for metabolic depression that is conserved both between tissues in an organism and across taxa (Martin et al. 1999, Bishop and Brand 2000, Guppy et al. 2000, St. Pierre et al. 2000c, Barger et al. 2003, Brown et al. 2007). The mechanism for the reduction in mitochondrial respiration appears to lie in the inhibition of substrate oxidation, as seen in experiments on isolated cells from both frogs and snails. In turn, this causes a drop in membrane potential and a subsequent decrease in proton leak and ATP turnover (involving processes such as ion transport and protein synthesis) (Bishop et al. 2002).

In eukaryotic cells, the synthesis of proteins for the assembly and functioning of mitochondria is a result of the cooperation between the mitochondrial and nuclear genomes. The majority (90%) of proteins that are found in mitochondria are synthesized in the cytoplasm by the nuclear genome and transported into the mitochondria. These proteins are found in all four of the mitochondrial compartments (inner and outer membranes, matrix and intermembrane space), and are key players in all aspects of mitochondrial function. For example, they are linked with mitochondrial transcription and translation, lipid synthesis, substrate oxidation by the TCA cycle, oxidative phosphorylation and electron transport (Poyton and McEwen 1996). The reduction of protein synthesis has been exhibited at the cellular level in response to hypoxia in goldfish (Smith et al. 1996), oscars (Chapter Four) and turtles (Bailey and Driedzie 1996,

Fraser et al. 2001) and in response to low temperature in the cunner (Chapter Three). Additionally, a reduction in mitochondrial protein synthesis was observed in the hearts of hypoxia exposed turtles (Bailey and Driedzie 1996) but the response in hypometabolic fish has yet to be elucidated. In fact, despite the growing number of examples of hypometabolic teleosts, there has been very little investigation into the physiological role of mitochondria in hypometabolic teleosts.

The cunner (*Tautoglabrus adspersus*) experiences active metabolic depression in response to seasonal hypothermia in its North Atlantic habitat, which has previously been described at the behavioural (Green and Farwell 1971), whole animal physiological (Haugaard and Irving 1948, Curan 1992, Costa 2007) and cellular levels (Chapter Three). In addition, cunner also display a decrease in basal metabolic rate in response to graded hypoxia (Corkum 2007). To our knowledge, cunner do not normally experience hypoxia in their natural environmental and as such, it poses the question: Are animals that have evolved the capacity for metabolic depression able to institute the same cellular responses when challenged with an environmental challenge that does not occur in its natural environment? Through measurement of protein synthesis in the whole tissue and mitochondrial protein pools, in cunner challenged with both acute hypothermia and hypoxia, it will be possible to determine: i) if the adaptation of decreased protein synthesis is conserved across environmental stressors, and ii) if the adaptive response of metabolic depression is conserved across levels of cellular organization. Two tissues have been chosen for the analysis of protein synthesis, the heart (an anaerobic tissue under hypoxia due to its position in the circulatory system) and the gill (an aerobic tissue due to its direct interface with environmental oxygen). Based on previous observations

that protein synthesis at the whole tissue level is depressed during winter dormancy in the cunner (Chapter Three), it is hypothesized that protein synthesis in the mitochondrial pool will be suppressed in both heart and gill in response to acute low temperature stress. In contrast, when challenged with acute hypoxia it is predicted that rates of mitochondrial protein synthesis will be reduced in the mitochondria of heart of the cunner, similar to the depression observed in mammalian (McKee et al. 1990) and turtle hearts under hypoxia (Bailey and Driedzic 1996) but defended in gill as this tissue plays a central role in survival as it is the primary organ for gas and ion exchange (Lyndon and Houlihan 1998).

## **5.2 Materials and Methods**

### *5.2.1 Animals*

Cunner were caught by baited hoop net in Portugal Cove (Newfoundland, Canada) in September 2006 and were held at the Ocean Sciences Centre, Memorial University of Newfoundland in 1 m<sup>2</sup> tanks. Fish utilized for this experiment were maintained on a flow through seawater system, with heated seawater ranging in temperature between 8 and 10°C and were exposed to a natural photoperiod. All animals were fed to satiation on a diet of chopped frozen herring, offered three times a week, with feeding being withheld one week prior to sampling. All fish were treated in accordance to Canadian Council of Animal Care guidelines.

### 5.2.2 Experimental Procedure

During April and May 2007 fish were randomly divided into three groups of nine fish: i) normoxic 8°C control, ii) acute temperature challenge, iii) acute hypoxia challenge. On a given sample day three fish were removed from the holding tank, weighed and tagged for individual recognition and placed in the experimental tank (40 L) 24 hours prior to the experiment. Immediately before beginning the experimental procedure a 30% water change was performed. Each treatment was repeated three times to give a total sample size of nine fish per treatment. Fish in the normoxic control group were held at 8°C ± 0.5°C, 90-100% water O<sub>2</sub> saturation. To challenge the fish with an acute hypothermia, temperature was decreased in a step wise manner of 1°C per hour from 8°C to 4°C under normoxic conditions. These temperatures were chosen as they represent the extremes of protein synthesis rates with respect to metabolic depression as results from the seasonal study (Chapter Three) indicated at 8°C proteins are being synthesized at the normal rate, where as at 4°C, protein synthesis was depressed to its lowest level in heart and gill tissue.

In the hypoxia treatment, fish were exposed to a gradual decrease in water oxygen saturation. Water oxygen levels were stepped down from 100 to 70, 50, 30, 20 and 10%, through the bubbling of nitrogen directly into the water. Oxygen levels were maintained for one hour at each level and temperature was held at 8°C ± 0.5°C. Once at the desired experimental point, fish were injected intraperitoneally, with 1.0 ml · 100 g<sup>-1</sup> of [2, 3-<sup>3</sup>H] phenylalanine (Amersham International) solution following the flooding dose methodology of Garlick et al. (1980) described in detail in previous chapters. Fish were sampled at 6, 8 and 10 hours-post injection; these particular time points have been

previously determined to fall within the linear range of phenylalanine incorporation in this species (Chapters Two and Three). One fish was sampled at each time point, at which time blood was drawn via caudal puncture with heparinized syringes for analysis of plasma lactate. Fish were killed by a severe blow to the head followed by a severing of the spine. Heart and gill tissue were immediately excised for subcellular fractionation as described below.

### *5.2.3 Isolation of Mitochondria*

Preliminary work was conducted to determine the appropriate protocol for the separation of subcellular fractions by differential centrifugation. Fractionation procedures were based upon the theory described by Ballantyne (1994) and the methodology of Treberg et al. (2006). After removing lamellae from the gill rakers and separating the ventricle from whole hearts, the resulting tissue was weighed and subsequently diced using scissors and a razor blade in a small volume of isolation media. The isolation media consisted of 250 mM Sucrose, 10 mM HEPES (pH 7.4), 1 mM EDTA, pH 7.4 at 20°C. Diced tissue was added to individual glass homogenization tubes with nine volumes of ice-cold isolation media containing 0.5% (w/v) fatty acid free bovine albumin. Tissue was homogenized on ice by three passes of a loose fitting and three passes of a tighter fitting motor-driven Teflon pestle. An aliquot of crude homogenate was taken for the analysis of whole tissue protein synthesis rates. The remaining homogenate used to obtain the subcellular fractions by differential centrifugation. All centrifugation was performed using a benchtop centrifuge at 4°C, for 10 minutes. Crude homogenate was centrifuged at 600 g to remove the myofibrils and other cellular debris and the supernatant was further

centrifuged at 9000 g to separate the mitochondria (pellet) from the cytosol (supernatant). The mitochondrial pellet was washed two times by resuspending the pellet in 1 ml of isolation media and gently pipetting up and down, followed by centrifugation at 9000 g.

#### *5.2.4 Marker Enzymes*

The purity of the mitochondrial fraction was determined by preliminary marker enzyme analysis. All enzyme analyses were performed on fresh homogenate at 15°C and run in duplicate with increasing sample volumes to ensure activity was linear with protein. Lactate dehydrogenase (LDH) and NAD<sup>+</sup>-linked glycerol-3 phosphate dehydrogenase (GPDH) were run as cytosolic markers, succinate-cytochrome C reductase (SCCR), an electron transport chain enzyme, was used as a mitochondrial membrane marker and citrate synthase (CS), an enzyme of the mitochondrial matrix, was used as an indicator of mitochondrial intactness. LDH (EC 1.1.1.27) and GPDH (EC 1.1.1.94) activity was determined at 340 nm (extinction coefficient of 6.22) in an assay buffer containing 50 mM imidazole (pH 7.4 at 20°C), 1.0 mM KCN, 0.2 mM NADH. The LDH reaction was initiated with 1.0 mM pyruvate and the GPDH reaction was initiated with 2 mM DIIAP. The SCCR (1.10.2.2) activity was determined at 540 nm (extinction coefficient of 19.2) in an assay buffer containing 60 mM K<sub>2</sub>HPO<sub>4</sub>, 40 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM KCN, 3 mM EDTA, 0.1 mM cytochrome C (pH 7.4), the reaction was initiated with 20 mM succinate. CS (EC 2.3.3.1) activity was determined at 412 nm (extinction coefficient of 14.1) in an assay media containing 75 mM Tris (pH 8.2 at 20°C), 0.2% Triton X-100, 0.1 mM DTNB, 0.3 mM acetyl-CoA and the reaction was initiated with 0.5 mM oxaloacetate.

### 5.2.5 Analysis of Protein Synthesis

Precipitation of proteins was achieved through the addition of four volumes of ice-cold 6% perchloric acid (PCA), except for the mitochondrial fraction in which nine volumes of PCA were added. Samples were mixed by vortexing and allowed to settle on ice for ten minutes. Samples were then centrifuged at 15,600 g for 5 minutes to separate out the protein pellet. The resulting supernatant was decanted and frozen at -20°C for further analysis of free pool phenylalanine content by fluorometric microplate assay (Gerasimova et al. 1989) and specific activity through scintillation counting. The protein pellets were washed with 1 ml 6% PCA until the radioactivity in the discarded supernatant was at background levels to ensure only protein bound <sup>3</sup>H phenylalanine was being measured in the protein pellet. The protein pellet was solubilized in 0.3 M NaOH in a water bath at 37°C. The dissolved protein was stored at -20°C until analysis for total protein content using the BioRad Dc assay and protein bound radioactivity through scintillation counting. The incorporation of phenylalanine into protein was expressed as a ratio of the protein bound radioactivity of the subcellular component to the specific radioactivity of the free phenylalanine pool in the whole tissue (dpm · mg pr<sup>-1</sup> per dpm · nmol phe<sup>-1</sup>).

### 5.2.6 Lactate Analysis

Plasma samples were diluted in three volumes of cold 6% PCA for lactate analysis in microplate spectrophotometer. Lactate content was determined in samples and standards at 340 nm via the reduction of NAD<sup>+</sup> to NADH at 340 nm using a Sigma diagnostics kit.

### *5.2.7 Statistical Analysis*

Comparison of the marker enzyme activity in the subcellular fractions and rates of protein synthesis were performed by one way ANOVA, followed by Tukey's post hoc (p<0.05 significant).

## **5.3 Results**

### *5.3.1 Marker Enzymes*

Table 5.1 shows the specific activities of the marker enzymes measured in the crude homogenate as well as in the cytoplasmic and mitochondrial fractions. These results give an indication of the relative purity of each of the subcellular fractions by demonstrating enhancement of the activity of the marker enzyme for that fraction compared to the activity in crude homogenate. The mitochondrial marker enzymes SCCR and CS were enriched from 64% to 107% in the mitochondrial fraction as compared to the crude homogenate in both heart and gill (Table 5.1). In addition, the low levels of CS activity (enzyme of mitochondrial matrix) in the cytoplasmic fraction in both heart (10-fold lower) and gill (15-fold lower), as compared to crude homogenate, indicate the subcellular fractionation procedure was successful in separating intact mitochondria from the cytoplasm. GPDH was added as a secondary cytosolic marker enzyme as LDH levels were elevated in gill mitochondrial fractions despite various modifications of the fractionation protocol. As activity levels of GPDH were four-fold higher in heart and two-fold higher in gill crude homogenate than in the mitochondrial fraction it demonstrates that there was little cytoplasmic contamination of the mitochondrial fraction. Based on these results, adequate, albeit only partial enrichment of the various

subcellular fractions was achieved though the differential centrifugation methods allowing investigation into rates of protein synthesis in enriched subcellular components.

### 5.3.2 Protein Synthesis in the Whole Homogenate

Under control (normoxic 8°C) conditions the rates of phenylalanine incorporation (expressed as  $\text{nmol phe} \cdot \text{mg pr}^{-1} \cdot \text{hr}^{-1}$ ) in heart ventricular and gill lamellar tissue were  $0.54 \pm 0.08$  and  $0.57 \pm 0.11$   $\text{nmol phe} \cdot \text{mg pr}^{-1} \cdot \text{hr}^{-1}$ , respectively. The exposure to an acute temperature decrease, from 8°C to 4°C, resulted in a significant reduction in protein synthesis in both heart ( $0.11 \pm 0.02$   $\text{nmol phe} \cdot \text{mg pr}^{-1} \cdot \text{hr}^{-1}$ ,  $p < 0.001$ ) and gill ( $0.13 \pm 0.03$   $\text{nmol phe} \cdot \text{mg pr}^{-1} \cdot \text{hr}^{-1}$ ,  $p = 0.0013$ ). The extremely high  $Q_{10}$  values of 53 and 40 calculated for heart and gill, respectively, suggest that this decrease was part of an active depression in metabolism and not due to temperature effects alone. Exposure to acute hypoxia resulted in a significant decrease in ventricular protein synthesis ( $0.27 \pm 0.07$   $\text{nmol phe} \cdot \text{mg pr}^{-1} \cdot \text{hr}^{-1}$ ,  $p = 0.024$ ). There was no significant difference in gill ( $0.32 \pm 0.10$   $\text{nmol phe} \cdot \text{mg pr}^{-1} \cdot \text{hr}^{-1}$ ,  $p = 0.18$ ) however, the average rate was 40% lower under hypoxic than control conditions. In addition, exposure to acute hypothermia caused a greater reduction in protein synthesis (~80% in both tissues) than did exposure to acute hypoxia (~40% in heart) (Figure 5.1).

**Table 5.1** Specific activity of subcellular marker enzymes ( $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg pr}^{-1}$ ) for heart and gill tissue from cunner (*Parutogobius aospersus*). Significant differences between the specific activities of marker enzymes are indicated by different letters,  $p < 0.05$ . Values are presented as mean  $\pm$  SEM.

<b>Enzyme Activity (<math>\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg pr}^{-1}</math>)</b>			
<b>Enzyme</b>	<b>Homogenate</b>	<b>Cytoplasmic</b>	<b>Mitochondrial</b>
<i>Heart</i>			
<b>LDH</b>	$1271.5 \pm 20.1^a$	$1246.9 \pm 28.8^a$	$354.2 \pm 4.9^b$
<b>GPDH</b>	$289.7 \pm 10.6^a$	$223.1 \pm 4.1^a$	$73.5 \pm 7.5^b$
<b>SCCR</b>	$1.1 \pm 0.04^a$	$0.4 \pm 0.02^b$	$1.8 \pm 0.06^a$
<b>CS</b>	$90.1 \pm 3.2^a$	$9.1 \pm 0.2^b$	$150.7 \pm 7.8^c$
<i>Gill</i>			
<b>LDH</b>	$282.5 \pm 3.4^a$	$254.9 \pm 5.1^a$	$268.2 \pm 7.5^a$
<b>GPDH</b>	$52.9 \pm 2.1^a$	$159.2 \pm 5.3^b$	$23.3 \pm 0.7^c$
<b>SCCR</b>	$1.3 \pm 0.06^a$	$0.5 \pm 0.01^b$	$2.3 \pm 0.06^c$
<b>CS</b>	$32.1 \pm 0.9^a$	$2.2 \pm 0.09^b$	$66.7 \pm 1.6^c$

### *5.3.3 Protein Synthesis in the Cytosolic Fraction*

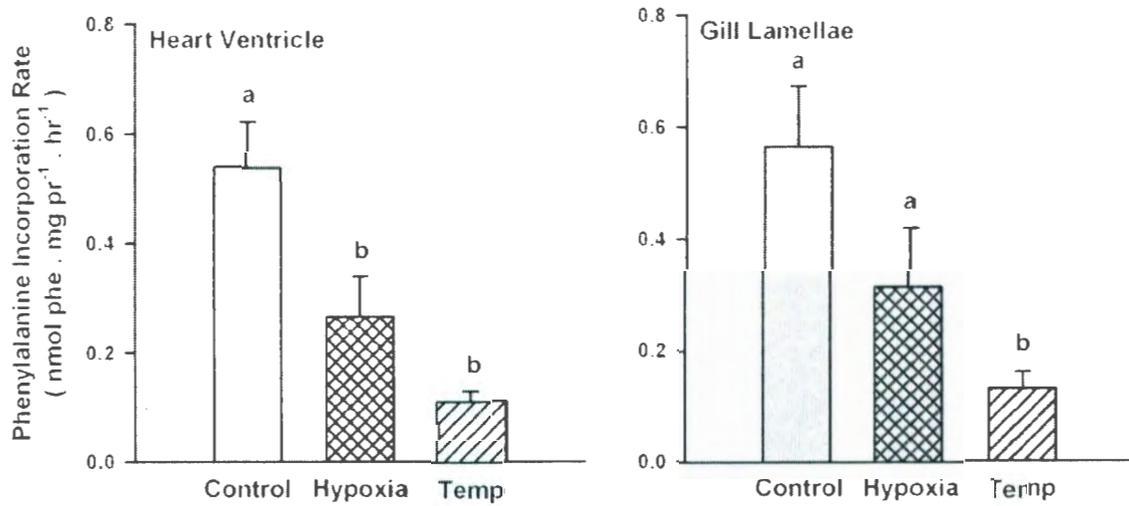
Protein synthesis rates of the cytosolic fractions of both heart and gill exhibited a similar response pattern as observed in the whole homogenate. The control rates of phenylalanine incorporation in the heart ( $0.40 \pm 0.05$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>) and gill ( $0.40 \pm 0.012$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>) were reduced by approximately 80% under low temperature stress to  $0.07 \pm 0.01$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup> ( $p < 0.001$ ) in heart and to  $0.08 \pm 0.02$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup> ( $p=0.018$ ) in gill. As in the whole homogenate, only heart cytosolic proteins showed a significant decrease in synthesis under hypoxia ( $0.20 \pm 0.07$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>,  $p=0.034$ ). The average rate for gill cytosolic protein synthesis under hypoxia was  $\sim 55\%$  lower ( $0.18 \pm 0.06$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>); however this decrease was not significantly different from control rates ( $p=0.12$ ) (Figure 5.2).

### *5.3.4 Protein Synthesis in the Mitochondrial Fraction*

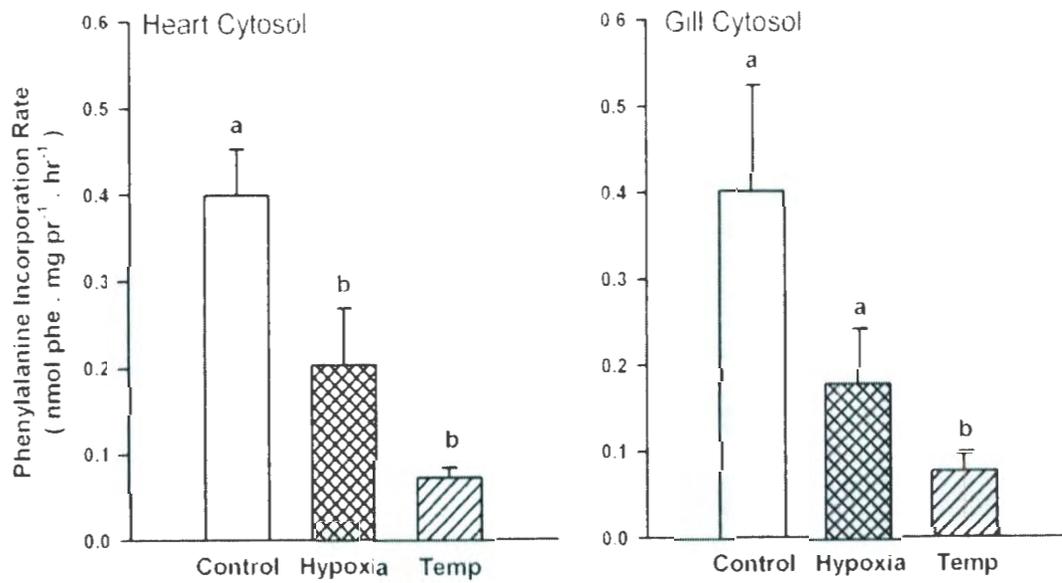
Rates of protein synthesis in the mitochondrial fraction were  $0.90 \pm 0.13$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup> for heart ventricular tissue and  $0.74 \pm 0.18$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup> for gill lamellar tissue in control fish. Rates of protein synthesis in the mitochondrial protein pool of the heart were depressed by approximately 60% when exposed to both low temperature ( $0.37 \pm 0.083$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>,  $p=0.003$ ) and hypoxic stress ( $0.32 \pm 0.087$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>,  $p=0.002$ ). In contrast, mitochondrial protein synthesis in gill lamellar tissue was defended under both low temperature ( $0.61 \pm 0.20$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>,  $p=0.64$ ) and hypoxic stress ( $0.68 \pm 0.24$  nmol phe  $\cdot$  mg pr<sup>-1</sup>  $\cdot$  hr<sup>-1</sup>,  $p=0.85$ ) (Figure 5.3).

### 5.3.5 Plasma Lactate

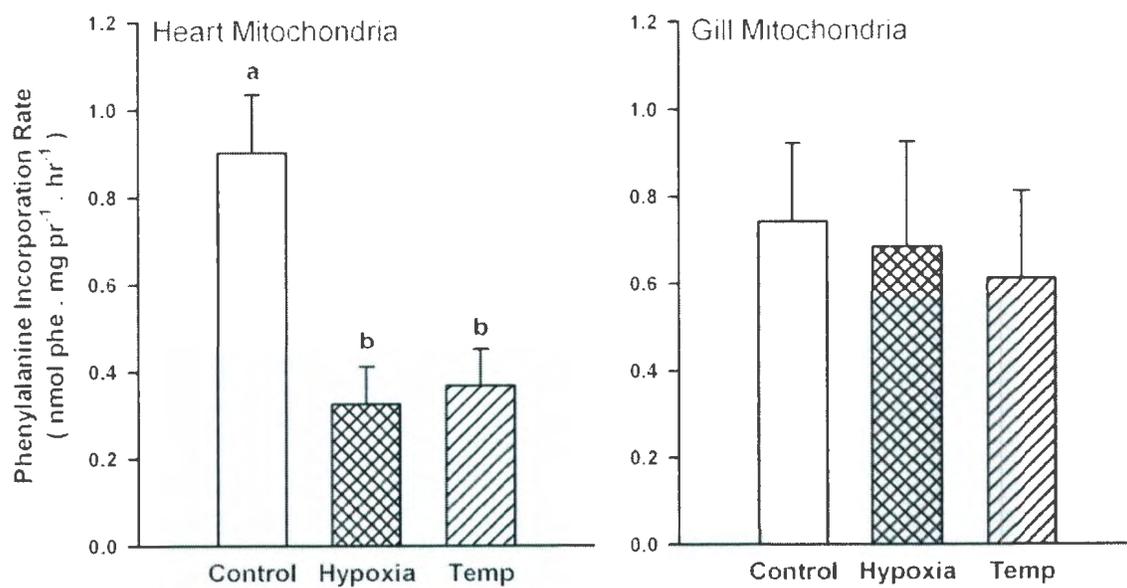
Levels of plasma lactate were below detectable levels in both the control (8°C) group (n = 9) and the acute temperature challenge group (4°C) (n = 9). However, exposure to hypoxia stimulated the production of lactate as levels increased to  $0.57 \pm 0.04$  mM (n = 9). Samples from all three time points were pooled as there was no significant change in lactate levels with increasing exposure time to hypoxia.



**Figure 5.1** Phenylalanine incorporation rates (nmol phe · mg pr<sup>-1</sup> · hr<sup>-1</sup>) in whole homogenate of heart ventricular and gill lamellar tissue under control conditions (normoxia, 8°C), acute hypoxia stress (10% O<sub>2</sub> SAT, 8°C) and acute low temperature stress (normoxia, 4°C). Rates expressed as mean ± SEM, n = 9 for each treatment; significance between treatments indicated by different letters, p < 0.05.



**Figure 5.2** Phenylalanine incorporation rates (nmol phe · mg pr<sup>-1</sup> · hr<sup>-1</sup>) in the cytosolic proteins in heart ventricular and gill lamellar tissue under control conditions (normoxia, 8°C), acute hypoxia stress (10% O<sub>2</sub> SAT, 8°C) and acute low temperature stress (normoxia, 4°C). Rates are expressed as means ± SEM, n = 9 for each treatment. Significant differences between treatments are indicated by different letters, p < 0.05.



**Figure 5.3** Phenylalanine incorporation rates (nmol phe · mg pr<sup>-1</sup> · hr<sup>-1</sup>) in the mitochondrial fraction of heart ventricular and gill lamellar tissue under control conditions (normoxia, 8°C), acute hypoxia stress (10% O<sub>2</sub> SAT, 8°C) and acute low temperature stress (normoxia, 4°C). Rates are expressed as means ± SEM. n=9 for each treatment. Significant differences between treatments are indicated by different letters. p<0.05.

## 5.4 Discussion

### 5.4.1 Subcellular Fractionation

The elevated protein specific levels of the mitochondrial marker enzymes (SCCR and CS) and lower levels of GPDH (cytoplasmic-marker) in the resulting pellet from the high speed centrifugation steps (9000 x g) indicate the differential centrifugation procedure was successful separating out relatively pure mitochondrial and cytosolic fractions. Thus, we can discuss rates of protein synthesis in this fraction as those of mitochondrial protein synthesis (translated in both the nuclear genome and transported into the mitochondria and the synthesis of proteins by mitochondrial ribosomes). Low levels of SCCR and CS in the first high speed supernatant referred to as the "cytosolic" fraction imply a trivial mitochondrial contamination. The relative enrichment of the cytosolic and mitochondrial fractions in this study are comparable to those obtained by Brooks and Storey (1993), despite the differences in the fractionation protocol, in which the mitochondrial marker enzyme was enriched by two-fold and the cytosolic marker enzyme was enriched by three-fold, in their respective sub-cellular fractions. The high levels of LDH in the mitochondrial fraction, particularly in gill tissue, may suggest the presence of a mitochondrial linked LDH but further investigation into this area is needed before this can be said with certainty. To our knowledge, the presence of a mitochondrial linked LDH is a novel finding in teleosts, but the existence of an intracellular lactate shuttle and a mitochondrial linked LDH has been described in mammals and yeast (Brooks et al. 1999). The role of a mitochondrial LDH in mammals appears to be linked to cellular lactate oxidation in liver, skeletal and cardiac muscle of rats to facilitate high clearance rates of lactate during exercise via an intracellular lactate shuttle.

#### 5.4.2 Physiological Response to Hypothermia Hypoxia

The exposure of both acute hypothermia and hypoxia caused a decrease in activity levels of the cunner, which were found sequestered amongst the cooling coils and in the corners of the experimental tank. Even at the lowest level of hypoxia (10% O<sub>2</sub> saturation) fish did not become agitated and there were no mortalities over the 10 hour experimental hypoxia exposure (personal observations). The ability of cunner to survive extended hypoxia exposure (up to 10 hours at 10% O<sub>2</sub> saturation) suggests they may employ similar physiological responses to low oxygen as other hypoxia-tolerant ectotherms, such as the carp (*Cyprinus carpio*), the oscar (*Astronotus ocellatus*), the common frog (*Rana temporaria*) and the turtle (*Chrysemys picta*) (Boutilier et al. 1997, Lutz and Nilsson 1997, Muusze et al. 2001). Plasma lactate levels in the cunner, which were below detectable levels under normoxic conditions, increased to 0.57 mM in hypoxia exposed cunner indicating that anaerobic metabolism had been stimulated. This increase in plasma lactate is on par with the response in other hypoxia-tolerant teleosts. For example, the exposure to three hours of hypoxia at 28°C caused plasma lactate to increase from 0.04 to 1.13 mM in the oscar (*Astronotus ocellatus*) (Chapter Four), exposure to six hours of anoxia at 15°C resulted in a 2.5-fold increase in whole body lactate concentration in crucian carp (*Carassius carassius*) (Johnston and Bernard 1983) and exposure to eight hours of anoxia resulted in an 8-fold increase in whole body lactate in goldfish (*Carassius auratus*) (van Waversveld et al. 1989). In facultative anaerobes, such as the aforementioned teleosts, the switch to anaerobic metabolism is not immediately detrimental as they are able to use the ATP generated from the glycolytic pathway to sustain reduced rates of energy turnover and slow down the use of limited endogenous

fuels (Boutilier and St. Pierre 2000) and in the case of cyprinids they are able to prevent large levels of lactate accumulation by converting pyruvate to ethanol in order to prevent acidosis from accumulation of lactic acid (Shoubridge and Hochachka 1980; Johnston and Bernard 1983).

#### *5.4.3 Protein Synthesis in the Whole Tissue and Cytosolic Protein Pools*

As protein synthesis is an energetically costly process, consuming 20-30% of ATP coupled cellular respiration (Rolfe and Brown 1997) it is often down-regulated during metabolic depression in order to conserve energy. In the current study, exposure to acute low temperature results in a significant depression in phenylalanine incorporation into both heart ventricular and gill lamellar whole tissue and cytosolic pools (~80%) in the cunner. The reduction in protein synthesis in response to acute hypothermia is greater than the response in cunner which entered a hypometabolic state under natural decline in temperature, in which a depression of 55% in heart and 66% in gill is observed over the same temperatures (Chapter Three). This substantial decrease in protein synthesis in the heart and gill in response to an acute temperature challenge ( $Q_{10}$  values of 53 for heart and 40 for gill) provides further evidence that the reduction in protein synthesis in response to seasonal hypothermia is part of an active metabolic depression and not just due to temperature effects alone.

As predicted, rates of protein synthesis in the heart were significantly reduced in response to acute hypoxia exposure (Figure 5.1). The 55% reduction in ventricular protein synthesis in the cunner is comparable to the 48% reduction observed in crucian carp hearts after 48 hours exposure to anoxia (Smith et al. 1996) and the 60% reduction

in ventricular protein synthesis in oscar exposed to three hours of acute hypoxia (Chapter 4). A substantial metabolic depression is also observed in cardiomyocytes from hypometabolic anurans exposed to acute hypoxia, however, a decrease in metabolism is not apparent in cardiomyocytes isolated from frogs exposed to long term hypoxic submergence (Currie and Boutilier 2001). The results from this study demonstrated that acute hypoxia stimulates an active depression in protein synthesis and suggests that the cellular responses involved in the whole animal metabolic depression are conserved across environmental stressors. Despite the 40-50% decrease in rates of protein synthesis in response to hypoxia in the whole homogenate or cytosolic pool of gill lamellae, this reduction was not statistically significant. These results were unexpected as previous studies have demonstrated that hypoxic exposure resulted in a 50% decrease in gill protein synthesis in the oscar (Chapter Four) and caused a repression of genes encoding for protein translational machinery, such as ribosomal proteins and subunits in zebrafish (van der Meer et al. 2005). One interpretation of the current data is simply that the volume of homogenate obtained from the tissue per individual and sample size was too small relative to individual variation to lead to statistically significant difference and that the trends shown by the decreases in average rates of protein synthesis under hypoxia are biologically significant. However, alternative interpretation that no change occurred deserves consideration. Tissue specific responses of protein synthesis have been shown to occur in hypometabolic teleosts and are often linked to adaptations that have evolved to extend survival time while in an energetically compromised state. For example, despite the substantial decrease in protein synthesis in the majority of tissues, levels of protein synthesis in the brain are maintained at normoxic levels in carp (Smith et al.

1996) and only minimally depressed in oscar (Chapter Four). These tissue specific responses in the brain have been linked to the maintenance of low levels of activity for predator avoidance. In the present study, the possible defense of lamellar protein synthesis in the whole tissue protein pool during hypoxia could be related to the central role this tissue plays in the survival of the fish as it is the primary organ for respiratory and ionic exchange (Lyndon and Houlihan 1998). In addition, as branchial protein synthesis only accounts for approximately 5% of whole body protein synthesis maintenance of this process during metabolic depression would not be energetically expensive when considered in the context to the whole animal (Lyndon and Houlihan 1998).

#### *5.4.4 Protein Synthesis in the Mitochondrial Protein Pool*

In the current study, rates of sub-cellular protein synthesis were determined *in vivo* and are representative of the translation of protein that occurs in both the nuclear-cytoplasmic compartment, and subsequently transported into the mitochondrion, as well as the translation of protein on ribosomes in the mitochondrial component itself. These proteins are found in all four of the mitochondrial compartments (inner and outer membranes, matrix and intermembrane space), and are key players in all aspects of mitochondrial function (Poyton and McEwen 1996).

When exposed to acute low temperature stress, protein synthesis in the mitochondrial pool of the ventricle was reduced by 60%. Although the reduction in the mitochondrial pool is less than the response in the whole tissue or cytoplasmic pools (80% reduction), a  $Q_{10}$  value of 9.2, calculated between 8°C and 4°C, indicates that it is

still a result of an active reduction in protein synthesis. These results suggest the response of metabolic depression, indicated by active depression of protein synthesis, is conserved across levels of biological organization in the ventricular tissue of cunner. Little work has been conducted on the adaptive responses of mitochondrial physiology in hypometabolic teleosts, and work on other hypometabolic ectotherms has focused mainly on changes to the oxidative capacity of the mitochondria (mitochondrial respiration and proton leak) during aerobic metabolic depression. As such, it is difficult to compare the effects of low temperature on the mitochondrial protein synthesis in the cunner to other hypometabolic ectotherms. Mitochondrial protein synthesis rates in rainbow trout (*Oncorhynchus mykiss*) were not influenced by a decrease in temperature from 25°C to 15°C, but when temperatures were further reduced to 5°C rates of mitochondrial proteins synthesis were substantially reduced (70%) (West and Driedzie 1999), which is comparable to the depression observed in hypothermia challenged cunner. McKee et al. (1990) demonstrated that in rat hearts, mitochondrial protein synthesis is coupled with aerobic respiration, however, in rainbow trout the substantial decrease in mitochondrial protein synthesis was not coupled to oxygen consumption in mitochondria (West and Driedzie 1999). In mitochondria from hypometabolic frog skeletal muscle, the decrease in respiration at the cellular level is also reflected at the mitochondrial level (St. Pierre 2000a). Although mitochondrial respiration was not measured in this study, it is speculated that protein synthesis would be tightly coupled with mitochondrial respiration in the heart of the cunner during metabolic depression. Cardiac muscles have a rich supply of mitochondria and are almost totally dependent upon aerobic metabolism to obtain energy needed for contraction. As such, it is not surprising that when cunner are

challenged with acute hypoxia the 50% reduction in protein synthesis in the whole tissue protein pool is accompanied by an even greater reduction in the pool of proteins destined for the mitochondria (65%). These results from the cunner are comparable to those from turtle (*Trachemys scripta elegans*) hearts, in which exposure to three hours of acute anoxia resulted in a three-fold decrease in protein synthesis in the whole tissue protein pool and a reduction of three to five-fold in the mitochondrial protein pool (Bailey and Driedzic 1996). Based upon these results from cunner and turtles, it appears that during hypoxia-induced hypometabolism mitochondria decrease both total ATP synthesis (cessation of oxidative phosphorylation) and the import of newly synthesized proteins. The decrease in mitochondrial protein synthesis observed in these animals may be a biochemical adaptation to decrease energy demand and subsequently extend anoxic survival (Bailey and Driedzic 1996).

The most important and novel finding of this experiment arises when examining subcellular protein synthesis rates in gill lamellar tissue. The synthesis of proteins destined for the mitochondria in gill are defended under acute hypoxia exposure, providing evidence that the defense of protein translation in the gill tissue is essential to the cunner's survival under anaerobic conditions. Gill mitochondrial protein synthesis was also defended in response to acute hypothermia, which was unexpected, particularly as there was a substantial inhibition of synthesis in the whole protein pool in this tissue (~80%). One likely explanation for the maintaining this ATP-consuming process in the mitochondrial despite the decrease in the whole tissue pool would be to ensure the continual synthesis of proteins slated for cellular turnover of epithelial and/or chloride cells of the gill (Lyndon and Houlihan 1998). The chloride cells of the gill lamellae are

mitochondrial rich and play a vital role in ionic regulation, i.e. site of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  ion exchange with the aquatic environment (Perry 1998). The reduction of mitochondrial respiration during hypometabolism is an excellent mechanism of energy conservation; however, the animal will inevitably be faced with severe challenges upon arousal if it doesn't maintain some capacity for oxidative phosphorylation (St. Pierre et al. 2000b). As proteins synthesized for the mitochondrial pool play key roles in electron transport and oxidative phosphorylation which supply the majority of energy (ATP) for eukaryotic cells in aerobic environments (Poyton and McEwan 1996), it is also possible that cunners are maintaining protein synthesis in the mitochondrial pools, during both hypoxia and hypothermia induced metabolic depression, in order to maintain oxidative capacity in gill lamellae. This would enable functional enzymes of the electron transport chain and TCA cycle to be available for the rapid reinstatement of oxidative phosphorylation upon the emergence from a metabolically depressed state.

## **5.5 Conclusion**

The present results demonstrated an active depression in protein synthesis in the whole tissue pool of heart and gill in response to acute hypothermia. These results further emphasize that the inhibition in protein synthesis in response to seasonal decreases in temperature is an intrinsic response that is part of an active metabolic depression. In addition, the substantial decrease in whole tissue and mitochondrial protein synthesis in the heart, in response to acute hypoxia, suggests that cunner are able to institute the same adaptive cellular responses that have evolved for hypothermia induced metabolic depression to lower the metabolic rate, and subsequently increase survival time when

exposed to an environmental stress that does not normally occur in the natural habitat. The ability of the cunner to decrease its metabolism in response to both low temperature and hypoxia makes it an ideal model organism to initiate the investigation of the role of mitochondrial physiology in hypometabolic teleosts. The response of protein synthesis in the heart is conserved across levels of cellular organization and exposure to varying environmental stressors. The novel finding of this experiment was that the synthesis of proteins slated for use in the mitochondria is defended under both hypoxia and hypothermia, despite being decreased at the whole animal level. As seen with seasonal metabolic depression, anomalies in the response of protein synthesis can be linked to adaptations that enable extended survival while in an energetically compromised state. The defense of protein synthesis in the mitochondrial cells of the gill is thought to be coupled with the vital role of this tissue in ion exchange with the aquatic environment. Also, the defense of synthesis of proteins for import into the mitochondria may play a role in maintaining oxidative capacity for a rapid reinstatement of mitochondrial respiration upon emergence from a metabolic depression.

## - CHAPTER SIX -

### 6.1 Summary

The objective of the thesis was to extend the study of metabolic depression in teleost fish beyond the behavioural and whole animal physiological level to include a description of the biochemical processes underlying regulated hypometabolism. Protein synthesis was chosen as the cellular mechanism to study as it contributes greatly to the overall ATP turnover of the cell (second only to active ion transport) and it is involved at various levels of physiological functioning of the animal from gene expression to growth at the whole animal level. Despite being equally as important as metabolic depression to the survival of the animal during environmental stress, the activities and roles of cellular processes during the post-dormancy recovery period are greatly understudied. Therefore, whenever possible investigation into protein synthesis during the recovery period was included in this thesis work.

In both the cunner and oscar, exposure to environmental stress triggers an active suppression in metabolic rate. The response of protein synthesis is tissue specific in both fish, but in general the whole animal metabolic depression is accompanied by a global inhibition of protein synthesis ranging from 50-80% of pre-dormancy/prehypoxic levels. This reduction in *in vivo* protein synthesis is similar to the reduction in protein synthesis that has been presented for other animals (Table 1.1). In the cunner, protein synthesis is actively depressed in both the naturally occurring winter dormancy and the metabolic depression that happens in response to an acute decrease in temperature. In the natural environment the trigger for metabolic depression is most likely a combination of both decreasing temperature and photoperiod (Figure 3.1). However, the active depression of

protein synthesis that also occurs when cunner are challenged with acute hypothermia, without decreasing photoperiod, indicates that there is a temperature trigger involved in initiating the regulated turning down of ATP turnover. The intrinsic properties of the hypometabolic response of the cunner are further emphasized through its ability to institute metabolic depression, exhibited by the depression in protein synthesis, in response to acute hypoxia. To our knowledge the cunner do not experience such low levels of water oxygen saturation in their habitat and would not have had the opportunity to become poised to respond to this environmental stress. The plasticity of the hypometabolic response in cunner provides an ideal model to investigate potential regulating mechanisms of metabolic depression and to determine if the regulatory loci are conserved across environmental challenges.

The importance of developing tissue-specific responses in protein synthesis to the survival of the animal in the face of environmental challenges becomes evident when drawing connections between the functions of those tissues to the physiological response at the whole animal level. During acute hypoxia exposure, levels of protein synthesis in the oscar brain undergoes a modest depression (20%) compared to that of the other tissues (50-60%) and to the whole animal metabolism. This maintenance of brain protein turnover is most likely linked to the preservation of neuronal activity. Oscars, like the cyprinids, do not enter the typical comatose-like state that is associated with metabolic depression in many other animals. Instead they sacrifice the loss in energy savings to allow the maintenance of low levels of activity. This is essential in the Amazonian floodplain environment as the avoidance of prevalent aquatic and aerial predators is key to the survival of the oscar. The cunner also exhibits a tissue specific protein synthesis

response during metabolic depression. During the seasonal metabolic depression, rates of protein synthesis in the liver along with the other tissues are initially inhibited. However, when seasonal water temperatures are at their lowest (-1.9 to 0°C) there is a substantial upregulation in liver protein synthesis. As cunner are known to produce antifreeze proteins at this time of year, this upregulation in the liver is suggestive of the synthesis of *de novo* proteins, slated specifically for freeze avoidance and stress protection. At the subcellular level, mitochondrial protein synthesis in the cunner is defended in the gill, but not the heart, in response to both acute hypothermia and hypoxia. This defense of protein synthesis in mitochondria despite the decrease observed in the whole homogenate and cytosol, particularly in response to hypothermia, is thought to be linked to vital role of gills in ionic exchange with the aquatic environment. As well, as proteins synthesized in and for the mitochondria play key roles in the electron transport chain and oxidative phosphorylation it is thought that defending synthesis of these proteins would allow the rapid reinstatement of oxidative phosphorylation upon emergence from a metabolically suppressed state. The tissue specific nature of the response of mitochondrial protein synthesis in the cunner warrants further investigation in order to determine if other biochemical processes such as mitochondrial respiration are defended in gill mitochondria during metabolic depression, and if so, how the regulatory mechanisms in gill mitochondria differ from other tissues. Mitochondrial protein synthesis in encysted *Artemia* embryos has been extensively studied in response to varying pH and oxygen levels. These studies have begun to elucidate potential regulating mechanisms and have suggested that changes in intracellular pH may provide an intracellular signal that integrates metabolic depression in both the mitochondria and cytoplasmic compartments

(Kwast and Hand 1993). As well, it has been suggested that anoxia induced dormancy is mediated through the presence of a molecular oxygen sensor within mitochondria (Kwast and Hand 1996 ab). The investigation of mitochondrial protein synthesis in other hypometabolic animals has been very limited and an exciting avenue for future research will be to determine if similar regulatory mechanisms, as described in dormant *Artemia*, also occur in higher animals. The use of the cunner as a study organism would provide an ideal departure point as it would be possible to investigate possible regulating loci under both hypothermia and hypoxia, allowing us to determine if the regulatory mechanisms of metabolic depression are conserved or if they vary depending on the specific challenge presented to the organism based on the environmental trigger.

The ability to facilitate a rapid recovery from metabolic depression is equally as important to the survival of the animal as metabolic depression itself. Despite this, few studies have investigated the cellular responses associated with the restoration of cellular processes in hypometabolic animals. This thesis described the role of protein synthesis in the recovery period after the extended winter dormancy of the cunner and the acute hypoxia exposure of the oscar. Based on these two examples and from previous work on anoxia tolerant turtles and crucian carp, a pattern emerges. As a hyperactivation in protein synthesis occurs after hypothermic stress in the cunner but not post-hypoxia stress in the oscar (nor in the studies on anoxic turtles or carp) it appears the response of protein synthesis during recovery may be stress specific. However, when the length of the hypometabolic period is taken into consideration, with cunner remaining hypometabolic for months compared to the acute challenges with hypoxia (hours) in the oscar, carp and turtle, it seems more likely that the amount of time spent in a hypometabolic state, i.e. the

size of the protein debt that is accumulated is more likely the dictating factor on whether or not protein synthesis will be hyperactivated during recovery. A comparison of recovery rates of protein synthesis in cunner challenged with acute hypoxia and low temperature could determine whether the response is related to the type of environmental stress or the amount of time spent in a hypometabolic state.

## **6.2 Future Directions**

The majority of early work on metabolic depression was conducted on hibernating mammals and still much of the work investigating the regulating factors behind the coordinated shutting down of energy turnover is on mammalian cell lines. Through this thesis work and other recent studies it is becoming more evident that metabolic depression is an adaptive response that is used frequently and effectively by teleosts. It is possible to find a teleost species that has evolved ways to cope with practically every environmental stress (i.e. hypoxia – oscar, cyprinids; hypothermia – cunner; anaerobiosis – lungfish) and in some cases the ability to cope with multiple environmental challenges exists (i.e. hypoxia and hypothermia - cunner and cyprinids). The Krogh principle states “For a large number of problems there will be some animal of choice or a few such animals on which it can be conveniently studied”. Following this principle, the adaptive plasticity of teleosts makes them excellent models on which future investigation into metabolic depression can be conducted.

With each additional study it is becoming more apparent that the cellular processes and molecular mechanisms behind metabolic depression are conserved across phylogenetic lines. The next step is to unveil the regulating loci and the signals that

initiate the coordinated 'shutting down' of energy consuming and producing processes. Recently, a 'hibernation induction trigger' has been described in hypometabolic mammals (Horton et al. 1998); it will be interesting to determine if similar extracellular signals (hormones) are present in other hypometabolic organisms and if the same triggers are instituted in response to varying stressors. As well, the control loci that regulate the depression of protein synthesis during metabolic depression still remain elusive. Initial studies have begun looking towards modifications in transcription, mRNA, translation factors and ribosomes as potential sites (Frerichs et al. 1998). It is becoming evident that in hibernating mammals, that protein synthesis is likely controlled at the elongation stage through the increased phosphorylation of the initiation factor eIF2 (Frerichs et al. 1998). Similar results are beginning to emerge from studies on invertebrates, with quiescent *Artemia* embryos (Hofmann and Hand 1994). To present, there is limited to no information on potential regulatory loci in metabolically depressed teleosts, but a study on control of protein synthesis in cold acclimated toadfish has suggested that in liver, protein synthesis is regulated by the activity of aminoacyl transferase (Hasehemeyer 1969). The cunner would make an ideal model species in which it could be determined if similar control of elongation via initiation factors and enzymes occurs in hypometabolic teleosts. Another area that deserves considerable attention is the post-hypometabolic arousal or recovery period. As seen in the hypometabolic teleosts studied in this thesis, the recovery period is often accompanied by a hyperactivation of metabolic processes. The question arises "how are animals that are energetically deprived and lacking in endogenous stores capable of fueling such energetically demanding process". The ability of the animal to institute a rapid and complete recovery from a metabolically suppressed

state may be just as or even more important to an animal's survival than metabolic depression itself. The recent development of molecular and genomic techniques allow a comparison of the differential expression of genes involved in the 'turning down' and 'turning up' of metabolism. I believe it is through investigation into the recovery response and the mechanisms controlling the hyperactivation of cellular processes that we will be able to elucidate key regulatory loci and triggers associated with the hypometabolic response in animals.

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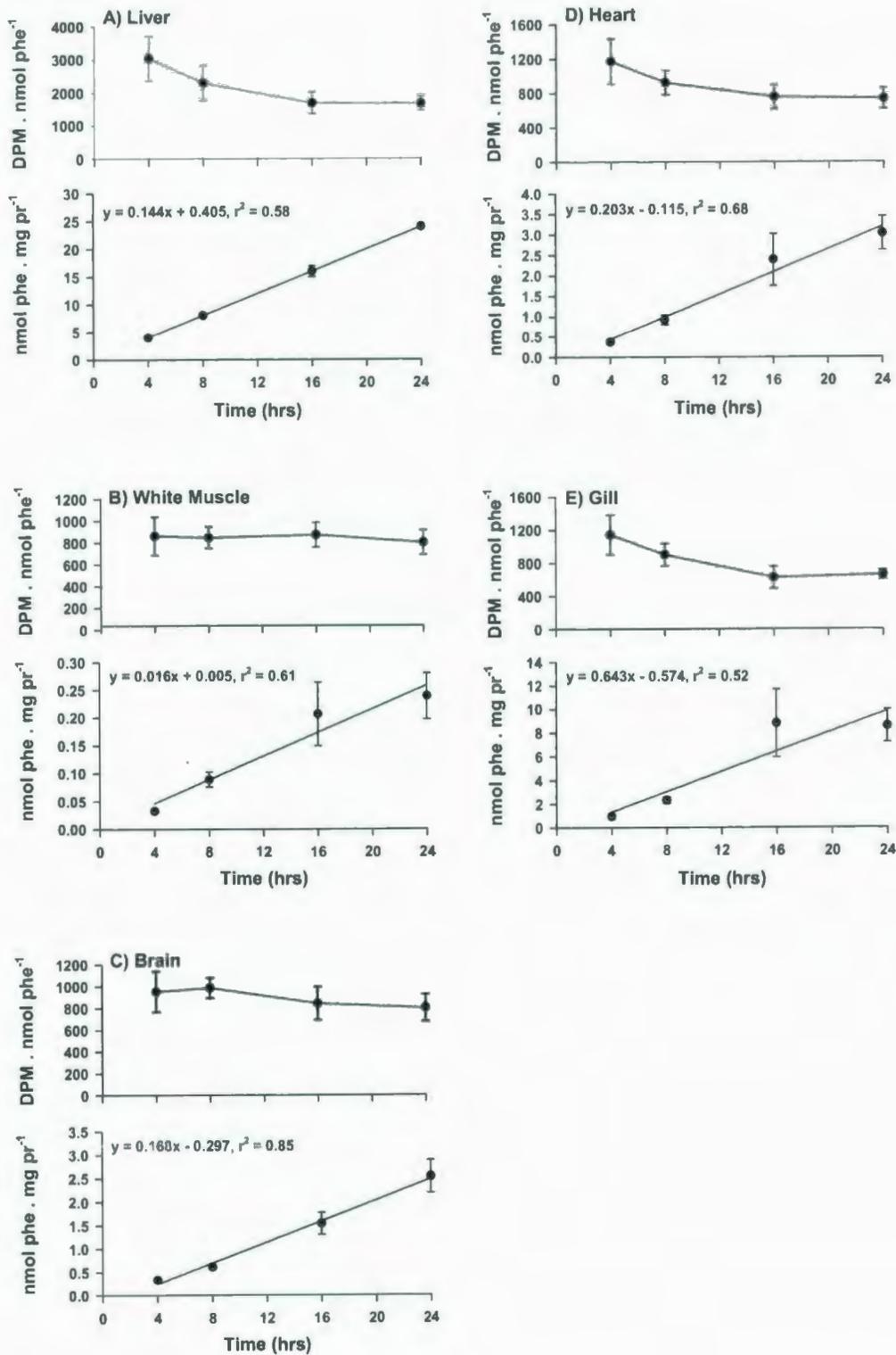
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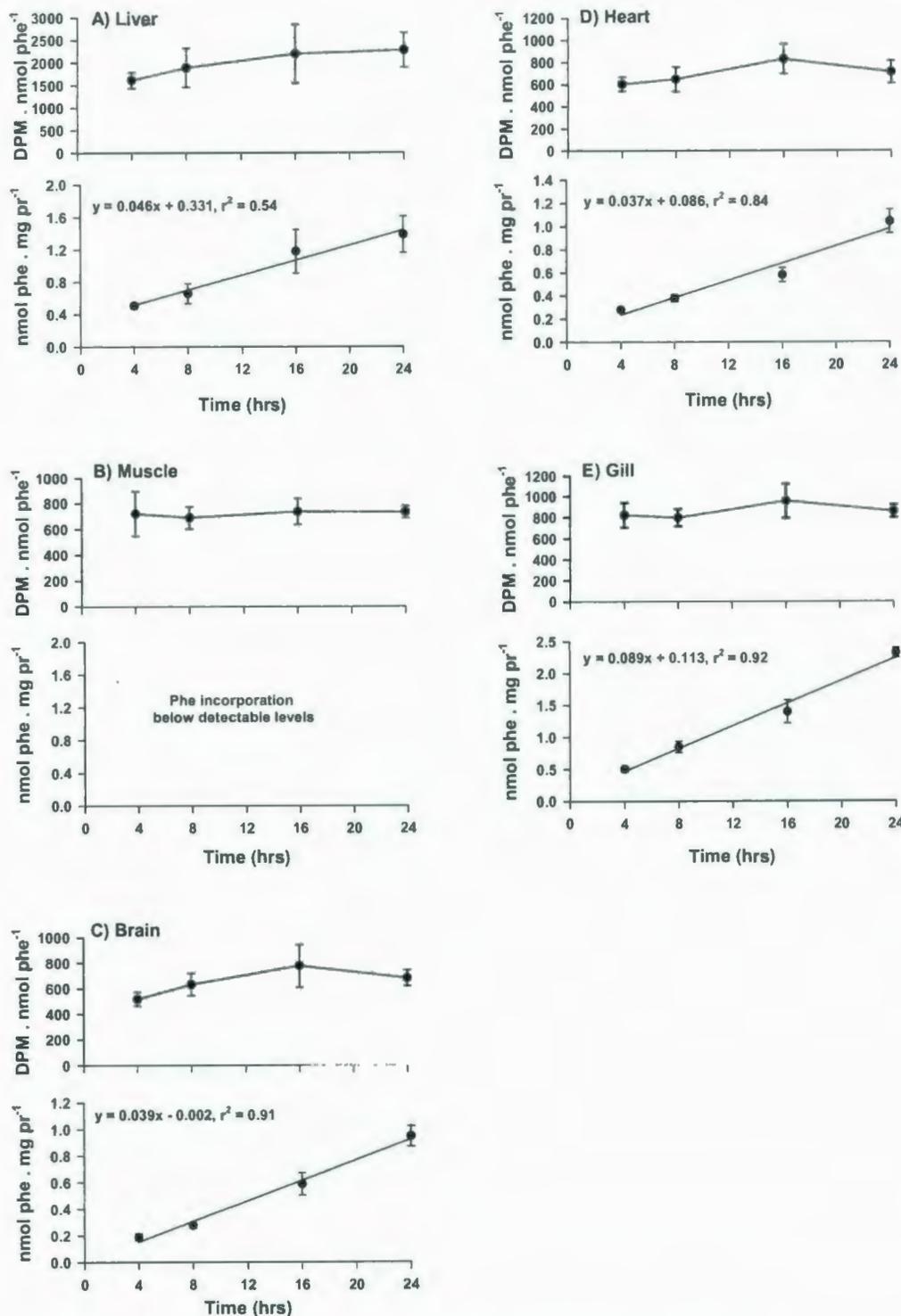
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## Appendix 1

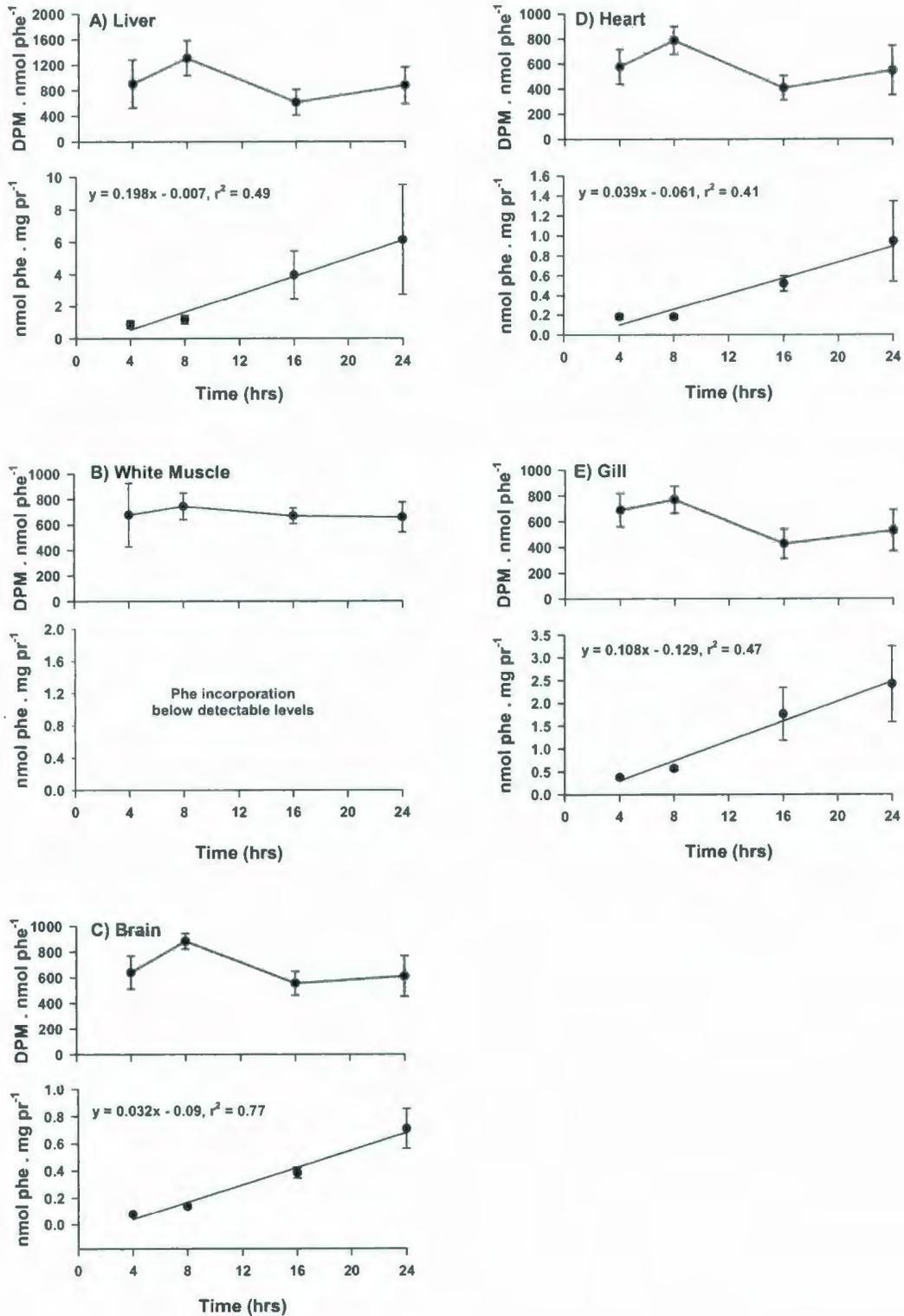
Validation Figures for Protein Synthesis in *Tautogolabrus adspersus* measured  
between 8°C November and 8°C July.



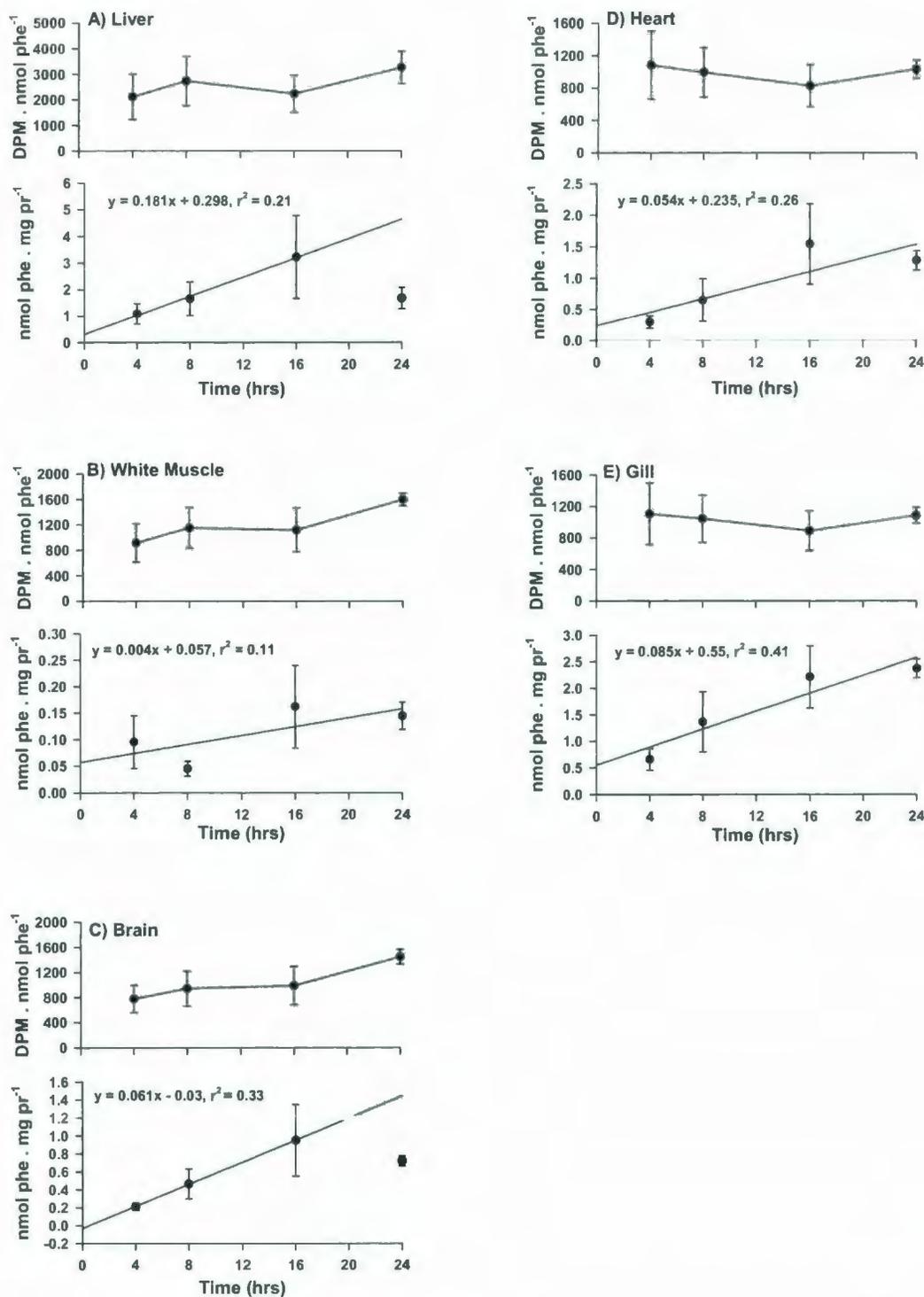
**Figure A1.** Post-injection time course for specific radioactivity of free pool (DPM . nmol phe<sup>-1</sup>, top figure) and incorporation of radiolabeled phenylalanine into protein (nmol phe . mg pr<sup>-1</sup>, bottom figure) in cunner at 8°C (November) for A) liver, B) white muscle, C) brain, D) heart and E) gill tissues. Values are means ± SE, n=4 fish at each sample time. Regressions calculated over 4 – 24 hrs for all tissues.



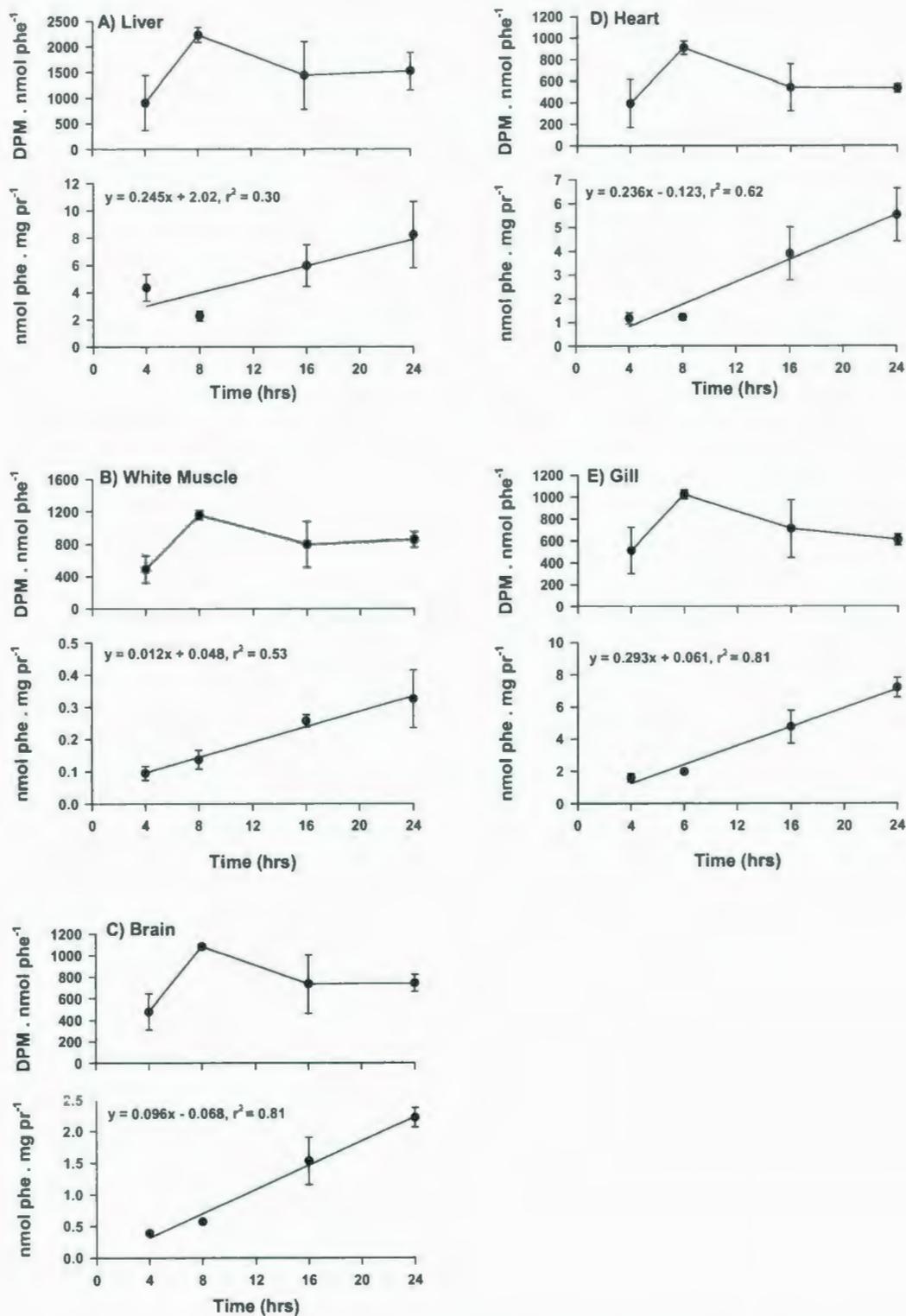
**Figure A2.** Post-injection time course for specific radioactivity of free pool (DPM . nmol phe<sup>-1</sup>, top figure) and incorporation of radiolabeled phenylalanine into protein (nmol phe . mg pr<sup>-1</sup>, bottom figure) in cunner at 4°C (December) for A) liver, B) white muscle, C) brain, D) heart and E) gill tissues. Values are means  $\pm$  SE, n=4 fish at each sample time. Regressions calculated over 4 – 24 hrs for all tissues.



**Figure A3.** Post-injection time course for specific radioactivity of free pool (DPM . nmol phe<sup>-1</sup>; top figure) and incorporation of radiolabeled phenylalanine into protein (nmol phe . mg pr<sup>-1</sup>, bottom figure) in cunner at 0°C (March) for A) liver, B) white muscle, C) brain, D) heart and E) gill tissues. Values are means ± SE, n=4 fish at each sample time. Regressions calculated over 4 – 24 hrs for all tissues.



**Figure A4.** Post-injection time course for specific radioactivity of free pool (DPM . nmol phe<sup>-1</sup>; top figure) and incorporation of radiolabeled phenylalanine into protein (nmol phe . mg pr<sup>-1</sup>, bottom figure) in cunner at 4°C (June) for A) liver, B) white muscle, C) brain, D) heart and E) gill tissues. Values are means ± SE, n=4 fish at each sample time. Regressions calculated over 4 -24 hrs for white muscle, heart and gill tissues and over 4 - 16 hrs for liver and brain tissues.



**Figure A5.** Post-injection time course for specific radioactivity of free pool (DPM . nmol phe<sup>-1</sup>; top figure) and incorporation of radiolabeled phenylalanine into protein (nmol phe . mg pr<sup>-1</sup>, bottom figure) in cunner at 8°C (July) for A) liver, B) white muscle, C) brain, D) heart and E) gill tissues. Values are means ± SE, n=4 fish at each sample time. Regressions calculated over 4 – 24 hrs for all tissues.







