THE EFFECT OF TEMPERATURE ON PROTEIN METABOLISM AND ANTIOXIDANT ACTIVITY IN THE SPOTTED WOLFFISH, *Anarhichas minor*.

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The effect of temperature on protein metabolism and antioxidant activity in the spotted wolffish, *Anarhichas minor*.

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Abstract

Temperature has profound effects on the rate of physiological processes in ectothermic vertebrates. Protein metabolism is no exception and the effects of temperature have mostly been studied with respect to protein synthesis. Temperature generally has a parabolic effect on protein synthesis with a maximum rate being observed at optimal growth temperature. The effect of temperature on protein degradation is poorly understood. The 20S proteasome is mainly responsible for the degradation of short-lived and oxidatively modified proteins. It has been recently identified as a potentially good proxy for protein degradation in fish.

In the first experiment, the relationships between the rate of protein synthesis, 20S proteasome activity, oxidative stress markers and antioxidant capacity in white muscle of juvenile spotted wolffish (*Anarhichas minor*) acclimated at three temperatures (4, 8 and 12 °C) were examined. The rate of protein synthesis was lower at 4 °C than at 8 °C while it was intermediate at 12 °C. Despite the decrease in protein synthesis at low temperature, 20S proteasome activity was maintained at a high level, reaching 130% of that of fish acclimated at 8 °C when measured at a common temperature. The oxidative stress markers

TBARS and carbonyl-protein content did not change amongst temperature groups, but the concentration of reduced glutathione was higher in cold acclimated fish suggesting a higher antioxidant capacity in this group. The data suggest that lower growth rate at cold temperatures results from both high 20S proteasome activity and a reduced rate of protein synthesis.

In a second experiment, the relationship between specific growth rate (SGR) and 20S proteasome activity in heart ventricle, liver and white muscle was assessed in fish acclimated at 4 and 12 °C in order to determine if protein degradation via the proteasome pathway could impose a limitation on somatic growth in fish of weight ranging from 150 to 1500 g in mass. The data show that white muscle 20S proteasome activity is negatively correlated to SGR (partial Pearson's r = -0.609) in white muscle at the cold acclimation temperature (4 °C) but not at 12 °C or heart and liver at either temperature. Contrary to the first experiment, the white muscle 20S proteasome activity was not higher in the group acclimated at 4 °C. This observation suggests that the effect of temperature on protein degradation may change during fish ontogeny. Nevertheless, the results from the first two experiments suggest that interindividual variation of 20S proteasome activity has an impact on SGR.

The third part of this study documents the effects of acclimation to

high and low temperature (4 and 12 °C) on mitochondrial and antioxidant capacities in white muscle, heart ventricle and liver of spotted wolffish. Following an acclimation period of 51 days, mitochondrial capacity was measured as the activities of the Complex I of the mitochondrial electron transport system (CPLXI) and citrate synthase (CS). Glutathione disulfide reductase (GR) and catalase (CAT) activities as well as glutathione concentration were also measured to estimate antioxidant capacities. Following acclimation to 4 °C, mitochondrial capacities were compensated in liver and heart ventricle but not in white muscle. GR activity was increased at cold temperature in the three tissues while CAT activity was increased at the higher acclimation temperature in heart and white muscle. The relationships between mitochondrial and antioxidant enzyme activities, when observed, were always positive. In white muscle only, the activity of 20S proteasome was positively related to the complex I ($r^2 = 0.450$) and to CS ($r^2 = 0.411$) activities. Also, only in white muscle, positive relationships were observed between 20S proteasome, CAT and GSH (only at 12 °C for the latter) activities. These results suggest a connection between mitochondrial capacity and protein degradation by the 20S proteasome but whether or not this link is mediated by the necessity to degrade protein oxidatively modified by mitochondrial ROS production remains an open question.

A supplementary section presents a simple method devised to measure the fractional rate of protein synthesis in fish using the stable isotope

labelled tracer (ring-D_5L-phenylalanine) instead of radioactive phenylalanine. The method takes advantage of the increasingly available technology of liquid chromatography with tandem mass spectrometry detection (LC-MSMS). The technique was validated by measuring the fractional rate of protein synthesis in the gills of goldfish (*Carrassius auratus*). The modified technique requires fewer steps compared to previously available procedures and allows studies on fish protein metabolism to be carried out in situations where the use of radioactivity is not possible, such as in free living animals.

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1

Introduction and overview

1.1 Growth and protein metabolism

The ultimate goal of most living organisms is to pass on their genes. To achieve this, animals have to reach a minimal body size that makes it possible for them to reproduce. As a consequence, growth is a fundamental process in virtually all living animals. Faster growth rate is associated with reduced probabilities of predation, therefore, positively correlating with fitness. In aquaculture, growth is of paramount importance given that the costs of production are directly related to the time and quantity of feed needed for the fish to reach commercial size.

In general, growth is evaluated in terms of weight gain and any weight gain is often interpreted as growth. This wrongly assumes that tissue composition is constant and any weight increase is made in an organized way. Tissue composition may vary according to physiological condition, and thus weight increase is not necessarily representative of an increased amount of organized matter (Jobling,

1994). Growth should be defined as an increase in energy content of an organism (Winberg, 1960) and in these terms, ingested energy that is neither lost as faecal or excretory products, nor used for energy production, is available for growth (Jobling, 1994). From this, the growth rate of animals is indeed highly dependent on food intake but also their ability to channel the food energy into growth. This is particularly important for the aquaculture industry as it is directly related to the amount of feed necessary to bring a stock of fish to commercial size. This is also important for fisheries and aquatic ecology in the modeling of energy fluxes within an ecosystem.

In a recent review, Fraser and Rogers (2007) estimated the cost of synthesising the body components of the Antarctic teleost *Notothenia coriicep.* Of that cost, proteins represent about 98% of the total energy needed and this estimate assumes a protein retention efficiency of 100%, which neglects protein turnover thus even 98% may be an underestimation. Accordingly, in the study of physiological processes of growth, processes involved in protein metabolism are of foremost importance. In all living organisms, cellular proteins are in a constant state of renewal. Animals are producing soft tissues by synthesising protein and retaining a proportion for growth, with the remainder being degraded (Fraser and Rogers, 2007). Protein metabolism refers to the inter-related processes of protein synthesis, degradation and growth. The incessant cycle of protein synthesis and protein breakdown is referred to as protein turnover. More specifically, protein turnover refers to the cycle of intracellular proteins being hydrolysed to their component amino acids and replaced by an equal amount of freshly synthesized protein (Hawkins, 1991). Positive growth occurs when the

rates of synthesis and deposition surpass the rate of degradation and in that case, protein turnover equates the rate of protein degradation. Conversely, in the case of negative growth, protein turnover is equivalent to the rate of protein synthesis (Hawkins, 1991; Sugden and Fuller, 1991; Houlihan *et al.*, 1995).

There are a wide range of factors affecting growth: temperature, feeding regime, nutrition, heterozygocity and social interactions (Jobling, 1994). These factors either have a direct or indirect effect on protein synthesis, protein degradation or both. Therefore, to understand the underlying processes controlling growth, it is important to understand *separately* protein synthesis, degradation and growth (Fraser and Rogers, 2007).

1.2 Protein synthesis

Protein synthesis is the process by which messenger RNA (mRNA) templates are read by the ribosome, and translated into peptides composed of amino acids. The translation of mRNA into protein can be divided into four steps that are tightly choreographed: *initiation*, when the start codon of the template mRNA is identified and the two ribosomal subunits form the functional 70S ribosome; *elongation*, during which the amino acids are condensed into a peptide chain in the sequence read from the mRNA template; *termination*, when the reading frame of the 70S ribosome reaches the termination codon, the freshly formed peptide is released and the two ribosomal subunits separate; *post-translational processing*, when specific enzymes remove the initiating residue and signal sequences, modify the terminal residue, attach phosphate, methyl, carboxyl, carbohydrates or

prosthetic groups (Nelson and Cox, 2005). With regards to understanding the underlying processes of protein metabolism, the rate of protein synthesis has received by far the most attention in the study of several intrinsic (species, weight, exercise, ontogenetic stage, nutritional condition, heterozygocity) and extrinsic parameters (temperature, seasonality, pollutants, oxygen and salinity) (reviewed in Carter and Houlihan, 2001; Fraser and Rogers, 2007). This is not surprising since in the study of growth, the "construction" phase, is likely to control most of the process. This also reflects the relative simplicity by which the rate of protein synthesis is measured in comparison to protein degradation. Despite the number, and the complexity of the chemical reactions involved in protein synthesis, the measurement of the rate of protein synthesis is greatly simplified by the fact that proteins are only synthesised via a single process. The vast majority of studies on fish use the flooding dose technique developed by Garlick *et al.* (1980) for small mammals. This technique measures the incorporation of a labelled amino acid into protein. Despite being relatively simple, this technique generally uses radioactive tracers which make it mostly unusable in commercial aquaculture facilities or the natural environment. The supplemental section introduces a simple modification of this technique using a deuterium labelled amino acid instead of the radioactive tritium, thus circumventing radiation safety issues.

1.3 Protein degradation

Although little is known about the importance of protein degradation, strong indications that it is a very important process in determining growth can be found in the literature (Houlihan *et al.*, 1995; Dobly *et al.*, 2004; Fraser and Rogers,

2007). In animals, three major systems are responsible for protein degradation: the lysosomal cathepsins, the ubiquitin-proteasome system and the calpain system.

1.3.1 Lysosomal protein degradation

The lysosomal system is confined within the lysosome; a small membrane bound organelle containing acid proteases that degrades any proteins that are brought in via autophagy. Proteolytic enzymes are mainly cathepsins (cysteine proteases) that are activated by the low pH prevailing in the lysosome. Cathepsins are ubiquitous lysosomal proteases that are classified according to their active site. Structural differences between various cathepsins result in variations in their substrate specificity and mechanism of action. Several forms of cathepsin exist with B, D and L being the most common forms in fish lysosomes (Aoki *et al.*, 2000). Cathepsins play an important role in the turnover of extracellular proteins that are imported into the lysosomes via endocytosis. In fish, cathepsins seem to play an important role in the recruitment of proteins as energy substrate in periods of food deprivation (Martin *et al.*, 2001; Guderley *et al.*, 2003; Mommsen, 2004; Salem *et al.*, 2007). This phenomenon contrasts with the finding that in higher vertebrates, it is the proteasome pathway that is up-regulated during food shortage (Martin *et al.*, 2002; Fraser and Rogers, 2007).

1.3.2 The calpain system

The calpain system is composed of two enzymes, μ-calpain and m-calpain, and their specific inhibitor calpastatin (Goll *et al.*, 2003). The calpain system appears

to be specific to skeletal muscle and is principally involved in the separation of the surface proteins of the myofibrils without impairing fibril integrity (Goll *et al.*, 1998). This mechanism would leave functionally intact myofibrils and the released myofilament could either be reassembled back on the myofibril or be degraded by the proteasome or lysosomal system (Delbarre-Ladrat *et al.*, 2004). The calpastatin inhibitory activity is increased following the administration of β-adrenergic agonist, suggesting a regulatory role of the inhibitor in the activity of this protein degradation pathway (Goll *et al.*, 2003). In starved rainbow trout (*Oncorhynchus mykiss*), a down regulation of calpastatin has been reported (Salem *et al.*, 2007), strengthening the idea of the controlling role of this protein. During development, calpain activity is also associated with an increased number of myoblasts that would result in larger number of muscle nuclei and potentially to larger muscle fibres in mature muscle (Goll *et al.*, 1998).

1.3.3 The ubiquitin—proteasome system

Selective or targeted protein degradation occurs in the proteasome, a large protein complex located in the nucleus and cytosol. Selective degradation implies that proteins have to be marked for destruction before being degraded. The labelling of a protein for degradation is accomplished by covalent attachment of multiple ubiquitin molecules, a highly evolutionary conserved protein. Conjugation of ubiquitin to the protein is realized via a three-step cascade mechanism involving three classes of enzymes, E1, E2 and E3. The first two classes are necessary to activate and transfer the ubiquitin to the substrate recognised by E3. Then, by successively adding activated ubiquitin moieties to the previously conjugated

ubiquitin, a polyubiquitin chain is synthesized and eventually recognised by the 26S proteasome complex. The proteasome degrades polyubiquitinated proteins to small peptides. It is composed of two subcomplexes: the 20S core particle (hereafter called 20S proteasome) that carries the proteolytic activity and the 19S regulatory particle (RP). The 20S proteasome is a barrel-shaped structure composed of four stacked rings with the catalytic activity taking place inside the barrel. Each extremity of the 20S proteasome can be capped by a regulatory particle that is responsible for the recognition of the polyubiquitinated substrate. After recognition of the degradation signal, the RP unravels the substrate protein into the proteolytic chamber of the proteasome. The unravelling of the substrate is an energy consuming process that is driven via ATP hydrolysis. After degradation of the substrate, short peptides are released, as well as reusable ubiquitin (Glickman and Ciechanover, 2002). In starving rainbow trout, the hepatic activity of the 20S proteasome was found to decrease, most probably as a result of lower protein turnover (Martin *et al.*, 2002). Dobly *et al.* (2004) found that 20S proteasome activity is negatively correlated with specific growth rate in rainbow trout liver. In the latter study, the authors were comparing two groups of fish, displaying high or low growth efficiency. Fish from the first (high efficiency) group had a higher growth rate, lower fractional rate of protein synthesis, higher protein deposition efficiency and lower 20S proteasome activity. This study was the first to show an inverse relation between the activity of the 20S proteasome and growth.

1.4 Acclimation to temperature

Life is found at temperatures ranging from -80 to slightly more than 100°C. Ectothermic vertebrates exploit a wide range of habitats where temperature may vary from -2°C in the Antarctic to 44°C in tidewater pools. Ectothermic animals mainly select their habitat according to how temperature is changing on spatial and temporal scales. The spatial scale represents latitude, altitude, subtidal vs intertidal for marine habitat, etc. On a temporal scale, habitat selection may change according to season of the year and/or time of the day. In this regard, most ectothermic vertebrates select their preferred temperature via seasonal migrations and/or diurnal cycles. Living organisms are thus geographically distributed according to patterns that reflect temperatures prevailing in their habitats (Hochachka and Somero, 2002). The effects of temperature are manifested at every level of biological organization, from molecular motion to animal behaviour. At the molecular level, temperature affects membrane fluidity (Hazel, 1995), enzyme flexibility and stability, as well as DNA and RNA stability (Somero, 1995; Portner, 2001; Hochachka and Somero, 2002; Somero, 2004). The major consequence of these thermal effects on cellular functions necessitates that organisms must, on an evolutionary time scale, be adapted to cope with environmental temperature. Moreover, most ectothermic organisms experience marked daily and seasonal thermal fluctuations, especially eurythermic species that represented the vast majority of ectothermic species (Portner, 2001). Responses to thermal changes are often behavioural, but ectothermic organisms also demonstrate the capacity to acclimate by adjusting their physiological rates, as well as their exact biochemical composition. Acclimation at the cellular level in

response to temperature change occurs following alterations in protein concentrations, intracellular pH, ion concentrations, and membrane composition, with most of these processes controlled at the transcription level (Guderley, 1990; Hochachka and Somero, 2002; Guderley, 2004b; Podrabsky and Somero, 2004). When faced with a reduction of temperature, animals can either: 1) passively slow down their physiological processes by submitting to Q_{10} effects; 2) enhance the effects of temperature on the rate of physiological processes by entering into metabolic depression; or 3) use compensatory modifications to offset the effects of decreased temperature and remain active.

Compensatory responses, during cold acclimation, include adjustments to membrane composition in order to maintain their fluidity and dynamic nature. Membrane fluidity is typically adjusted by modifications in the level of unsaturation and the proportions of long chain polyunsaturated fatty acids. The dynamic nature of membranes is maintained by adjusting the relative content of phospholipids head groups (phosphatidylcholine and phosphatidylethanolamine) such that membranes are capable of phase transitions, enabling fusion events associated with normal membrane traffic, while being stable enough to prevent these processes from occurring in an unregulated fashion (Hazel, 1995). It is also generally observed that during acclimation to low temperature there is a concomitant increase of mitochondrial volume density (the number and/or size of mitochondria per cell) and/or an increase in mitochondrial cristædensity (reviewed by Guderley and St-Pierre, 2002). Both these mechanisms result in enhanced mitochondrial activity per gram of tissue at low temperature. Increasing mitochondrial volume density has the detrimental effect of reducing the space available for contractile

function and thus reduces the power output of the muscle fibres whereas increasing the cristædensity would not have this negative effect (Guderley and St-Pierre, 2002).

1.5 Effect of temperature on growth and protein metabolism

Temperature acts as a controlling factor of growth, and is therefore one the most significant abiotic factors affecting the physiology and growth of ectothermic vertebrates (Brett, 1979b). In fish, growth rate increases with temperature up to its thermal limit, after which it rapidly decreases (Brett, 1979b; McCarthy *et al.*, 1998; McCarthy *et al.*, 1999; Fraser and Rogers, 2007). In Atlantic wolffish (*Anarhichas lupus*), McCarthy *et al.* (1999) showed that the whole body protein synthesis increases along with temperature while protein growth is maximized at 11 °C and decreases thereafter. Whole body protein retention efficiency thus has a parabolic relationship with temperature, being maximized at optimal growth temperature and decreasing at both lower and higher temperature. The same trends were reported in white muscle. The effect of temperature on protein synthesis in wolffish contrasts with earlier work in cod (*Gadus morhua*) by Foster *et al.* (1992). Overall growth rate and tissue protein synthesis rates were not significantly different for cod acclimated at 5 or 15 °C for 40 days. Interestingly, however, in a second study on cod, growth rate and the rate of protein synthesis increased with temperature (Treberg *et al.*, 2005). The difference between these studies may be associated with the feed ration in the two experiments (Fraser

and Rogers, 2007). Fish were fed a fixed ration irrespective of temperature in Foster *et al.* (1992), while fish were fed to satiation in Treberg *et al.* (2005).

Information on the thermal effect on protein degradation in fish is limited, with only a few studies measuring the effect of temperature on enzyme activities of proteolytic pathways (Fraser and Rogers, 2007). The scarcity of information on the effect of biotic and abiotic factors on protein degradation is mainly due to the multitude of degradation pathways that exist. Moreover, it appears that different pathways may be used preferentially under specific circumstance (Martin *et al.*, 2001; Houlihan and Martin, 2002; Martin *et al.*, 2002; Salem *et al.*, 2006; Salem *et al.*, 2007). The majority of data available on protein degradation are estimated by calculating the difference between protein growth and the rate of protein synthesis. The problem with this type of estimate is that protein growth is measured over weeks while protein synthesis is measured over a few hours. The assumption that the rate of protein synthesis measured is representative of the protein synthetic activity during the growing trial is difficult to meet (Fraser and Rogers, 2007).

There is a growing body of evidence coming from the field of transcriptomics that mRNA transcripts of the ubiquitin proteasome pathway are upregulated at low temperature. Two studies are worth mentioning: one on common carp (*Cyprinus carpio*)(Gracey *et al.*, 2004) and a second one on the annual killifish (*Austrofundulus limnaeus*) (Podrabsky and Somero, 2004). In both experiments, the effect of temperature on the expression levels of thousands of genes was examined. The majority of transcripts that increased in expression upon cooling

are in agreement with our general understanding of cold acclimation such that the organisms compensate for the rate-depressing effects of cold temperature by synthesizing more enzymes to increase biochemical performance (Hochachka and Somero, 2002). These included genes involved in transcriptional regulation, RNA splicing, translation, mitochondrial production of ATP, membrane lipid adaptation (such as Δ6-fatty acyl desaturase and polyunsaturated fatty acid elongase), and heat shock protein classes involved in protein stabilization during temperature stress. Genes involved in ubiquitin-dependent protein catabolism and proteasomal function were also shown to be upregulated in the cold (Gracey *et al.*, 2004; Podrabsky and Somero, 2004). An upregulation of the genes involved in protein degradation at low temperature is surprising since one would intuitively expect the expression level of these genes to be down-regulated or at least unchanged provided that protein synthesis decreases at lower temperature, as shown by McCarthy *et al.* (1999). It would be coherent if protein degradation was regulated accordingly. A logical explanation of this upregulation could be that proteasome activity is highly thermosensitive and upregulation is necessary to maintain sufficient activity. Information on the thermosensitivity of proteasome activity in vertebrates is scarce, and nonexistent for fish. Indications that proteasome 20S might be a highly thermosensitive proteolytic enzyme comes from a study on the golden-mantled ground squirrel (*Spermophilus lateralis*). In that study, proteolysis at the proteasome level, as a function of assay temperature, showed a very steep decrease and very low or no activity below 10 °C (Velickovska *et al.*, 2005). The upregulation of transcripts from the ubiquitin proteasome pathway might then be a consequence of this strong temperature dependence but this hypothesis remains to be tested.

1.6 Reactive oxygen species and proteins

Following acclimation to cold temperature, organisms generally have an increased mitochondrial capacity in white muscle (Guderley, 2004). Reactive oxygen species (ROS) are a byproduct of the mitochondrial respiration (Boveris and Chance, 1973). For the purpose of this discussion, ROS encompass a variety of diverse chemical species including superoxide anion (O_2^-), hydroxyl radicals (OH·) and hydrogen peroxide (H_2O_2). Some of these species, such as superoxide or hydroxyl radicals, are extremely unstable, whereas others, like hydrogen peroxide, are freely diffusible and relatively long-lived. These various radical species can either be generated exogenously or produced intracellularly from several different sources. Most estimates suggest that the majority of intracellular ROS production is derived from the mitochondria. The production of mitochondrial superoxide radicals occurs primarily at two discrete points in the electron transport chain, namely at complex I (NADH dehydrogenase) and at complex III (ubiquinone-cytochrome c reductase; reviewed by Finkel and Holbrook, 2000). These ROS may damage all types of biological molecules but, because of their high relative abundance in tissue composition, proteins are a major target (Dalle-Donne *et al.*, 2003; Levine *et al.*, 1990; Shacter, 2000). To avoid undue damages to cellular structures, organisms use a wide variety of antioxidant systems, some active (enzymatic systems: superoxide dismutase, catalase, etc.) and some passive (non-enzymatic: glutathione, tocopherols, ascorbic acid, etc.). There is however a distinct possibility that organisms exposed to adverse conditions experience a

higher rate of oxidative damage to their protein pool and, accordingly, have to replace more proteins at a higher rate (i.e. have a higher protein turnover).

1.7 Research questions and hypotheses

Evidence that protein degradation by the proteasome pathway could set a limit on growth rate emerged from the study of Dobly *et al.* (2004). The importance of this protein degradation pathway, especially at cold temperatures, is also emphasized by transcriptomics studies that showed an increased number of mRNA transcripts following exposure to cold temperature. The information brought about by the transcriptomics studies must, however, be validated with measurements of enzyme activities. Whether or not the activity of the proteasome pathway is upregulated *in vivo* during cold acclimation remains an open question. My work will focus on the relationships between acclimation temperature, growth rate, protein synthesis and protein degradation by the proteasome pathway.

The first experiment (chapter 2) investigates the relationships among acclimation temperature, growth rate, protein synthesis, the activity of proteasome 20S and some markers of oxidative stress in the white muscle of juvenile ($\sim$ 5 g) spotted wolffish. This is, to my knowledge, the first attempt to measure both the rate of protein synthesis and the activity of 20S proteasome in the same animal so that they can both be related to growth rate. This experiment also addresses the question of whether 20S proteasome activity is highly thermosensitive, as a high sensitivity of its proteolytic activity could explain the upregulation of the

mRNA transcripts at colder temperature. The major finding of this first experiment is that the activity of 20S proteasome is increased following acclimation to cold temperature and is negatively related to the specific growth rate of juvenile spotted wolffish.

In the second experiment (chapter 3), I explore the effect of high and low temperature acclimation (i.e. the link observed in the first experiment) on the relationship between growth rate and 20S proteasome activity. These measurements were conducted on three tissues that have different rates of protein turnover (heart ventricle, liver and white muscle) and a wider size range of spotted wolffish (60 to 1500 g). It is known that as fish weight increases, their growth rate decreases as a result of a decrease of protein synthesis (Houlihan *et al.*, 1986). However, nothing is known about the activity of the 20S proteasome in relationship to fish mass and how this relation, if it exists, is influenced by acclimation temperature. Following acclimation to low temperature, 20S proteasome activity was not increased as expected from the first experiment. However, a negative relationship between 20S proteasome activity and specific growth rate was again observed at 4 °C. The absence of effect of acclimation temperature on proteasome activity suggests that the impact of temperature on proteasome activity changes during the ontogeny of spotted wolffish.

The first two experiments linked reduced growth rate and white muscle 20S proteasome activity when fish are acclimated at low temperature. The activity of the 20S proteasome is known to be highly responsive to oxidative stress. Accordingly, I hypothesized that the high capacity for protein degradation at cold tem-

perature could be related to mitochondrial capacity and its potentially associated reactive oxygen species (ROS) production. The third experiment thus focuses on the relationships between 20S proteasome activity and mitochondrial capacity by measuring key enzymes of energy production pathways (citrate synthase and complex 1 of the electron transport system) in the heart, liver and white muscle of fish acclimated at high and low temperature. The activities of two antioxidant enzymes (catalase and glutathione reductase) were also measured to assess the link between mitochondrial capacity and ROS production in spotted wolffish. The relationships between mitochondrial capacity, antioxidant enzymes and 20S proteasome activities were examined. If the negative relationship observed between growth rate and 20S proteasome activity at low acclimation temperatures in Chapter 3 is a result of inter individual variation in mitochondrial ROS production, it should be possible to directly correlate mitochondrial oxidative capacity and 20S proteasome activity. Following the acclimation period, the mitochondrial capacity was higher at cold temperature (4 °C) in liver and heart but not in white muscle. The relationships between mitochondrial and antioxidant enzymes were not consistent among tissues but those observed were always positive, as expected. A relatively strong relationship was observed between 20S proteasome activity and mitochondrial capacity only in the white muscle. It suggests that there is a connection between oxidative capacity and protein turnover, at least in the white muscle. Whether or not this relationship is mediated by mitochondrial ROS production is also discussed.

1.8 Wolffish as a model species

Wolffishes display several assets for use as a model fish species for the study of the relationships among protein metabolism, growth and temperature. The wolffish's fractional rate of protein synthesis is high compared to other fish species (McCarthy *et al.* 1999). Further, this species also displays very low spontaneous swimming activity, thus allowing it to allocate more energy to somatic growth than active metabolism (McCarthy *et al.*, 1999; Le François *et al.*, 2004). Finally, wolffish are relatively stenothermic as they can't survive at temperatures below -2 °C or above 16 °C. Accordingly, a relatively small difference in temperature greatly affects physiological rates including growth.

2

Protein synthesis is lowered while 20S proteasome activity is maintained following acclimation to low temperature in juvenile spotted wolffish (*Anarhichas minor*, Olafsen)

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2.1 Abstract

The effects of temperature on protein metabolism have been studied mostly with respect to protein synthesis. Temperature generally has a parabolic effect on protein synthesis, with a maximum rate being observed at optimal growth temperature. The effect of temperature on protein degradation is poorly understood. The 20S proteasome is mainly responsible for the degradation of short-lived and oxidatively modified proteins and has been recently identified as a potentially good proxy for protein degradation in fish. The aim of this experiment was to examine the relationships among the rate of protein synthesis, activity of the 20S proteasome, oxidative stress markers and antioxidant capacity in white muscle of juvenile spotted wolffish (*Anarhichas minor*) acclimated at three temperatures (4, 8 and 12 °C). The rate of protein synthesis was lower at 4 °C than at 8 °C while it was intermediate at 12 °C. Despite the decrease of protein synthesis at low temperature, the activity of 20S proteasome activity was maintained high in fish acclimated at lower temperature (4 °C), reaching levels 130 % of that of fish acclimated at 8 °C when measured at a common temperature. The oxidative stress markers TBARS and protein-carbonyl content did not change among temperature groups, but reduced glutathione concentration was higher in cold-acclimated fish, suggesting a higher antioxidant capacity in this group. The data suggest that lower growth rate in cold temperature results from both high 20S proteasome activity and a reduced rate of protein synthesis.

2.2 Introduction

Protein turnover refers to the continuous degradation and renewal of intracellular proteins. These are hydrolyzed to their component amino acids and usually replaced by an equal amount of freshly synthesized protein (Hawkins, 1991). Positive growth occurs when the rate of synthesis surpasses the rate of degradation (Houlihan *et al.*, 1995; Sugden and Fuller, 1991). Conversely, in the case of negative growth, protein degradation surpasses the rate of protein synthesis (Hawkins, 1991). Factors that have an effect on growth necessarily affect (directly or indirectly) protein synthesis, protein degradation or both. The rate of protein synthesis has received the most attention in the study of several intrinsic (e.g. species, mass, exercise, ontogenetic stage, nutritional condition, heterozygocity) and extrinsic factors (e.g. temperature, pollutants, oxygen levels and salinity) (for a review, see Carter and Houlihan, 2001; Fraser and Rogers, 2007). Although little is known about protein degradation, there is a strong indication that it is a very important process in the determination of growth rate (Dobly *et al.*, 2004; Fraser and Rogers, 2007; Houlihan *et al.*, 1995). For instance, in Atlantic wolffish (*Anarhichas lupus*), white muscle and whole-body fractional rates of protein synthesis (Ks; % day^{-1}) were shown to increase linearly with temperature (McCarthy *et al.*, 1999). However, the fractional rate of protein growth (Kg; % day^{-1}) and protein retention efficiency (Kg/Ks) increased with temperature until they reached their optimal temperature and then decreased rapidly. As the upper thermal limit was approached, protein degradation increased while retention efficiency and growth decreased (Fraser and Rogers, 2007; McCarthy *et al.*, 1999).

2.2 Introduction

The inherent complexity of measuring protein degradation comes from the multiple pathways that are involved. Protein degradation is often estimated from the difference between protein synthesis and protein growth. This method, however, assumes that protein synthesis measured over a few hours is representative of growth rate measured over weeks. It also assumes that there is no change in tissue composition during the experiment. For now, however, it is the only method available to measure *in vivo* protein degradation (Fraser and Rogers, 2007). Several studies have focused on the activity of enzymes involved in protein degradation in fish experiencing muscle wasting conditions, such as food restriction, starvation (Guderley *et al.*, 2003; Martin *et al.*, 2002; Martin *et al.*, 2001; Salem *et al.*, 2007), migration and spawning in salmonids (Mommsen, 2004; Salem *et al.*, 2006) that is also associated with starvation. In such situations, it is mainly the cathepsin and calpain systems that are responsible for protein mobilization as energetic substrate and/or as material for reproductive investment (Martin *et al.*, 2002; Mommsen, 2004). To my knowledge, only one study has linked growth rate and the activity of one pathway of protein degradation in fish growing without food restriction or the complicated aspect of sexual maturation. In rainbow trout (*Oncorhynchus mykiss*), specific growth rate was negatively linked with the hepatic activity of 20S proteasome, and high proteasome activity was linked to decreased growth efficiency (Dobly *et al.*, 2004). In a second study on starving rainbow trout, the activity of 20S proteasome was shown to decrease after 14 days of starvation in rainbow trout, most likely as a result of reduced protein turnover (Martin *et al.*, 2002). These results indicate that the activity of 20S proteasome could be a good proxy to protein degradation in fish growing in *normal* conditions.

The proteasome pathway was mainly responsible for degradation of oxidatively damaged proteins (Friguet, 2006; Perepechaeva *et al.*, 2006; Poppek and Grune, 2006). This concept motivated me to assess if its activity is linked to the levels of markers of oxidative stress and the level of an antioxidant. Protein-carbonyls and thiobarbituric acid reactive substances (TBARS) were used as indicators of oxidative damage of protein and lipids, respectively. Reduced glutathione concentration was measured as an indicator of antioxidant capacity.

The aim of this study is to measure the impact of three temperatures (4, 8 and 12 °C) on the rate of protein synthesis, activity of 20S proteasome and levels of oxidative stress markers and antioxidant in white muscle of juvenile spotted wolffish (*Anarhichas minor*), a close relative of Atlantic wolffish. The use of three acclimation temperatures within the species thermal range was utilized to generate variability in growth rate without introducing undue stress to fish. To my knowledge, there are no studies that have concurrently measured the rate of protein synthesis and 20S proteasome activity in an ectothermic animal.

2.3 Materials and methods

2.3.1 Growth trial

The growth trial and the measurement of protein synthetic rate were carried out in the aquaculture facilities of the Centre Aquacole Marin de Grande-Rivière (QC, Canada). The juvenile spotted wolffish used were the offspring of the domestic broodstock maintained at this facility since 1999. Prior to the experiment,

fish were held at 8 °C and fed to satiety according to established protocols. The growth trial took place during the period of April through May 2006 for 41 days using fish with a mean initial mass and length of 1.72±0.20 g and 5.86±0.25 cm, respectively. The fish were individually marked using visible implant elastomer (VIE, Northwest Marine Technology, Shaw Island, WA, USA) on the top of the head, and their initial mass and length were recorded before they were randomly transferred (12 fish per tank) into one of the three low-level rearing units (1.5 litre volume) supplied with oxygenated sea water (0.5 $l \cdot min^{-1}$) at 4 °C, 8 °C or 12 °C (3.98±0.56 °C; 8.09±0.5 °C and 11.94±0.30 °C) for the duration of the trial. Water temperature was controlled by mixing cold seawater with heated water in a mixing tank. Water depth was adjusted to 2 cm in order to facilitate feed ingestion (Strand *et al.*, 1995). Fish were fed an adapted commercial feed (Gemma 1.0; Skretting, Bayside NB, Canada) to satiety daily for 41 days, and water quality was monitored daily. Fish were exposed to a 12 h:12 h L:D photoperiod, mean salinity was 29 ‰ and oxygen concentration was always over 80 %.

Specific growth rate was calculated using SGRm=[log(Mf)−log(M0)]x100/t, where Mf and M0 are final and initial mass (g) and t is the length in days of the experiment. For SGRl, Mf and M0 were replaced by lf and l0, the final and initial length (cm), respectively. Fulton condition index (K) was calculated using, $K = M \cdot l^{-3}$, where M is fish weight and l is fish length (Ricker, 1975). Rates of protein synthesis, 20S proteasome activity, antioxidants and markers of oxidative stress were all measured on the same fish.

2.3.2 Protein synthesis

Protein synthetic rate was measured based on the principles of the flooding dose of radiolabeled phenylalanine method developed for rats (Garlick *et al.*, 1980). In this experiment, I report the protein synthetic rate as the incorporation of nanomoles of phenylalanine per mg of protein per hour as expressed in other studies (Lewis and Driedzic, 2007; Treberg *et al.*, 2005). Fish were starved for 24 h prior to experimentation. Each fish was injected intraperitoneally with 1 ml · 100 g^{-1} of the tracer solution. No anesthetic was used during this procedure and the fish were immediately returned to their tank, thus protein synthetic rate is measured at acclimation temperature. The tracer solution was composed of 135 $mmol \cdot l^{-1}$ phenylalanine containing L-[2,3,4,5,6-^{3}H]phenylalanine (GE Healthcare, Mississauga, ON, Canada) at a dosage of 1.85 MBq ml^{-1} in a buffered solution consisting of (in $mmol \cdot l^{-1}$) 150 NaCl, 5 KCl, 5 $NaPO_4$, 2 $CaCl_2$, 10 $NaHCO_3$, 2.0 Na_2HPO_4, 1.0 $MgSO_4$, 5 D-glucose, and 5.0 Hepes at pH 7.6 (Treberg *et al.*, 2005). An incorporation period of about three hours was adopted following the results of McCarthy *et al.* (1999) on Atlantic wolffish in the same size and temperature range. Fish were thereafter killed by a blow to the head, and the peritoneal cavity was opened and thoroughly rinsed with distilled water. A sample of white muscle from the deep dorsolateral region was dissected (care was used not to sample red muscle), immediately frozen on dry ice and transported to the laboratories in Rimouski (QC, Canada) within 24 h after dissection to be stored at −80 °C until further laboratory work. Frozen muscle samples were pulverized in liquid nitrogen using a stainless steel mortar and pestle, and samples of the resulting powder were stored in sealed cryogenic tubes at −80 °C.

2.3.3 Tissue preparation and scintillation counting

Powdered muscle samples were homogenized in 10 volumes of ice-cold 0.2 mol·l^{-1} perchloric acid (PCA) using a Heidolph Diax 900 homogenizer (3x10 s) and incubated for 10 min on ice prior to being centrifuged at 15,000 g for 5 min at 4 °C (Thermo IEC Micromax RF benchtop centrifuge, Waltham, MA, USA). Supernatant (for free pool phenylalanine) was delicately removed, taking care not to include any lipid located on top of the microcentrifuge tube. The protein pellet was then washed three times by resuspending it in 1 ml of 0.2 mol·l^{-1} PCA, incubating on ice for 10 min and centrifuging for 10 min at 15,000 *g*. The protein pellet was then dissolved in 2 ml of 0.3 mol·l^{-1} NaOH (less than 2 h at 37 °C). The determination of labelled phenylalanine in proteins and in the free pool was conducted by mixing samples of the pellet dissolved in NaOH or PCA supernatant, respectively, in 10 ml ScintiVerse II (Fisher Scientific Canada, Ottawa, ON, Canada). This mixture was then counted in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Fullerton, CA, USA). The fluorometric method of McCaman and Robins (1962) was used for the determination of total phenylalanine in the free pool, allowing for the determination of free-pool phenylalanine specific activity. Briefly, phenylalanine forms a highly fluorescent compound with ninhydrin in the presence of L-leucyl-L-alanine after 2 h incubation at 60 °C. Fluorescence was then measured in a Hitachi F-2500 spectrofluorometer (Hitachi High Technologies, San Jose, CA, USA) set at excitation 365 nm and emission 515 nm. A standard curve ranging from 0 to 120 pmol·l^{-1} was prepared for each assay using 0.2 mol·l^{-1} PCA as solvent. Protein synthesis was calculated from radioactivity in the protein bound pool divided by specific activity in the free pool. Data were

normalized to tissue protein content. Protein concentration in the sample was determined with a standardized colorimetric assay (Bio-Rad, Mississauga, ON, Canada).

2.3.4 20S proteasome assay

The chymotrypsin-like activity of 20S proteasome was assayed following Shibatani and Ward (1995). The powdered sample was homogenized in five volumes of lysis buffer using a Potter–Elvehjem (PTFE pestle and glass tube) and centrifuged at 20,000 g at 4 °C for 1 h. The lysis buffer was composed of 0.1 mmol·l^{-1} EDTA, 1.0 mmol·l^{-1} β-mercaptoethanol in a 50 mmol·l^{-1} Tris buffer (pH 8.0). The assay used the proteasome-specific synthetic substrate LLVY-AMC (Biomol International, Plymouth Meeting, PA, USA) and is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate. Briefly, 50 μg of protein from the supernatant was incubated at 15 °C with 40 μmol·l^{-1} LLVY-AMC and 0.0475% SDS in 100 μl of 100 mmol·l^{-1} Tris buffer (pH 8.0) for 30 min. The reaction was stopped with 300 μl of 1% SDS and 1 ml of 0.1 mol·l^{-1} sodium borate (pH 9.1). Fluorescence was determined at excitation/emission wavelengths of 370/430 nm. A standard curve was prepared for each assay. Blanks were prepared by stopping the reaction prior to incubation, and parallel samples were supplemented with 50 μmol·l^{-1} MG-115 and 50 μmol·l^{-1} MG-132 (Biomol International), two potent inhibitors of the chymotrypsin-like activity of the proteasome. The inhibitor-sensitive activity is hereby reported as 20S proteasome activity using pmol AMC per hour per 50 μg protein as a unit. Protein concentration was determined as described for protein

synthesis. The activity was linear for at least 60 min, and the assays never lasted more than 40 min (data not shown).

The *in vitro* thermosensitivity of the wolffish 20S proteasome chymotrypsin-like activity was evaluated by measuring the activity in samples as above but at different temperatures (4, 8, 16, 24, 32 °C). Replicates of white muscle samples of five fish averaging 10 g and acclimated to 8 °C were used. A linear model was fitted on the $\log_{10}$-transformed activities and used to convert the activities measured at 15 °C to the fish's acclimation temperature. The Q_{10} was calculated using $Q_{10} = 10^{(slope \times 10)}$.

2.3.5 Quantification of protein-bound carbonyls

Reactive oxygen species are the cause of many cellular damages, one of which is the oxidation of amino acid residues on proteins, forming protein carbonyls. The protein-carbonyl content was measured by the method of Levine *et al.* (1990) with slight modifications. Briefly, powdered muscle samples were homogenized in ice-cold phosphate buffer saline (PBS; 3.2 mmol·l^{-1} Na_2HPO_4, 0.5 mmol·l^{-1} KH_2PO_4, 1.3 mmol·l^{-1} KCl, 135 mmol·l^{-1} NaCl, pH 7.4), and 50 μl of this homogenate was incubated with 500 μl of 10 mmol·l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in 2 mmol·l^{-1} HCl or 500 μl of 2 mmol·l^{-1} HCl (control) for 1 h. The tubes were vortexed every 10 min. The proteins were then precipitated by adding 500 μl of 20% (w/v) trichloroacetic acid (TCA). After an incubation on ice for 10 min, the tubes were centrifuged at 10,000 g for 10 min. To remove any unbound DNPH, the resulting pellet was washed with 1 ml of 10% TCA and then washed

three times with 1 ml ethanol:ethyl acetate (1:1, v/v). After each wash, the supernatant was carefully aspirated and discarded. The final pellet was dissolved by incubating it in 500 μl of 6 mol·l^{-1} guanidine hydrochloride at 37 °C for 1 h. Absorbance at 370 nm was read on a Lambda 11 spectrophotometer (PerkinElmer, Woodbridge, Canada), and the molar absorption coefficient of 22,000 M^{-1}·cm^{-1} was used to quantify the levels of protein-carbonyls. Protein concentration was determined as described for protein synthesis.

2.3.6 TBARS assay

Free radical damage to lipids results in the generation of malonedialdehyde (MDA), which reacts with thiobarbituric acid (TBA) to form a fluorescent compound. The thiobarbituric acid reactive substances (TBARS) were used as an index of lipid peroxidation in the white muscle samples. The assay was performed using a commercially available kit (Zeptometrix, Buffalo, NY, USA). The kit was used as recommended by the manufacturer except that the assay was downscaled to be performed in microtubes. Muscle samples were homogenized in 10 volumes of ice-cold PBS as above, and 20 μl was pipetted into labeled tubes, 20 μl of the supplied SDS solution was added, followed by 500 μl of the TBA reagent. The tubes were incubated at 95 °C for 60 min along with a standard curve of MDA (0–4 nmol ml^{-1}). After cooling, the tubes were centrifuged at 10,000 g for 5 min, the fluorescence of the supernatant at excitation/emission wavelengths of 530/550 nm was recorded and the MDA equivalent concentration interpolated from the standard curve. The results are presented as the average of three replicates and expressed in nmol·g^{-1} tissue.

2.3.7 Determination of reduced glutathione

Glutathione (GSH) is an intracellular low-molecular-mass thiol that plays a critical role in the cellular defence against oxidative stress. The GSH concentration was measured according to Kamencic *et al.* (2000). Reduced glutathione is combined with monochlorobimane (mCB) by the enzyme glutathione-S-transferase to form a fluorescent GSH–mCB adduct that can be quantified by spectrofluorometry. Muscle samples were homogenized in 10 volumes of ice-cold PBS as above. The homogenate was incubated in triplicate for 30 min at room temperature with mCB and glutathione-S-transferase at a final concentration of 100 μmol $l^{-1}$and 1 U ml^{-1}, respectively. After centrifugation (5,000 *g*, 5 min) the fluorescence of the supernatant was recorded (excitation/emission: 380/470 nm) and the GSH concentration was extrapolated from a standard curve (0–100 μmol·l^{-1}).

2.3.8 Statistical analysis

All data are presented as means ± 95% confidence interval. The GLM procedure of Systat 11 (Systat software, Chicago, IL, USA) was used to examine the effect of temperature on growth, protein synthesis, proteasome activity, protein-carbonyl, TBARS and GSH. When a significant effect of temperature was detected, the multiple comparison test of Tukey was performed. Regression analysis was used to examine the relationship between protein synthesis or 20S proteasome activity and SGR. Finally, a multiple regression was used to analyze the combined effect of protein synthesis and 20S proteasome activity on SGR. Equality of variances was tested using Levene's test, and residual normality was tested using Kolmogorov–Smirnoff test with the correction of Lilliefors. All tests were two-tailed with a

significance level of 0.05.

2.4 Results

There were no mortalities during the time of the experiment. The final mass of the group acclimated at 4 °C was significantly lower than the mass of the other two groups ($F_{2,28}$=17.93, P<0.001); the final masses were 3.44±0.51 g, 4.72±0.39 g and 4.89±0.79 g for fish acclimated at 4, 8 and 12 °C, respectively. The final length was significantly different among the three groups ($F_{2,28}$=28.28, P<0.001); the final lengths were 6.9±0.4 mm, 7.6±0.3 mm and 8.0±0.4 mm for 4, 8 and 12 °C, respectively. At 4 °C, SGRm was significantly lower than at 8 and 12 °C and there was no significant difference between 8 and 12 °C (Fig. 2.1) ($F_{2,28}$=36.11, P<0.001). There was a strong trend for higher length increase at 12 °C (Tukey's P=0.06), which yielded a significantly lower condition index, i.e. fish that were more slender compared with fish acclimated at 4 and 8 °C (Fig. 2.2) ($F_{2,28}$=8.96, P=0.001).

2.4.1 Protein synthesis

The protein synthetic activity was measured in white muscle at the end of the growth trial. The effect of water temperature on the rate of protein synthesis is presented in Fig. 2.3. At the lowest temperature (4 °C), the rate of protein synthesis was significantly lower compared with the group acclimated at 8 °C ($F_{2,23}$=3.150, P=0.026) while acclimation at 12 °C led to an intermediate rate with no significant difference between 4 and 12 °C groups (Fig. 2.3).

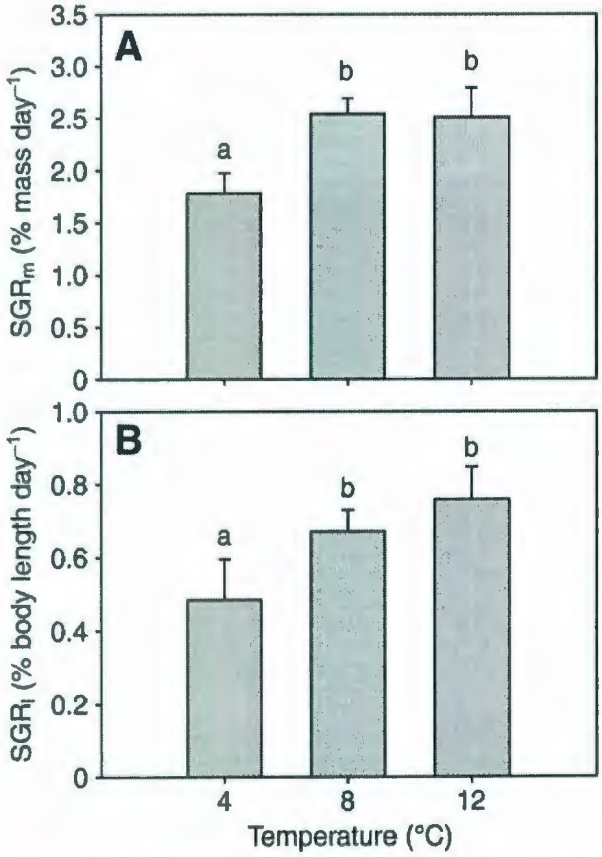

Figure 2.1: The effect of temperature on specific growth rate (SGR) in (A) body mass per day and (B) body length per day of juvenile spotted wolffish. Values are means ± 95% CI (N=10); different letters indicate significant difference (P<0.05) -

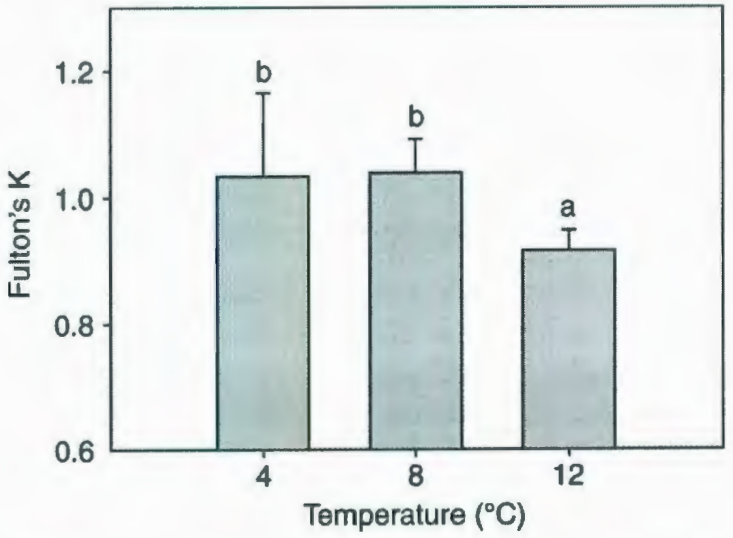

Figure 2.2: Fulton's condition index of spotted wolffish growing at three temperatures. Values are means ± 95% CI (N=10); different letters indicate significant difference (P<0.05).

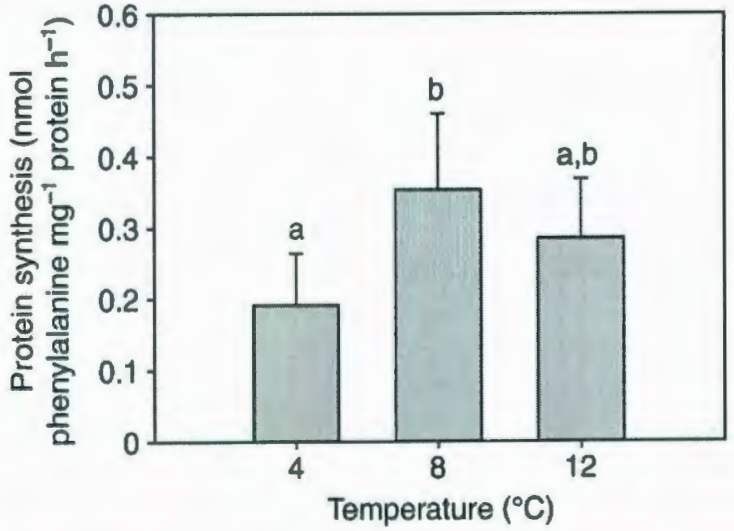

Figure 2.3: Rate of protein synthesis expressed as incorporation of phenylalanine in white muscle proteins of juvenile spotted wolffish acclimated at three temperatures. Values are means ± 95% CI (N=8–10); different letters indicate significant difference (P<0.05).

2.4.2 20S proteasome activity

The chymotrypsin-like activity of the 20S proteasome was affected by acclimation temperature. When measured at a common temperature, the activity in fish acclimated at 4 °C was higher than in fish acclimated at 8 °C, the latter also displaying a higher activity than those at 12 °C (Fig. 2.4) ($F_{2,26}$=12.619, P<0.001). There was no effect of acclimation temperature on the level of non-specific activity in the reactions supplemented with MG-115 and MG-132; the averages were 3.47±1.03, 3.52±0.51 and 2.51±0.61 pmol AMC·h^{-1}·50 μg^{-1} protein for fish acclimated at 4, 8 and 12 °C, respectively ($F_{2,26}$=2.196, P=0.12).

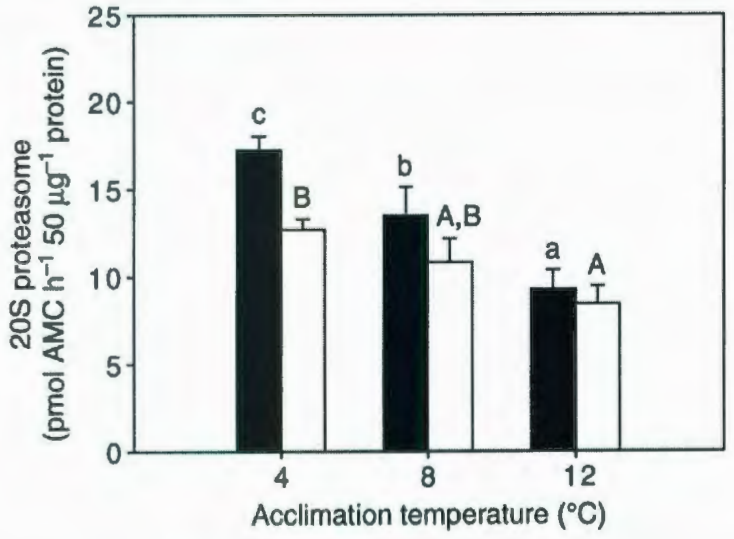

Figure 2.4: Activity of 20S proteasome in white muscle of juvenile spotted wolffish acclimated at different temperatures. The black bars represent activity measured at 15 °C while the open bars represent the activity calculated at acclimation temperature (see text for details). Values are means ± 95% CI (N=8–10); different letters indicate significant difference (P<0.05)

Catalytic rate of the chymotrypsin-like activity of 20S proteasome at physiological temperatures (Fig. 2.4, white bars) was estimated by first determining

activity at different assay temperatures for homogenates from fish acclimated to 8 °C. The relationship between proteasome activity and temperature, determined at temperatures ranging from 4 °C to 32 °C, was linear (Fig. 2.5). This reveals that nothing untoward with respect to thermal sensitivity is occurring over the temperature range of study. The expected activity at acclimation temperature was calculated from the activity measured at 15 °C using the calculated Q_{10} of 1.33. When the activities were reported at their expected values at acclimation temperature, the direction of the differences remains the same but the group acclimated at 8 °C was no longer significantly different from the extremes while the 4 and 12 °C groups remained significantly different from each other (Fig. 2.4, open bars) ($F_{2,26}$=5.069,P=0.015).

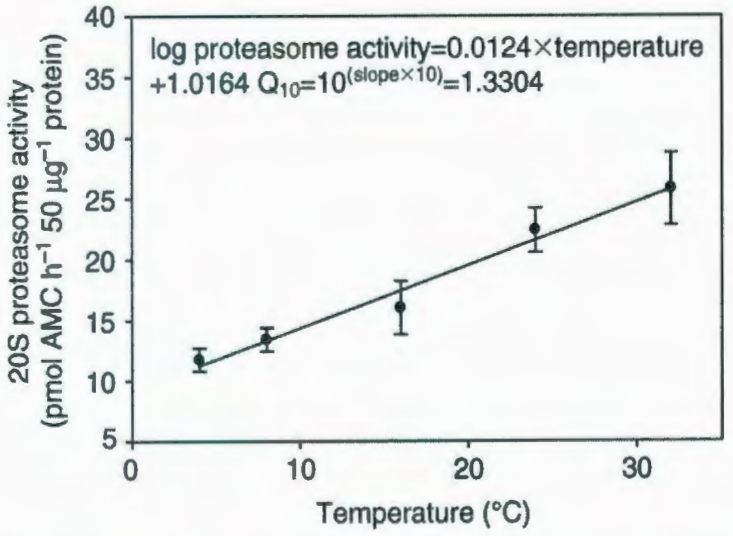

Figure 2.5: Activity of 20S proteasome in relation to assay temperature in white muscle of spotted wolffish acclimated at 8 °C. The top equation describes the linear relation between log-transformed proteasome 20S activity and temperature (r^2=0.680, P<0.001). The slope of this relationship was then used in the second equation to determine Q_{10}. Each data point represents the mean activity of five fish ± SD

In an attempt to describe growth data using protein synthesis and 20S proteasome activity, simple and multiple regression analyses were performed on pooled groups. Growth rate was well described by protein synthesis according to the following equation: SGRm = 1.714 + 2.065 × protein synthesis (r^2=0.343, P=0.001). The relationship between growth rate and 20S proteasome activity (adjusted to acclimation temperature) was best described by: SGRm = 2.926 − 0.048 × proteasome (r^2=0.253, P=0.006). A multiple regression best described growth rate using both protein synthesis and 20S proteasome activity: SGRm = 2.22 + 2.040 × protein synthesis − 0.039 × proteasome (r^2=0.55, P<0.001).

2.4.3 Quantification of protein-bound carbonyls

Oxidative protein damages in the white muscle were measured as protein-bound carbonyls. There was no significant effect of acclimation temperature on protein-bound carbonyls ($F_{2,24}$=1.520, P=0.242). The protein-bound carbonyl content varied between 0.364 and 1.879 nmol·mg^{-1} protein and averaged 1.074 nmol·mg^{-1} protein.

2.4.4 Quantification of TBARS and reduced glutathione

Lipid peroxidation was measured as the content of TBARS. The TBARS concentrations varied between 0.087 and 0.263 μmol·g^{-1} white muscle and averaged 0.154 μmol·g^{-1} white muscle. There was no significant effect of temperature on TBARS content ($F_{2,25}$=1.50, P=0.245). Acclimation temperature had a significant effect on GSH concentration (Fig. 2.6). Fish acclimated at 4 °C had a higher concentration of GSH than those acclimated at 12 °C while fish acclimated at 8

°C had intermediate values ($F_{2,25}$=7.01, P=0.005).

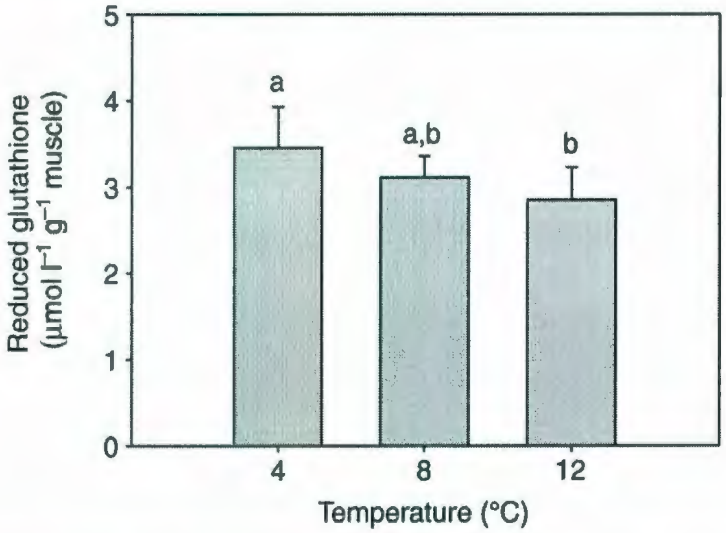

Figure 2.6: White muscle concentration of reduced glutathione in juvenile spotted wolffish acclimated at three temperatures. Values are means ± 95% CI (N=8–10); different letters indicate significant difference (P<0.05).

2.5 Discussion

Wolffish possess several assets that make them useful as a model species for the study of relationships linking protein metabolism and growth. The effect of temperature on muscle fractional rate of protein synthesis was described in fish as a log-linear relationship for many species, and in wolffish, protein synthesis occurs at higher rates compared with other species (McCarthy *et al.*, 1999). Wolffish also display very low spontaneous swimming activity and aggressive behaviour, allocating more energy to somatic growth than active metabolism (Le François *et al.*, 2004; McCarthy *et al.*, 1998; McCarthy *et al.*, 1999; Savoie *et al.*, 2008), which contributes to a reduced level of intraspecific growth variability. This, in

turn, allows for a better assessment of the links among temperature, growth rate and protein metabolism measurements.

As expected, acclimation at the coldest temperature had a significant negative effect on growth rate, mean SGRm being 70% of SGRm of fish acclimated at 8 °C. Spotted wolffish acclimated at 12 °C did not show significantly different growth rate when compared with the 8 °C group, as found in Atlantic wolffish (McCarthy *et al.*, 1998; McCarthy *et al.*, 1999). However, Savoie *et al.* (2008), studying newly hatched spotted wolffish, reported the highest growth rate at 12 °C compared with 8 °C during the first two weeks post-hatch, then a sharp decrease of growth rate at 12 °C occurred. Our results are also in accordance with other published growth rates of juvenile spotted wolffish (Hansen and Falk-Petersen, 2002; Imsland *et al.*, 2006a; Imsland *et al.*, 2006b). The SGRm data presented here are, to my knowledge, the first published for spotted wolffish in the range of 2 to 5 g. The absence of mortality and generalized positive growth during the growth trial is indicative of the good health of the experimental fish but also, shows that the selected experimental temperatures were well within the species thermal range. Acclimation to the highest temperature (12 °C) seems to have an effect on the shape of fish, as suggested by the reduced Fulton's condition index (Fig. 2.2). This phenomenon might be indicative of the difficulty of retaining energy reserves at higher temperature due to higher maintenance costs. It might also be indicative of different thermosensitivity of growth rate and developmental processes.

2.5.1 Protein synthesis

In this study, the rate of protein synthesis was maximal at 8 °C, lower at 4 °C and intermediate at 12 °C. At 12 °C, the rate of amino acid incorporation tends to be lower than at 8 °C. This contrasts with the results for Atlantic wolffish (McCarthy *et al.*, 1999), where the fractional rate of protein synthesis increased linearly with temperature. In juvenile barramundi (*Lates calcarifer*), protein synthesis was shown to display an asymmetrical relation with temperature, as it tends to in the present study (Katersky and Carter, 2007). It has been suggested that, under satiation feeding, protein synthesis is maximal at optimum growth temperature (Carter and Houlihan, 2001; Loughna and Goldspink, 1985). This seems to be the case in the present study as protein synthesis tends to be higher at 8 °C, which is generally recognized as the optimal growth temperature of spotted wolffish (Foss *et al.*, 2004; Hansen and Falk-Petersen, 2002; Imsland *et al.*, 2006a; Imsland *et al.*, 2006b).

2.5.2 Proteasome activity

The thermosensitivity of the chymotryptic-like activity of 20S proteasome was the same for the white muscle and liver (data not shown). The relatively low Q_{10} observed in spotted wolffish contrasts with findings of other studies, mostly on mammals, where reported Q_{10} are between 1.6 and 4 (Velickovska *et al.*, 2005; Woods and Storey, 2005). The skeletal muscle chymotryptic-like activity of hibernating thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) has a lower Q_{10} than that of conspecific euthermic animals (1.6 *vs* 2.1) and is much lower than that of the mouse (*Mus musculus*), which has a Q_{10} of 2.9 [calculated

from fig. 2.3 of Woods and Storey (2005)]. In general, Q_{10} increases at lower temperature, but this phenomenon was not observed in the range of temperature studied. This does not rule out the possibility of a very large Q_{10} between 4 °C and lower temperatures but this is beyond the scope of this study. It is worth mentioning that, in the wild, spotted wolffish experience temperatures that range from −1 to 7 °C (Barsukov, 1959). Consequently, it is not surprising that a low Q_{10} is measured even at cold temperatures. It is not clear whether wolffish 20S proteasome is adapted to be more efficient at cold temperature or if animals living at higher temperatures have evolved a protection mechanism that limits protein degradation during short-term decreases of temperature, such as in hibernating squirrels (Velickovska *et al.*, 2005).

Protein degradation is a tightly regulated process (Hershko *et al.*, 2000) and, in eukaryotic cells, three major protein degradation systems exist. The lysosomal system is composed of a vacuole-bound acidic environment where the cathepsins hydrolyze the proteins in a non-specific manner. The concentrations of the different cathepsins are under tight control (Aoki *et al.*, 2000; Mommsen, 2004). The calpain system is composed of two calcium-activated proteolytic enzymes (the m-calpain and the μ-calpain) and their specific inhibitor (the calpastatin). This system is thought to be principally involved in the cleavage of cytoskeletal/membrane attachments and presumably signal transduction (Goll *et al.*, 2003; Goll *et al.*, 1998). The ubiquitin/proteasome system, examined here, is involved in the specific degradation of tagged proteins. Tagging of a protein for destruction is accomplished by covalent attachment of multiple ubiquitin moieties, a highly evolutionary conserved protein. Conjugation of ubiquitin to the protein is

realized *via* a three-step cascade mechanism involving three classes of enzymes (E1, E2 and E3) that activate ubiquitin, transport it and attach it to substrate protein, respectively (Attaix *et al.*, 2001). The 20S proteasome is composed of four stacked rings that form a barrel-like structure hosting proteolytic activities. Both ends of the 20S proteasome can be capped by a 19S regulatory particle that is responsible for substrate recognition and its translocation into the lumen for degradation (Attaix *et al.*, 2001; Braun *et al.*, 1999; Glickman and Ciechanover, 2002).

This is the first study to measure 20S proteasome activity in fish acclimated at different temperatures. There is a clear indication of thermal compensation of proteasome activity in fish acclimated at 4 °C. Proteasome activity at 4 °C was 130% higher than the activity level measured at 8 °C, while in fish acclimated at 12 °C, activity was 87% of that of 8 °C (calculated using activities at acclimation temperature). The use of Q_{10} to calculate proteasome activity at acclimation temperature was preferred to measuring the enzyme activities at acclimation temperature for practical considerations. The effect of temperature on enzyme activity was linear in the studied range of temperature and there is no reason to believe that the fish express different isoforms of proteasome at different temperature. Therefore, the temperature sensitivity of the proteasome should be the same between acclimation groups and the calculated activities should represent reality. The assays were performed on tissues extracted at least 24 h after the last meal and the activities are likely to represent the basal rate of protein degradation in white muscle of juvenile spotted wolffish. Our results are in accordance with many transcriptomics studies that show an increase of the genes coding for 20S pro-

teasome and/or ubiquitinating enzymes during acclimation to cold temperatures. In a study on gene expression level of annual killifish (*Austrofundulus limnaeus*) during acclimation to low and high temperature, Podrabsky and Somero (2004) found that a subunit of 26S proteasome was strongly upregulated during cold acclimation. In the common carp (*Cyprinus carpio*), acclimation to cold temperature also led to upregulation of 21 genes involved in the ubiquitin-proteasome pathway (Gracey *et al.*, 2004). Upregulation of genes of the proteasome pathway was also observed, along with an apparent increase in the ubiquitin-conjugated (Ub-conjugated) level of some proteins in common carp (McLean *et al.*, 2007). Todgham *et al.* (2007) found a higher level of Ub-conjugated protein in Antarctic fish species than in fish inhabiting temperate waters of New Zealand. The authors concluded that cold waters placed higher physiological constraints on maintaining proteins in their native state (Todgham *et al.*, 2007). These authors also suggested that the accumulation of Ub-conjugated proteins could be a result of a lower efficiency of the proteasome at lower temperature. This interpretation is now challenged by our results, given the compensation of 20S proteasome activity at lower acclimation temperature. Our results, combined with those of Todgham *et al.* (2007), indicate that cold temperatures place an important physiological constraint on protein metabolism and that the ubiquitin-proteasome pathway seems to be upregulated in such conditions. It is noteworthy that the 20S proteasome activities reported in the present study reflect the capacity of degradation of a synthetic peptide and not the degradation of proteins *per se*. This is, for now, the closest we can get to protein degradation by the proteasome pathway.

Lower protein synthesis retention efficiency (PSRE) was observed in Atlantic

wolffish at lower temperature (McCarthy *et al.*, 1999). PSRE was also found to be lower at low temperature in juvenile barramundi (Katersky and Carter, 2007). Low PRSE indicates that a lower proportion of synthesized proteins is successfully retained for growth, in other words it is indicative of a high protein turnover and therefore of a low growth efficiency. Our results show that the proteasome pathway is a potentially significant component of the low PRSE generally observed at low temperature. It appears that, at temperatures slightly higher than the optimal, growth rate is maintained by a combination of skeletal growth and slightly reduced muscular activity of proteasome, as shown by the Fulton's K and proteasome 20S activity, respectively. Since the higher acclimation temperature was not high enough to produce a detrimental effect on growth rate, it is not possible to evaluate the role of proteasome in the case of acclimation to adversely high temperature. In their study on barramundi, Katersky and Carter (2007) also found that PRSE was maintained over a wide range of temperature at the higher end of the experimental temperatures tested. To date, protein metabolism (synthesis, growth and degradation) has not been measured in animals acclimated at temperatures clearly above their optimal temperature. Such information would provide further data to explore the relationships between protein metabolism and growth rate.

2.5.3 Protein turnover

A significant positive relationship was found between the rate of protein synthesis in white muscle and SGRm while a negative relationship was found between 20S proteasome activity and SGRm. A significant multiple regression described

SGRm by using protein synthesis and proteasome activity. As shown by the equation of this regression, white muscle 20S proteasome activity negatively impacts growth rate. Dobly *et al.* (2004) also found a negative relationship between growth rate and 20S proteasome activity in rainbow trout liver but not in white muscle. This is probably related to the different experimental approach they used to study the relationship between protein metabolism and growth rate and also to the sedentary swimming behaviour of spotted wolffish compared with rainbow trout. The multiple regression was computed on pooled fish without using temperature as a factor for two reasons; first, I wanted to avoid the use of too many parameters and their interactions. More importantly, since the selected acclimation temperatures were within the thermal range of spotted wolffish, it was considered as a mild treatment influencing growth rate in comparison to food restriction. Keeping in mind that during positive growth, protein turnover is equal to protein degradation (Hawkins, 1991), turnover appeared to be maintained high in slow-growing fish acclimated at lower temperature. Fish acclimated at the higher temperature maintained growth rate similar to the group acclimated at 8 °C while having a slightly lower rate of protein synthesis and 20S proteasome activity.

2.5.4 Protein damage

Protein cold denaturation and/or protein misfolding have been suggested as causal factors of the high levels in Ub-conjugated protein content reported in cold-water-adapted fish species (Todgham *et al.*, 2007). Although, as pointed out by these authors, millions of years of evolution in a cold environment should have

led to adaptive modifications of proteins in order to maintain their stability under natural conditions. However, acclimation to cold temperature generally leads to increased mitochondrial capacity in white muscle (Guderley, 2004), and reactive oxygen species (ROS) are a byproduct of the mitochondrial respiration (Boveris and Chance, 1973). These ROS may damage all types of biological molecules but, because of their high relative abundance in tissue composition, proteins are a major target (Dalle-Donne *et al.*, 2003; Levine *et al.*, 1990; Shacter, 2000). A generally recognized role of the proteasome pathway is the degradation of oxidatively damaged proteins. Oxidatively damaged proteins are tagged by enzymes of the ubiquitinating pathway, and proteasome activity is also known to be highly responsive to oxidative stress (Friguet, 2006; Perepechaeva *et al.*, 2006; Poppek and Grune, 2006). A higher level of oxidative modification of proteins could perhaps explain the higher rate of protein degradation at cold temperature. In the present study, neither protein-carbonyl nor TBARS contents were higher at low temperature; however, GSH concentration was significantly higher at low temperature. Unfortunately, the method used for GSH assay did not allow for assessment of GSSG, making it impossible to estimate the total glutathione pool, its redox state and thus the antioxidant status of the fish. However, GSH concentration was shown to increase as a response to mild oxidative conditions (hyperoxia) in gills (Ritola *et al.*, 2002a) and liver (Ritola *et al.*, 2002b) of rainbow trout. In the North Sea eelpout (*Zoarces viviparous*), GSH levels were shown to increase following stressful exposure to low temperature (Heise *et al.*, 2006). In the Antarctic eelpout (*Pachycara brachycephalum*), glutathione content is two to three times higher than in the North Sea eelpout, and the ratio GSSG/GSH of the Antarctic eelpout was among the highest reported in the literature. The

authors argued that such a high ratio of GSSG/GSH and the high concentration of GSH were both characteristic traits of some polar fish, such that metabolic processes, adapted to function at low temperatures, are also able to operate in a more oxidized redox environment (Heise *et al.*, 2007). In these studies, both GSH and GSSG were measured but, in all cases, the absolute content of GSH was increased following exposure to potentially stressful conditions. As such, the higher content of GSH in cold-acclimated spotted wolffish possibly indicates that the animal faces more oxidative conditions. The redox state of the glutathione pool following acclimation to different temperatures would definitely be more informative but the data obtained in this study present a valuable point of departure. Reduced glutathione was the only antioxidant investigated but it could well be that antioxidant enzymes are participating in ROS detoxification in cold-acclimated spotted wolffish. For instance, in common carp, along with the ubiquitin–proteasome pathway transcripts, the transcription levels of the antioxidant enzymes superoxide dismutase and glutathione-S-transferase were upregulated during cold acclimation (Gracey *et al.*, 2004).

2.6 Conclusion

20S proteasome activity seems to be a good proxy for protein degradation at suboptimal temperature in spotted wolffish. The effect of low temperature on the growth rate of juvenile spotted wolffish is attributed to the lower rate of protein synthesis and high proteasome activity resulting in lower protein retention efficiency and lower growth efficiency. High rate of protein degradation at low temperature is suggested as an adaptive response to keep the steady-state con-

centration of oxidatively modified proteins at an acceptable level. Further work on mitochondrial ROS generation and the antioxidant status of fish is nevertheless needed to strengthen this hypothesis. Adaptation to a colder environment could involve tradeoffs between increased mitochondrial capacity and lower protein retention efficiency due to higher oxidative damages incurred by proteins when ROS production is higher.

3

White muscle 20S proteasome activity is negatively correlated to growth rate at low temperature in the spotted wolffish (*Anarhichas minor*; Olafsen)

This chapter has been accepted in the Journal of Fish Biology subject to minor revision.

3.1 Abstract

The effect of temperature and mass on specific growth rate (SGR) was examined in spotted wolffish of different size classes (ranging from 60 to 1500 g) acclimated at different temperatures (4, 8 and 12 °C). The relationship between SGR and 20S proteasome activity in heart ventricle, liver and white muscle tissue was then assessed in fish acclimated at 4 and 12 °C in order to determine if protein degradation via the proteasome pathway could be imposing a limitation on somatic growth. In heart, 20S proteasome activity was not affected by acclimation temperature and fish mass. No relationship was observed between heart 20S proteasome activity and SGR. Hepatic 20S proteasome activity was higher at 12 °C but did not show any relation with SGR. Partial correlation analysis showed that white muscle 20S proteasome activity was negatively correlated to SGR (partial Pearson's r = -0.609) but only at cold acclimation temperature (4 °C). I suggest that acclimation to cold temperature involves compensation of the mitochondrial oxidative capacity which in turn leads to increased production of oxidized-damaged proteins that are degraded by the proteasome pathway, and ultimately negatively affects SGR at cold temperature.

3.2 Introduction

The wolffish family (Anarhichadidae) consists of five species of which two are found in the Pacific, and three in the Atlantic. The spotted wolffish (*Anarhichas minor*) is a bottom-dwelling species distributed in the North Atlantic and the Barents Sea that was looked upon as a promising marine fish species for cold

water aquaculture in Canada, Norway and Iceland (Falk-Petersen *et al.*, 1999; Le François *et al.*, 2002; Foss *et al.*, 2004). It is generally found at depths ranging from 25-550m and temperatures from -1 to 7 °C (Barsukov, 1959). Reported specific growth rate (SGR) of newly hatched wolffish is between 4.5 and 5.1 % $\cdot day^{-1}$ at temperatures between 12 and 14 °C (Hansen and Falk-Petersen, 2002; Savoie *et al.*, 2006; Savoie *et al.*, 2008). As fish grow larger, SGR decreases to about 1 % $\cdot$ day^{-1} in fish averaging 100 g and 0.4 % $\cdot$ day^{-1} in fish of 400 g (Imsland *et al.*, 2006). This reduction in growth rate with increasing size has been observed in several other fish species (Brett, 1979; Jobling, 1985; Houlihan *et al.*, 1986; Imsland *et al.*, 1996).

Growth is the net outcome of a series of behavioural and physiological processes that begins with food intake and concludes with accretion of protein and other biomolecules. Growth rate mostly results from the difference between protein synthesis and degradation, and both growth components are influenced by body size (Houlihan *et al.*, 1986). In rainbow trout (*Oncorhynchus mykiss*), higher fractional rates of protein synthesis and protein degradation are found during the early life history stages and both decrease with increasing body mass, with the fractional rate of protein synthesis decreasing at a faster rate than protein degradation (Houlihan *et al.*, 1986; Peragon *et al.*, 2001). The decrease of growth rate with increasing size is generally accompanied by a reduction of optimal growth temperature (T_{optSGR}) (Brett, 1979; Imsland *et al.*, 1996; Bjornsson *et al.*, 2007). For instance, during the first 60 days after hatching, T_{optSGR} of spotted wolffish is estimated to be between 10 and 12 °C (Hansen and Falk-Petersen, 2002). As the fish gain mass, T_{optSGR} decreases to 7.9 °C and 6.6 °C

in fish weighing 130-135 g and 360-380 g, respectively (Imsland *et al.*, 2006). In Atlantic wolffish (*A. lupus*), a close relative of the spotted wolffish, the fractional rate of protein synthesis was shown to increase with temperature, while protein retention efficiency presented a parabolic relationship, being highest at optimal growth temperature and decreasing at lower and higher temperatures (McCarthy *et al.*, 1999). Collectively these data suggest that protein degradation is a critical determinant of growth and is influenced by both temperature and body size in a fashion independent from that of protein synthesis.

One of the most important mechanisms of protein degradation is the ubiquitin-proteasome pathway (UPP) in which proteins are targeted for degradation by covalent bonding to ubiquitin. These proteins are then recognized and degraded by the proteasome. Genes involved in UPP were shown to be upregulated during cold temperature acclimation in the common carp (*Cyprinus carpio*) (Gracey *et al.*, 2004) and an annual killifish (*Austrofundulus limnaeus*) (Podrabsky and Somero, 2004). In rainbow trout, hepatic activity of 20S proteasome was negatively related to growth rate (Dobly *et al.*, 2004). More recently, in white muscle of $\sim$ 5g spotted wolffish, the rate of protein synthesis was shown to increase from 4 to 8 °C with no further increase from 8 to 12 °C. However, in the same individuals, 20S proteasome activity was maximal at 4 °C and decreased significantly in fish acclimated at 8 °C and 12 °C while growth rate was higher at 8 and 12 °C (Chapter 2). These findings suggest that, along with reduced protein synthesis, protein degradation by UPP could be responsible for the lower protein retention efficiency and growth rate observed at low temperature.

Information on the activity of UPP in fish is scarce, particularly for marine fish species. There is little or no information available on the activity of 20S proteasome in different tissues or on the scaling relation with fish mass. The objectives of the present study were to examine the relationships among SGR, temperature and fish mass as well as the relation between SGR and the activity of 20S proteasome in white muscle, liver and heart ventricle of spotted wolffish. Fish encompassing a wide range of mass (60 to 1500 g individuals) and acclimated at high and low temperature (12 and 4 °C, respectively) were utilized. I chose to study heart and liver because they have high protein turnover while white muscle makes up to 50% of total fish mass and its protein metabolism is closely related to whole body protein metabolism (McCarthy *et al.*, 1999).

3.3 Materials and methods

3.3.1 Growth trial

The growth trial was carried out in the aquaculture facilities of the Centre Aquacole Marin de Grande-Rivière (QC, Canada). The juvenile spotted wolffish (mixed sexes) were the offspring of a captive brood stock population maintained at this facility since the year 1999. Prior to the growth trial, fish were held at 8 °C and fed *ad libitum* according to established protocols. The growth trial took place during the period of October 17th to December 6th 2006 for 51 days using fish of four size classes with initial mass of 61.74±16.50 g, 125.3±17.6 g, 339.5±69.1 g and 1361±286 g. Growth data of fish having an initial mass of 1.72±0.20 g were included from a previous growth trial (Chapter 2). The fish

were individually marked using PIT tags (Avid Canada) at least one month prior to the growth trial in order to avoid any stress related to marking operations. At the beginning of the trial, fish were anaesthetized (benzocaine 50 mg L^{-1}), mass and length were recorded, and fish were randomly assigned (10 fish per size class per temperature) into one of the three shallow raceways supplied with aeratated sea water at 4 °C, 8 °C or 12 °C (4.41±0.23 °C; 7.81±0.07 °C and 11.73±0.44 °C) for the duration of the trial. The fish of different size classes were separated in the raceways by fences that were spaced to adjust fish densities to approximately 40 $kg \cdot m^3$. Fish were hand-fed several times a day to satiety until uneaten food was left at the bottom of the tank. Feed used were Europa 15 (Skretting, NB, Canada) for fish up to 400 g and on AquaBrood 10 (Corey, NB, Canada) for larger fish. Water quality was monitored daily for oxygen concentration and salinity. Fish were exposed to a 12/12 photoperiod, salinity was 29 ‰, and oxygen concentration was always over 80 % of saturation.

At the end of the growth trial, fish were anaesthetized (50 $mg \cdot l^{-1}$ benzocaine) and mass and length recorded. A muscle sample was taken from the deep dorsolateral region (care was used not to sample red muscle) and a sample of liver from the distal part of the bigger lobe was dissected. The heart was quickly excised, excess blood was washed out using phosphate buffered saline and all tissues were immediately frozen on dry ice and stored at -80 °C until laboratory analysis.

3.3.2 Proteasome activity

The chymotrypsin-like activity of 20S proteasome was assayed following Shibatani and Ward, (1995). Approximately 100 mg of tissue sample was homogenized in 10 volumes of lysis buffer for heart and liver and 5 volumes for white muscle using a Potter-Elvehjem (PTFE pestle and glass tube) and centrifuged at 20,000 g at 4 °C for one hour. The lysis buffer was composed of 0.1 mM EDTA and 1.0 mM β-mercaptoethanol in a 50 mM Tris buffer (pH 8.0). The assay used the proteasome specific synthetic substrate LLVY-AMC (Biomol International, Plymouth Meeting, PA) and is based on detection of the fluorophore 7-Amino-4-methylcoumarin (AMC) after cleavage from the labelled substrate. Briefly, 50 μg of protein from the supernatant was incubated at 15 °C with 40 μM LLVY-AMC and 0.0475% SDS in 100 μl of 100 mM Tris buffer (pH 8.0) for 30 min. The reaction was stopped with 300 μl of SDS 1% and 1 ml sodium borate (0.1 M pH 9.1). Fluorescence was determined at excitation/emission wavelengths of 370/430 with a F-2500 spectrofluorimeter (Hitachi). A fresh standard curve of AMC, ranging from 0 to 100 nM, was prepared for each assay. Blanks were prepared by stopping the reaction prior to incubation and parallel samples were supplemented with 50 μM of MG-115 and 50 μM of MG-132 (Biomol International, Plymouth Meeting, PA), two potent inhibitors of the chymotrypsin-like activity of the proteasome. The inhibitor sensitive activity is hereby reported as 20S proteasome activity using pmol AMC·hr^{-1}·50 μg protein^{-1} as a unit. In my previous study (Chapter 2), the thermosensitivity of 20S proteasome was measured in muscle and liver and a Q_{10} of 1.33 was obtained. The activity measured at 15 °C was then converted to that at the acclimation temperature as detailed in Chapter 2. Protein con-

centration in the sample was determined with a standardized colorimetric assay (Bio-Rad, Mississauga, Canada).

3.3.3 Statistical analysis

Results are presented as mean ± SD. Specific growth rate (SGR) was calculated using; $\mathrm{SGR} = [\ln(M_f) - \ln(M_0)] \cdot 100/t$, where Mf and $M0$ are final and initial mass and t (51 days) is the duration of the whole experiment. The effect of temperature on SGR was analyzed with a least squares second order polynomial regression where SGR was regressed against temperature for five size-classes. The regression formula takes the form of: $\mathrm{SGR} = aT^2 + bT + c$, where T = temperature, and a, b and c are constants determined by the regression. Optimal temperature for growth (T_{optSGR}) for each size-class was calculated using $T_{optSGR} = \left(\frac{-b}{2a}\right)$ from the parabolic regression. Maximum growth rate (G_{max}) was calculated by substituting T by T_{optSGR} in the parabolic equation for each size-class. The scaling relationship between mass and growth rate at each temperature was examined using regression analysis on the log transformed data using the following formula: $\mathrm{Log}Y = \mathrm{Log}a + b \times \mathrm{Log}X$, where X is body mass, Y is SGR, a is a constant and b is the mass exponent (Brett, 1979). The regression slopes were compared with covariance analysis (ANCOVA (Zar, 1999)).

The relation between 20S proteasome activities in various tissues and fish mass were examined by ANCOVA and data were log-transformed when necessary (determined by a residual analysis). A t test was used to compare the protein concentration in the supernatant used to measure 20S proteasome activity of

fish acclimated at 4 and 12 °C. To examine the relations between SGR and 20S proteasome activities, a partial correlation matrix was constructed to remove the effect of mass on SGR for fish acclimated at 4 and 12 °C. The significance of the partial correlation coefficients was determined according to Zar (1999). A multiple regression analysis was used to quantify the effects of fish mass and white muscle 20S proteasome activity on SGR. Equality of variances was tested using Levene's test and residual normality was tested using Kolmogorov-Smirnov test with the correction of Lilliefors. All tests were two-tailed with a significance level of 0.05. All statistical analyses were performed using Systat 11.

3.4 Results

3.4.1 Effect of temperature and mass on growth

No mortalities were observed during the experimental period with the exception of two fish from the 60 g group held at the 12 °C. The effect of temperature on SGR was described by a parabolic regression (Fig. 3.1). The equations of the regression lines were;

$SGR = -0.0212 \times T^2 + 0.443 \times T + 0.349$; $r^2 = 0.728$ $p < 0.001$ (5 g fish)

$SGR = -0.0075 \times T^2 + 0.060 \times T + 0.259$; $r^2 = 0.279$ $p = 0.017$ (150 g fish)

$SGR = -0.0185 \times T^2 + 0.258 \times T - 0.1892$; $r^2 = 0.482$ $p < 0.001$ (400 g fish)

$SGR = -0.0044 \times T^2 + 0.057 \times T + 0.165$; $r^2 = 0.088$ $p = 0.287$ (1500 g fish)

Optimal temperature for growth was highest for the 5 g group and decreased in bigger fish. There was a significant effect of temperature in all size-class groups

except the 1500 g group. In the 60 g group, it was not possible to generate a regression with a positive a parameter (having only one summit) and as such, T_{optSGR} could not be calculated. Fish from the 60 g size class were excluded from further analysis because there were mortalities in this group and because of the absence of temperature effect on growth rate.

Specific growth rates in relation to fish mass for all experimental temperatures (4, 8 and 12 °C) are presented in Figure 3.2. The relationships are linear on a log-log scale and the slope for fish at 12 °C is significantly different from that at 4 and 8 °C (ANCOVA, $F_{2,129} = 8.33$, $p<0.001$). The maximum growth rate (growth rate at optimal temperature G_max) decreased linearly with mass on a log-log scale. The fitted power model corresponds to $G\_max=aW^b$, where W is fish mass, the intercept $a = 4.82$ and the exponent $b = -0.37$ (Fig. 3.3).

3.4.2 20S Proteasome activity

The activity of the 20S proteasome was measured at the common temperature of 15 °C for white muscle, liver and ventricle of fish maintained at 4 and 12 °C (Fig. 3.4). The extreme acclimation temperatures were selected because these conditions were associated with the most pronounced difference in growth rate. White muscle presented the lowest activity, while ventricle and liver activities were in the same range and approximately 7-fold higher than white muscle. There was no significant interaction between the effect of mass and temperature for any tissue. The 20S proteasome activity in white muscle was marginally correlated to fish mass only at 12 °C (20S proteasome activity = -0.002 × mass + 9.185;

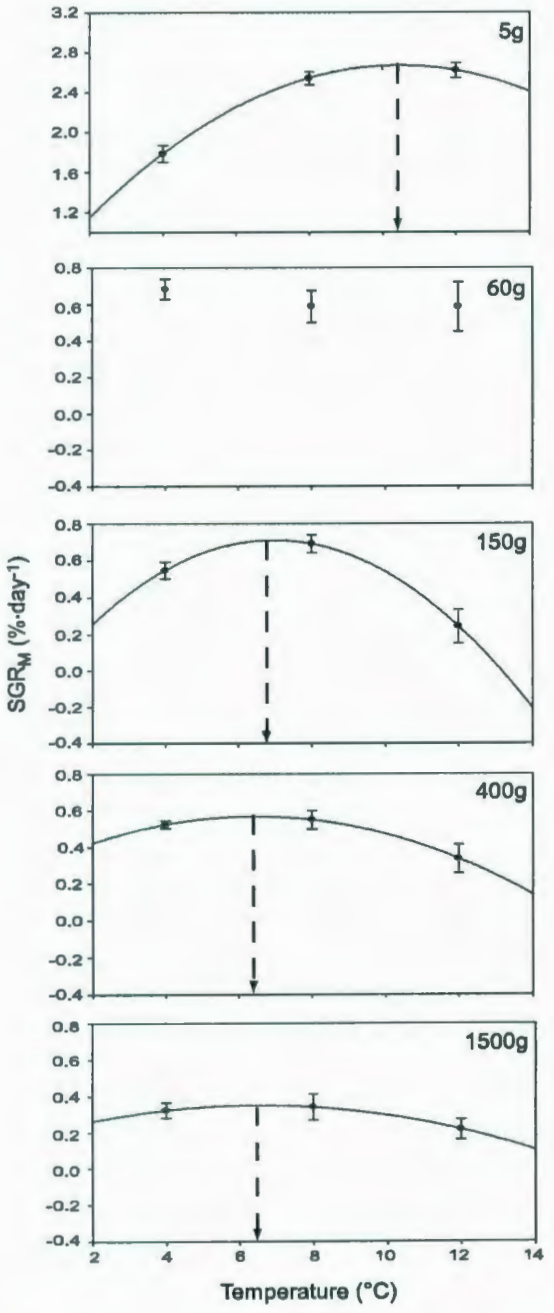

Figure 3.1: Changes in specific growth rate (SGR) with temperature for five different size classes of spotted wolffish. Approximate masses at the end of the trial are shown. The curves were fitted by the least squares second order polynomial: $G = aT^2 + bT + c$ where G is SGR, a, b and c are constants determined by the regression. Values are means ± SD of 10 fish. Arrows indicate optimum temperature for growth (T_{optSGR}) calculated from the regression. Data for fish of 5g are from Chapter 2.

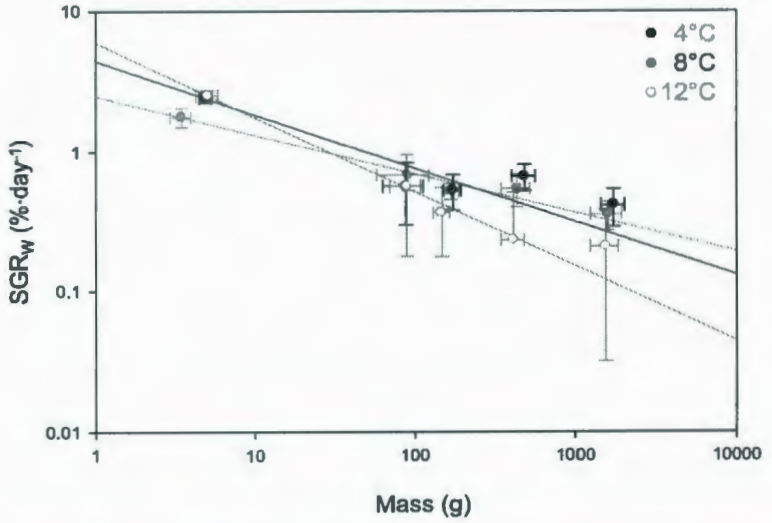

Figure 3.2: Specific growth rate in relation to mean weight in spotted wolffish acclimated at 4, 8 and 12 °C. Each dot represents the mean±SD of mass and SGR of ten fish. The equation for the regression lines were: logSGR = 2.46 X Mass-0.27 r^2= 0.81 (4 °C), logSGR = 4.55 X Mass-0.39 r^2= 0.72 (8 °C) and logSGR = 5.82 X Mass-0.51 r^2= 0.75 (12 °C) and $p<0.001$ in all case.

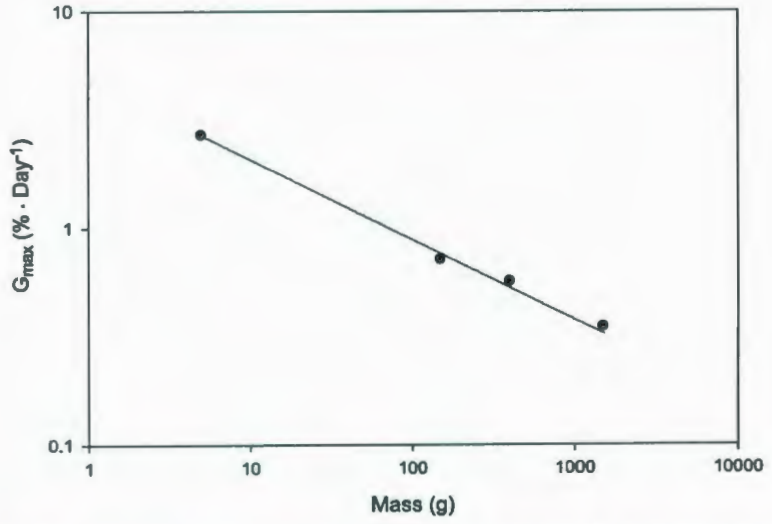

Figure 3.3: Maximum growth rate (*G_max*) in relation to fish mass in spotted wolffish at optimal temperature (T_{optSGR}). Rates at optimal temperatures were determined based on the 2^{nd} order polynomial relationship presented in Fig 3.1.

$r^2 = 0.154$) while no relationship was observed at 4 °C. The activity of the 20S proteasome in liver was higher at 12 °C (ANCOVA $F_{1,55}$=17.41 p<0.001) than 4 °C and slightly increased with mass (mass was log transformed, ANCOVA $F_{1,55}$=6.01 p=0.017). 20S Proteasome activity in ventricle did not change in relationship to either temperature or mass of fish.

3.4.3 Mass adjusted partial correlations and multiple regression

To examine the relation between 20S proteasome activity and SGR, the effect of mass on SGR was removed and a partial correlation matrix of SGR and 20S proteasome activity in ventricle, liver and white muscle was calculated for fish acclimated at 4 °C and 12 °C (Table 3.1). Specific growth rate was negatively correlated with white muscle 20S proteasome activity at 4 °C ($p < 0.002$) but not with any other combination of tissue or acclimation temperature.

Table 3.1: Partial correlation coefficients of 20S proteasome activity and SGR in tissues of spotted wolffish acclimated at 4 and 12 °C. The effect of fish mass was removed.

	Temperature	
Tissue	**4 °C**	**12 °C**
Ventricle	-0.187	-0.088
Liver	-0.001	-0.130
Muscle	-0.609*	-0.202

* p<0.002

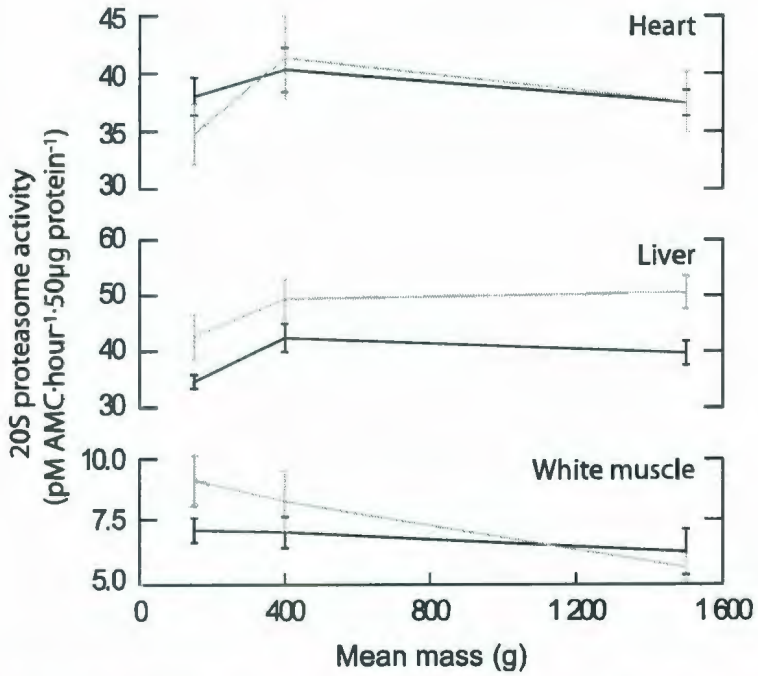

Figure 3.4: The activity of 20S proteasome in heart, liver and white muscle in relation to mean mass in spotted wolffish acclimated at 4 °C and 12 °C (black and gray lines, respectively); N = 8-10. Enzyme activity was measured at 15 °C and converted to acclimation temperature (see text for details).

3.5 Discussion

The primary aim of this study was to assess the relationship between 20S proteasome activity and growth in spotted wolffish. As such, it was necessary to assess if growth was modulated, as expected from previous work with respect to temperature and size. Growth rate was significantly influenced by temperature and fish mass. T_{optSGR} decreased substantially with size in spotted wolffish, from 10.4 °C for 5 g fish to 6.4 °C for fish > 400 g. The SGR and T_{optSGR} measured in the present study are very similar to what was previously reported for this species (Hansen and Falk-Petersen, 2002; Imsland *et al.*, 2006). To my knowledge this is the first report on the T_{optSGR} for fish over 1.5 kg and our results suggest that the value is approximately the same for 400 g fish; however, care should be used in the interpretation of these data as only three temperatures were used. As fish mass increases SGR decreases linearly on a log-log scale and this relation was influenced by temperature as the decrease in SGR was more marked in fish acclimated at 12 °C compared to fish acclimated at 4 and 8 °C. The growth rate at T_{optSGR} (G_{max}) also decreased with increasing fish mass on a log-log scale with a slope of -0.37. Similar relationships have been found for cod (-0.40 to -0.45, Bjornsson *et al.*, 2001; Bjornsson *et al.*, 2007), immature halibut (-0.46, Bjornsson and Tryggvadottir, 1996) and various salmonid species (-0.30 to -0.49, Brett, 1979). These similarities point toward a common functional mechanism for the reduction of G_{max} with increasing fish mass among cold-water fish species.

3.5.1 20S Proteasome activity

The cytosolic protein concentration in the supernatant following the 20,000 *g* centrifugation used to assay 20S proteasome was not significantly different among acclimation groups in all tissues. The chymotrypsin-like activity of 20S proteasome was approximately 7 times higher in liver and ventricle than in white muscle, reflecting the higher protein turnover in these tissues compared to white muscle (Houlihan *et al.*, 1986; Lewis *et al.*, 2007). The relationship between 20S proteasome activity and fish mass was tissue specific. The activity of 20S proteasome increased with body mass in the hepatic tissue, decreased in white muscle only at 12 °C and remained constant in cardiac tissue. 20S Proteasome activity generally decreases with mass and age in mammalian liver tissue (Conconi *et al.*, 1996; Shibatani *et al.*, 1996). However, wolffish shows the opposite as hepatic activity of 20S proteasome increased with fish mass at both temperatures. 20S proteasome activity in liver was not negatively linked to growth rate in the present study as it was shown for rainbow trout (Dobly *et al.*, 2004). The study on trout compared fish selected for high or low growth efficiencies at optimal temperature, whereas our study focused on fish acclimated at different temperatures without monitoring growth efficiency. Accordingly, results from both studies are not directly comparable. In the previous experiment, the activity of the 20S proteasome in white muscle of smaller wolffish (~5 g) was found to be higher in cold acclimated spotted wolffish (Chapter 2) while this pattern was not found in this experiment. Besides, the smaller fish displayed a thermal optimum for growth that was 3 to 4 °C above fish from the present study. These discrepancies between small and large fish could reflect changes of metabolic physiology during ontogeny of wolffish. It

suggests that the relative impact of protein degradation via 20S proteasome on growth rate at low temperature decreases as the fish grow. Nevertheless, the negative relationship observed between 20S proteasome activity and SGR in white muscle suggests that protein degradation via this pathway could also be a key limiting factor for growth of bigger fish (> 150 g) at cold temperature.

Despite having a lower 20S proteasome specific activity than liver and heart, white muscle makes up approximately 50% of fish mass, therefore, muscle contribution to total 20S proteasome activity is substantive. The activity of 20S proteasome was measured at a common temperature for practical reasons. Since it was previously shown that the chymotrypsin-like activity of 20S proteasome was linearly related to temperature (Chapter 2) and the measured activities were corrected accordingly, I deem the presented data to accurately reflect activity at acclimation temperature. Also, since the cytosolic protein concentrations did not differ between acclimation groups, the relationship between growth rate and 20S proteasome is not likely to be an artefact of the analytical procedure.

At cold temperature, the results show that growth rate is to some extent controlled by protein degradation. Accordingly, individuals having a higher 20S proteasome activity have a lower growth rate. The rationale behind this individual variability in the 20S proteasome activity is of course unknown but I can speculate on two aspects: 1) As nascent polypeptides undergo folding events that lead to the native state, there is a possibility that these proteins fold incorrectly or interact with other nascent polypeptides forming protein aggregates (Hochachka and Somero, 2002). This phenomenon could be exacerbated by acclimation to

cold temperature. 2) Proteins are prone to oxidative damage by reactive oxygen species (ROS) (Levine *et al.*, 1990; Shacter, 2000). ROS are for the most part a by-product of the mitochondrial metabolism (Boveris and Chance, 1973). Both misfolded and oxidatively damaged proteins are degraded by the proteasome pathway (Friguet, 2006; Perepechaeva *et al.*, 2006; Poppek and Grune, 2006). A recent study comparing Antarctic and temperate fish species showed that fish living in the permanent cold (Antarctic) have a higher level of ubiquitin-conjugated proteins, an index of misfolded or damaged proteins (Todgham *et al.* 2007). The inter-individual variability of the 20S proteasome activity could reflect the individual's capacity to synthesise and maintain natively folded proteins or variability in mitochondrial oxidative capacity and its associated ROS production.

3.6 Conclusion

The relationships among fish mass, growth rate and the activity of 20S proteasome in different tissues of spotted wolffish acclimated at high and low temperature were examined. The effects of temperature on growth rate of spotted wolffish of different size classes were similar to those previously reported. The relation between the maximum growth rate and fish mass described here was very similar to other fish species, suggesting that the effect of mass on growth rate is not species specific, at least for carnivorous species. In white muscle and at cold temperature, the specific growth rate is negatively associated with 20S proteasome and this suggests that growth at cold temperature is impaired by the proteasome pathway. Further studies are necessary to determine whether this relationship is mediated by individual variation in the capacity to maintain properly folded pro-

teins or variation in mitochondrial capacity and its associated ROS production.

4

Mitochondrial capacity correlates with glutathione reductase and 20S proteasome activities in white muscle of spotted wolffish (*Anarhichas minor*, Olafsen).

4.1 Abstract

This study documents the effects of acclimation to high and low temperature (4 and 12 °C) on mitochondrial and antioxidant capacities in white muscle, heart ventricle and liver of spotted wolffish. Following an acclimation period of 51 days, mitochondrial capacity was measured as the activities of the Complex I of the mitochondrial electron transport system (CPLXI) and citrate synthase (CS). Glutathione disulfide reductase (GR) and catalase (CAT) activities, as well as glutathione concentration were also measured to estimate antioxidant capacities. Following acclimation to 4 °C, mitochondrial capacities were compensated in liver and heart ventricle but not in white muscle. GR activity was also enhanced at cold temperature in the three tissues while CAT activity was increased at the higher acclimation temperature in heart and white muscle. The relationships between mitochondrial and antioxidant enzymes activities, when observed, were

positive (i.e. never negative). Only in white muscle was the activity of 20S proteasome positively related to the activity of complex I ($r^2 = 0.450$) and to CS ($r^2 = 0.411$) activities. Also, only in white muscle was a positive relationship observed between 20S proteasome and CAT activity and GSH concentration (only at 12 °C). These results suggest a connection between mitochondrial capacity and protein degradation by the 20S proteasome, but whether or not this link is mediated by the necessity to degrade protein oxidatively damaged by mitochondrial ROS production remains an open question.

4.2 Introduction

Many fish species exhibit positive thermal compensation at low temperature, a consequence of which is an increased capacity to consume oxygen and perform energetically demanding activities such as growth and swimming than would otherwise occur. Acclimation at cold temperature generally involves adjustments of membrane composition, increases in mitochondrial volume density (the number and/or size of mitochondria per cells) and/or an increase in mitochondrial cristæ density (Guderley, 2004b). All these modifications generally result in enhanced mitochondrial activity per gram of tissue at low temperature. However, there are potential negative consequences of enhanced mitochondrial volume/activity that must be handled. Mitochondria are the major site for reactive oxygen species (ROS) generation (Boveris and Chance, 1973). Consequently, given the higher solubility of oxygen in cold water and the higher proportion of unsaturated fatty acids in membranes, the drawback of increasing the mitochondrial capacity is the associated risk of raising ROS production and oxidative damages. ROS may dam-

age all types of biological molecules but because of their high relative proportion in tissue, proteins are a major target for oxidation (Levine *et al.*, 1990; Shacter, 2000; Dalle-Donne *et al.*, 2003). Some oxidation products can be repaired within protein by specific enzymes (Holmgren *et al.*, 2005) but the bulk of oxidized proteins has to be degraded by the proteasome pathway (Coux *et al.*, 1996; Friguet, 2006) and replaced by synthesis, increasing protein turnover at high metabolic cost (Fraser and Rogers, 2007). In the proteasome pathway, short-lived, misfolded and oxidized proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein. Ubiquitinated proteins are then recognized and degraded by the proteasome (Glickman and Ciechanover, 2002). The activity of the 20S proteasome in white muscle of spotted wolffish (*Anarhichas minor*) acclimated to cold temperature (4 °C) is associated with reduced specific growth rate (SGR) (chapter 2 and 3). Evidence points toward the possibility that high activities of 20S proteasome at 4 °C could be linked to an increased ROS production as the concentration of reduced glutathione (GSH) was also increased in cold acclimated fish (chapter 2).

The objective of the present study was to determine if there is a compensation of mitochondrial capacities during acclimation to high and low temperatures in wolffish, and if so, if it is associated with a concomitant adjustment of antioxidant enzymes and 20S proteasome activity. Following an acclimation period to cold and warm temperatures (4 and 12 °C, respectively), mitochondrial capacity, 20S proteasome activity and antioxidant defences were examined in white muscle, heart ventricle and liver of spotted wolffish. Mitochondrial capacity was assessed as the activity of citrate synthase (CS; EC 4.1.3.7) and complex I of the elec-

tron transport system (NADH:ubiquinone oxidoreductase, CPLXI; EC 1.6.5.3). Antioxidant defences were measured as the concentration of GSH, as well as activities of glutathione-disulfide reductase (GR; EC 1.8.1.7) and catalase (CAT; EC 1.11.1.6). The concentration of thiobarbituric reactive substances (TBARS) was used as an index of lipid peroxidation by the ROS. Finally, the link among mitochondrial capacity, antioxidant capacity and 20S proteasome activity was examined.

4.3 Materials and methods

4.3.1 Fish and sampling

Spotted wolffish (*Anarhichas minor*, Olafsen) were selected from a population of fish held at 8 °C and fed to satiety according to established protocols. Thereafter, fish were acclimated to either 4 °C or 12 °C for a period of 51 days. Fish of three size classes with initial mass of 125±17 g, 339±69 g and 1361±286 g were utilized. Details of animal husbandry are presented in chapter 3. Fish were anesthetised using benzocaine (50 $mg \cdot l^{-1}$) and samples of liver, heart and white muscle were immediately frozen on dry ice and transported to the laboratories in Rimouski (QC, Canada) to be stored at -80 °C until further analysis. The white muscle sample was dissected from the deep dorsolateral region and care was used not to include red muscle. For liver, the distant part of the bigger lobe was sampled and care was used to avoid contamination from bile. The heart was flushed with homogenization buffer (see below) prior to freezing to remove the blood and only the ventricle was used. All animal protocols were in accordance with the

recommendations of the Canadian Council of Animal Care and approved by the Animal Care Committee of the Université du Québec à Rimouski.

4.3.2 Tissue extraction

The three tissues were homogenized 3 X 10 seconds in 10 volumes of ice-cold homogenization buffer using a Heidolph Diax 900 (Heidolph Instruments, Germany). The homogenization buffer was a 10 mM phosphate buffer containing 137 mM of NaCl and 2.7 mM of KCl (pH 7.4). The homogenate was centrifuged at 5,000 *g* at 4 °C for 10 min (Thermo IEC Micromax RF benchtop centrifuge). Enzyme activities were immediately measured in the supernatant while aliquots were stored at -80 °C for the subsequent determination of protein content, TBARS and GSH. 20S proteasome activity was measured in a separate sample as it necessitates the use of a different homogenization procedure. The activity of 20S proteasome was measured as described in chapter 3 but the results were not adjusted to acclimation temperature using the Q_{10} as all enzymes activities compared here were measured at 15 °C.

4.3.3 Mitochondrial and antioxidant enzyme activities

CPLXI , CS, glutathione disulfide reductase (GR) and catalase (CAT) activities were measured using uv/vis spectrophotometers (Lambda 11, PerkinElmer, Woodbridge, Canada for CPLXI and CS; Ultrospec 3100 Pro, Biochrom, Cambridge, MA, USA for GR and CAT) both equipped with a circulating refrigerated water bath. All enzyme activities were assayed at 15 °C and the linearity of each method was ascertained in preliminary experiments (not shown). Mitochondrial

and antioxidant enzymes were assayed on 8 fish per size group per temperature.

The complex I is located in the inner mitochondrial membrane and catalyzes the transfer of electrons from NADH to coenzyme Q; it is the first step of the mitochondrial electron transport system. The activity of CPLXI was assayed at 600 nm in an incubation volume of 1.0 ml containing 25 mM potassium phosphate, 3.5 $g \cdot l^{-1}$ bovine serum albumin (BSA), 60 μM 2,6-dichloroindophenol (DCIP), 70 μM decylubiquinone, 1.0 μM antimycine-A, and 0.2 mM NADH, pH 7.8. After preincubating 20 μL of homogenate in a 960 μL reaction mixture without NADH for 3 min, 20 μl of 10 mM NADH was added and the change in absorbance at 340 nm recorded for 4 min. After 4 min, 1 μl rotenone (1 mM in dimethyl sulfoxide) was added and the absorbance was measured again for 4 min. The rotenone sensitive activity was considered to be the activity of CPLXI. The activity was expressed as U·mg $protein^{-1}$, in which 1 U of CPLXI activity equals 1 μmol DCIP reduced per min (extinction coefficient = 21.0 $mM^{-1} \cdot cm^{-1}$) (Janssen *et al.*, 2007).

Citrate synthase is the first step of the citric acid cycle; it catalyzes the condensation reaction of the two-carbon acetate residues from acetyl coenzyme A and oxaloacetate to form the six-carbon citrate. The activity was assayed in a reaction mixture containing 100 mM imidazole, 0.3 mM 5,5′dithiobis-2-nitrobenzoic acid (DTNB) and 0.3 mM acetyl-CoA, pH 8.0. The reaction was initiated by the addition of 40 μl of the tissue extract and 100 μl of oxaloacetic acid (0.1 mM final concentration). Absorbance at 412 nm was recorded over 4 min. One activity unit equals the formation of 1 μmol 5-thio-2-nitrobenzoic acid per min (DTNB extinction coefficient = 13.6 $mM^{-1} \cdot cm^{-1}$)(Srere, 1969).

Glutathione reductase is a flavoprotein catalyzing the NADPH-dependent reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH). The GR assay mixture consisted of 10 μM NADPH, 1 mM GSSG, 2 mM EDTA and 1.3 $g \cdot l^{-1}$ BSA in potassium phosphate buffer (0.2M pH 7.0). The assay volume was 500 μL and the decrease in absorbance at 340 nm was recorded for 4 min. A unit of GR activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH per minute (NADPH extinction coefficient = 6.22 $mM^{-1} \cdot cm^{-1}$; modified from Carlberg and Mannervik (1985).

Catalase catalyses the decomposition of hydrogen peroxide into molecular oxygen and water without the production of free radicals. The catalase assay mixture consisted of 50 mM potassium phosphate buffer containing 50 mM H_2O_2 (pH 7.0). The disappearance of hydrogen peroxide was directly followed at 240 nm for 1 min using extinction coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ (Chance and Maehly, 1955; Livingstone *et al.*, 1992). One activity unit was equal to 1 μmol of H_2O_2 decomposed per min.

4.3.4 TBARS and GSH

Free radical damage to lipids results in the generation of malonedialdehyde (MDA). The thiobarbituric acid reactive substances (TBARS), which are mainly MDA, were used as an index of lipid peroxidation in tissue samples. The assay was performed using a commercially available kit (Zeptometrix, Buffalo, NY, USA). The kit was used as recommended by the manufacturer except that the assay

was downscaled to be performed in microtubes; 20 μl of sample was added to 20 μl of the supplied SDS solution followed by 500 μl of the TBA reagent. The tubes were incubated at 95 °C for 60 min along with a standard curve for MDA (0 to 4 nmol·ml^{-1}). After cooling, the tubes were centrifuged at 10,000 g for 5 min; the fluorescence of the supernatant at excitation/emission wavelengths of 530/550 was recorded using a Hitachi F-2500 spectrofluorometer (Hitachi High Technologies, San Jose, CA, USA) and the MDA equivalent concentration extrapolated from the standard curve. The results are presented as the average of three replicates and expressed in nmol·g tissue^{-1}.

GSH is the principal intracellular low molecular weight thiol and plays a critical role in the cellular defence against agents that impose oxidative stress. The GSH concentration was measured according to Kamencic *et al.* (2000). Reduced glutathione is combined with monochlorobimane (mCB) by the enzyme glutathione-S-transferase (GST) to form a fluorescent GSH-mCB adduct that can be quantified by spectrofluorometry. The homogenate was incubated in triplicate for 30 min at room temperature with mCB and GST at a final concentration of 100 μM and 1 U·ml^{-1}, respectively. After centrifugation (5,000 g, 5 min) the fluorescence of the supernatant was recorded (excitation/emission wavelengths of 380/470 nm) and the GSH concentration was extrapolated from a standard curve (0-100 μM). To quantify the concentration of oxidized glutathione GSSG, one diluted aliquot from each sample was completely reduced by the addition of 15 μl of 4 M triethanolamine (TEA) to determine the total amount of GSH. To calculate GSSG, the amount of reduced GSH was subtracted from the TEA-treated sample and divided by two to account for the dimerization (Chapman *et al.*, 2005).

4.3.5 Statistical analysis

Analyses of covariance (ANCOVA) were used to examine the effects of fish mass and acclimation temperature on enzyme activities, TBARS and GSH concentration. The relationships between enzymes activities were examined (ANCOVA) using fish mass as a covariate and temperature as a factor. When it was necessary, fish mass was log transformed to fulfill the assumptions of residuals normality and independence with predictors. SYSTAT 11 (SYSTAT Software Inc.) was used to perform all statistical analyses.

4.4 Results

The growth results of the three size classes at 4 and 12 °C are presented in Chapter 3.

4.4.1 Enzyme activities, GSH and TBARS levels

No effect of fish mass on mitochondrial and antioxidant enzyme activities, GSH or TBARS concentrations was observed at the two temperatures. Acclimation to low temperature (4 °C) led to a significant compensation of CPLXI activity in ventricle ($F_{1,42}$ = 34.22 p<0.001) and liver ($F_{1,43}$ = 6.85 p=0.012) but not in white muscle (Table 4.1). In liver, there was a significant compensation of CS activity ($F_{1,43}$ = 29.48 p<0.001) that was not observed in heart or white muscle. In ventricle and white muscle, the ratio of CPLXI to CS activity (ratio CPLXI/CS) ratio was significantly higher in fish acclimated at low temperature (ventricle $F_{1,41}$ = 21.64 p< 0.001; white muscle $F_{1,45}$ = 9.54 p = 0.003) while no

effect of temperature was detected in liver.

The GR activity was higher ($F_{1,43} = 4.72$ $p = 0.035$), and CAT activity was lower ($F_{1,43} = 20.57$ $p<0.001$) in ventricle of fish acclimated at 4 °C (Table 4.2). The same observations were made in white muscle ($F_{1,48} = 5.07$ $p = 0.029$ and $F_{1,48} = 4.66$ $p = 0.036$, respectively). In liver, GR activity was also higher at low temperature ($F_{1,42} = 44.73$ $p<0.001$) but there was no effect of acclimation temperature on CAT activity.

Tissue levels of GSH and TBARS are presented in Table 4.3. There were no significant effects of temperature on GSH concentration in ventricle, liver and white muscle but there was a trend in white muscle for increased GSH concentration at 4 °C ($F_{1,53} = 3.92$ $p = 0.053$). The concentration of TBARS in the liver, ventricle and white muscle was unchanged following acclimation to high or low temperature. The reduction of the sample with TEA did not result in the detection of measurable levels of GSSG.

4.4.2 Relationships between mitochondrial capacity and antioxidant defences

ANCOVAs using fish mass as a covariate and temperature as a factor were used for the analyses of the relations between enzyme activities (or GSH concentration). In heart ventricle, no relationships were observed between GR activity and mitochondrial enzymes Complex I and CS (Fig 4.1a and b). There was no

Table 4.1: Activities of the mitochondrial enzymes citrate synthase (CS) and complex I, and the ratio of complex I vs. CS activity (CPLXI/CS) in heart ventricle, liver and white muscle of spotted wolffish acclimated at 4 and 12°C. The data represent the average of the three size classes combined but the statistical analyses included fish mass as a covariate.

	Temperature	
Enzyme	**4°C**	**12°C**
(a) ***Ventricle***		
Complex I	21.66 ± 5.68	12.64 ± 3.17*
CS	71.94 ± 10.08	68.91 ± 13.57
Ratio CPLXI/CS	0.30 ± 0.08	0.19 ± 0.06*
(b) ***Liver***		
Complex I	9.81 ± 4.69	6.67 ± 2.43*
CS	9.15 ± 1.49	6.69 ± 1.30*
Ratio CPLXI/CS	1.06 ± 0.47	1.06 ± 0.48
(c) ***Muscle***		
Complex I	1.98 ± 1.01	1.72 ± 0.87
CS	17.76 ± 8.81	22.14 ± 14.03
Ratio CPLXI/CS	0.12 ± 0.05	0.08 ± 0.03*

Enzyme activities are expressed in mean ± SD U·g $prot^{-1}$; N =25-30

*Significant difference between temperature groups (ANCOVA). The F-ratio and P values are provided in the text.

Table 4.2: Activities of antioxidant enzymes catalase (CAT) and glutathione reductase (GR) in heart ventricle, liver and white muscle of spotted wolffish acclimated at 4 and 12 °C. The data represent the average of the three size classes combined but the statistical analyses included fish mass as a covariate.

	Temperature	
Enzyme	**4°C**	**12°C**
(a) ***Ventricle***		
CAT	56.66 ± 16.38	86.55 ± 21.77*
GR	46.47 ± 6.83	40.23 ± 10.3*
(b) ***Liver***		
CAT	113 ± 35	123 ± 42
GR	20.4 ± 1.9	15.4 ± 3.2*
(c) ***Muscle***		
CAT	63.62 ± 27.49	90.83 ± 56.77*
GR	1.81 ± 0.52	1.48 ± 0.41*

Enzyme activities are mean ± SD expressed in U·g $prot^{-1}$ N = 25-30
*Significant difference between temperature groups (ANCOVA). The F-ratio and P values are provided in the text

Table 4.3: Concentration of reduced glutathione (GSH) and TBARS in ventricle, liver and white muscle of spotted wolffish acclimated at 4 and 12°C. The data represent the average of the three size classes combined but the statistical analyses included fish mass as a covariate.

	Temperature	
Enzyme	**4°C**	**12°C**
(a) ***Ventricle***		
GSH	9.37 ± 1.23	9.65 ± 2.02
TBARS	37.14 ± 8.31	39.49 ± 8.47
(b) Liver		
GSH	12.50 ± 2.18	12.98 ± 2.46
TBARS	38.81 ± 7.65	36.74 ± 8.73
(c) Muscle		
GSH	7.01 ± 1.52	6.20 ± 1.58
TBARS	12.79 ± 3.45	12.33 ± 4.62

Concentration in nmoles · mg prot^{-1}; Values are mean ± SD; n = 25-30

*Significant difference between temperature groups (ANCOVA). The F-ratio and P values are provided in the text.

relationship between GR and Complex I activities in the liver. At 12 °C, there was a highly significant positive relationship between CS and GR activities (r^2 = 0.657, $p < 0.001$) which was not observed at 4 °C (Fig 4.1d). In the white muscle, the activity of GR was positively related to Complex I activity and this relation was independent of weight and temperature (r^2 = 0.11, p = 0.011; Fig 4.1e). A tenuous relationship between GR and CS activities was observed at 12 °C (r^2 = 0.195; p = 0.031; Fig 4.1f), but not at 4 °C.

In heart, CAT did not display significant relationships with Complex I or CS (Fig 4.2a and b). In liver, a significant positive regression was found between CAT and CS activities. The slopes of the regressions were not different between the two temperatures but the elevation was higher at 12 °C (r^2 = 0.165 p = 0.017 and r^2 = 0.34, p = 0.014 for 4 and 12 °C, respectively; Fig 4.2d). In white muscle, there was a significant relationship between CAT and Complex I only at 12 °C (r^2 = 0.329, p = 0.002; Fig 4.2e).

A significant relationship between CS and GSH was observed in heart ventricle. This relationship was independent of mass or temperature (r^2 = 0.275, p = 0.002; Fig 4.3b). No relationships were observed between GSH and Complex I or CS in liver. Finally, in white muscle there was a positive relationship between Complex I activity and GSH concentration at 12 °C only (r^2 = 0.244, p = 0.004; Fig 4.3e).

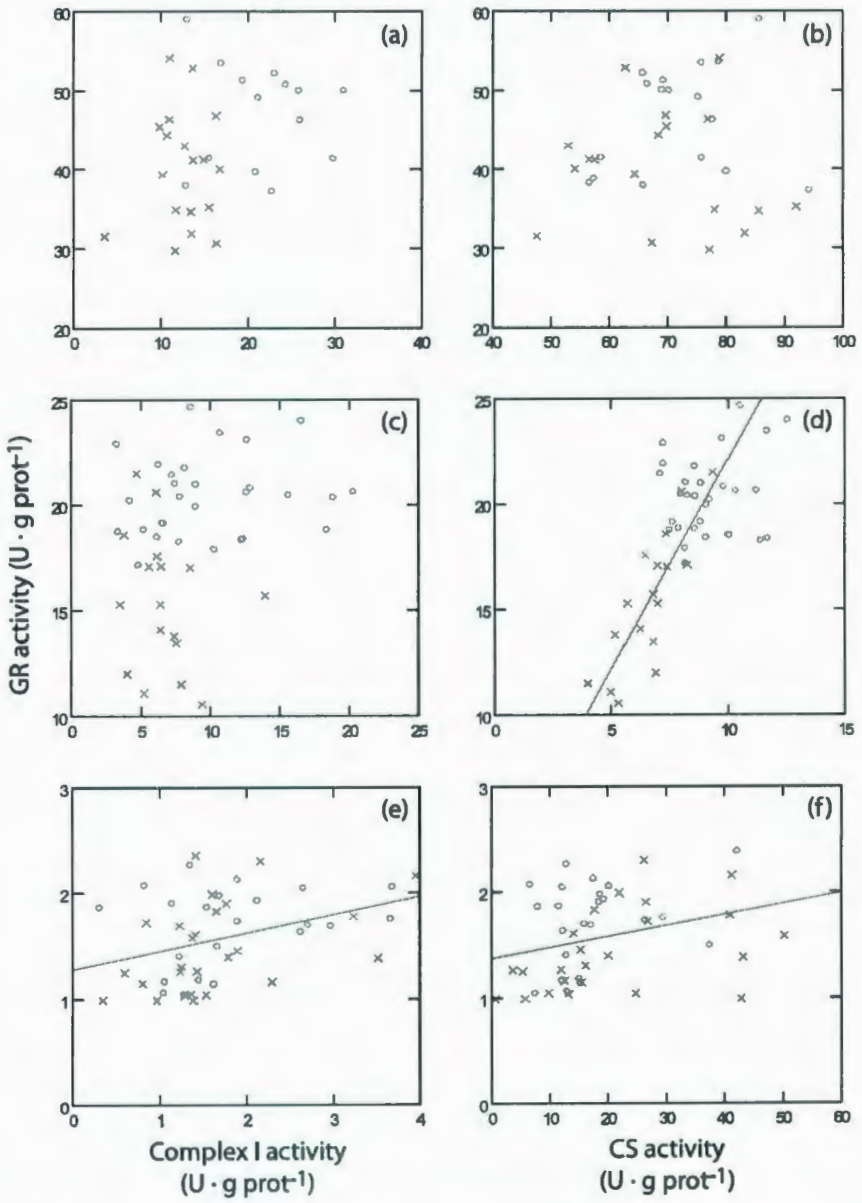

Figure 4.1: Activity of glutathione reductase (GR) in relation to Complex I and CS activities in heart ventricle (a and b), liver (c and d) and white muscle (e and f) of spotted wolffish acclimated at 4 and 12 °C (o and ×, respectively). The regression lines in (d) and (f) are for 12 °C only (see text for details) while it is for both temperatures in (e).

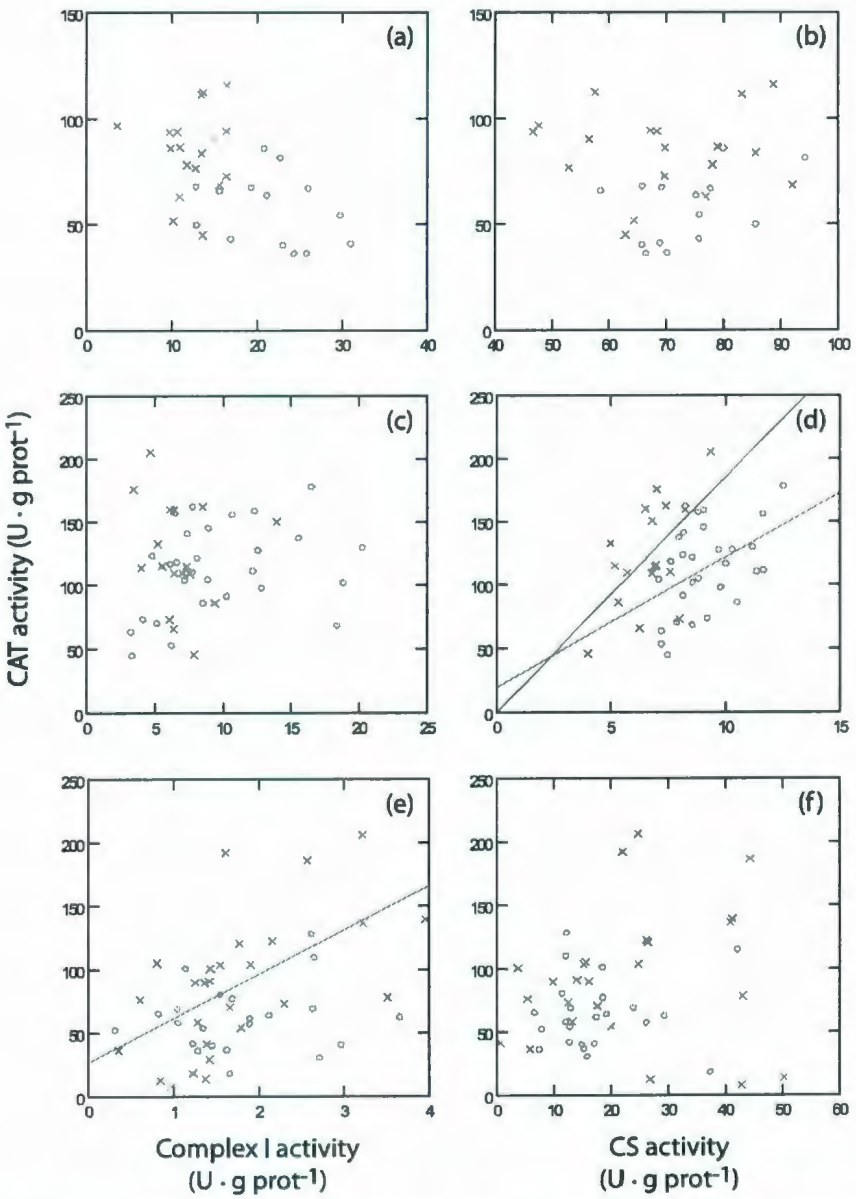

Figure 4.2: Activity of catalase (CAT) in relation to Complex I and CS activities in heart ventricle (a and b), liver (c and d) and white muscle (e and f) of spotted wolffish acclimated at 4 and 12 °C (o and ×, respectively). The regression line in (e) is for 12 °C only (see text for details).

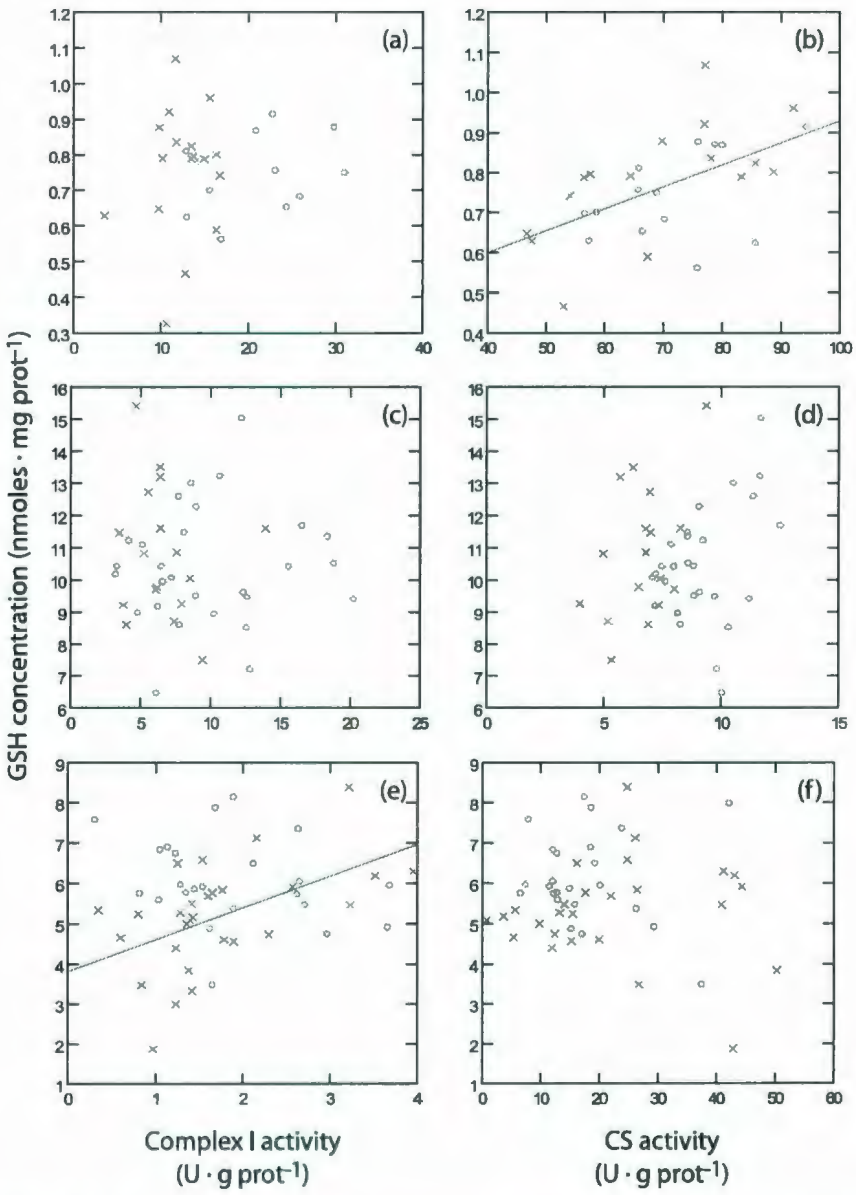

Figure 4.3: Reduced glutathione concentration (GSH) in relation to Complex I and CS activities in heart ventricle (a and b), liver (c and d) and white muscle (e and f) of spotted wolffish acclimated at 4 and 12 °C (o and ×, respectively). The regression line in (b) is for both temperatures while in (e) is for 12 °C only (see text for details).

4.4.3 20S proteasome activity in relation to mitochondrial and antioxidant enzyme activities

4.4.3.1 White muscle

20S proteasome activity for the three size classes of wolffish at two acclimation temperatures is presented in chapter 3 (Fig. 3.4). In white muscle, a multiple regression described the activity of 20S proteasome using complex I activity and fish mass (log). The relationship was independent of acclimation temperature and took the form of: 20S proteasome = 1.634 × Complex I – 2.003 × (log)mass + 10.382 ($r^2 = 0.450$, $p_{(ComplexI)} < 0.001$ where $p_{(ComplexI)}$ is the p value of the t test for Complex I as a predictor). The relationship between Complex I and 20S proteasome activity is shown in Fig 4.4a; it is, however, important to note that the effect of mass is not represented in that figure. A similar relationship was also detected at 4 °C between CS and 20S proteasome activities with the equation 20S proteasome = 0.102 × CS – 2.371 × (log)mass + 13.104 ($r^2 = 0.411$, $p_{(CS)}$) < 0.043, where $p_{(CS)}$ is the p value of the t test for CS as a predictor; Fig 4.4b). No relationships were observed between GR and 20S proteasome activities. At 12 °C there was a positive relationship between GSH concentration, fish mass and 20S proteasome activity; 20S proteasome = 0.983 × GSH – 2.976 × (log)mass + 10.159 ($r^2 = 0.466$, $p_{(GSH)} = 0.005$, where $p_{(GSH)}$ is the p value of the t test for GSH as a predictor; Fig. 4.4d). A positive relationship that was independent of mass was observed between 20S proteasome and CAT activities ($r^2 = 0.386$, $p < 0.001$; Fig 4.4e) at 12 °C.

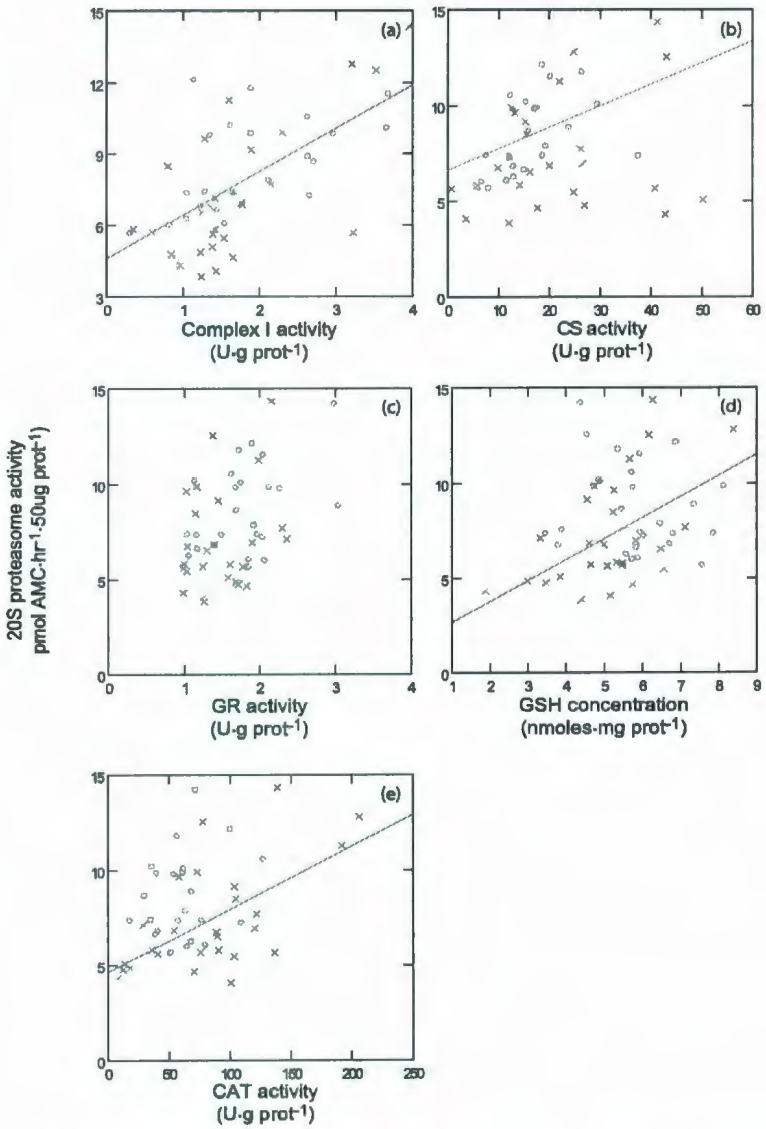

Figure 4.4: Activity of 20S proteasome in relation to Complex I activity (a), CS activity (b), GR activity(c), GSH concentration (d) and CAT activity (e) in white muscle of spotted wolffish acclimated at 4 or 12 °C (o and ×, respectively). Regression line in (a) is for 4 and 12 °C, only for 4 °C in (b) and 12 °C in (d) and (e) Details on the relationships are provided in the text.

4.4.3.2 Liver and heart ventricle

Relationships between 20S proteasome activity and antioxidant or mitochondrial enzymes were not detected in liver and heart. In liver, a negative relationship was observed among 20S proteasome activity, GSH concentration and weight. There was no significant effect of temperature and the equation of the regression was: 20S proteasome = -2.677 × GSH – 7.257 × (log)mass + 61.381 (r^2 = 0.403, p(GSH) < 0.001, where $p_{(GSH)}$ is the p value of the t test for GSH as a predictor; Fig. 4.5). In heart no relationships were detected between 20S proteasome and GSH level.

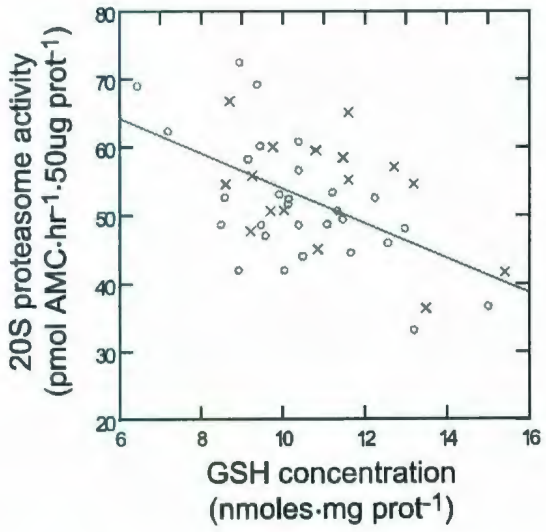

Figure 4.5: Activity of proteasome 20S in relation to GSH concentration in liver of spotted wolffish acclimated at 4 or 12 °C (o and ×, respectively). Details on the relationship are provided in the text.

4.5 Discussion

The present study investigated whether acclimation to low and high temperature in spotted wolffish led to compensation of mitochondrial oxidative capacity in different tissues and, if so, if there was a parallel increase in antioxidant enzyme and/or 20S proteasome activities.

4.5.1 Mitochondrial enzymes

Acclimation to cold temperature resulted in a compensation of mitochondrial capacity in heart ventricle and liver but not in white muscle. There is a difference in the way hepatic and cardiac muscles mitochondrial activity responded to temperature. In liver, there is an elevation of both CPLXI and CS activities without significant changes in the CPLXI/CS ratio. In the ventricle, acclimation to low temperature almost doubles (1.7 times) the activity of CPLXI while CS activity remains stable. This results in a significantly higher CPLXI/CS ratio in the cold acclimated wolffish. Although neither of the individual activities of CS or CPLX1 changed significantly in skeletal muscle, the CPLXI/CS ratio is significantly increased following cold acclimation. Such a change in the ratio of these two enzymes, as seen in muscle and heart, could be indicative of an ultrastructural change (i.e. increase in cristæ density relative to mitochondrial volume) in cold acclimated mitochondria as it was previously observed by St-Pierre et al. (1998). However, the links between enzyme activities and ultrastructure are tenuous and for now only speculative. Positive compensation of mitochondrial enzyme activities is, generally but not always, observed following cold acclimation in fish muscles (Blier and Guderley, 1988; Guderley and Gawlicka, 1992 and

Thibault et al., 1997; reviewed in Guderley, 1990). The reasons for these inconsistencies are not well understood but it could reflect the condition and strain of fish, the analytical methods (St-Pierre et al., 1998) or whether the studies focus on seasonal or artificial acclimation. The benthic and sedentary nature of wolffish could also explain the lack of compensation in white muscle. It suggests that there is no requirement for compensation of aerobic capacities in the white muscle of this species in this temperature range as is observed in heart and liver, two highly aerobic tissues.

4.5.2 Antioxidant systems

Glutathione is one of the primary low molecular weight antioxidants in the cell and forms the front-line defence against ROS. Reduced glutathione is oxidized to glutathione disulfide (GSSG) in the presence of ROS. This reaction can be either spontaneous or catalyzed by glutathione peroxidase (Meister and Anderson, 1983). The maintenance of a high GSH/GSSG ratio is of vital importance for the cell to maintain a proper redox state. The high GSH/GSSG ratio is maintained by enzymatic reduction of GSSG to GSH by the action of GR (Carlberg and Mannervik, 1985) and/or de novo synthesis of GSH. In the present study, acclimation to low temperature is not accompanied by a significant increase of GSH content in any tissue. This contrasts with the result presented in chapter 2 and might be an indication that oxidation status of smaller fish is more sensitive to cold temperature than bigger fish. In a study on the effects of temperature on GSH metabolism of killifish (*Fundulus heteroclitus*), Leggatt *et al.* (2007) observed GSH concentrations very similar to those noted in the present study.

These authors reported GSSG concentrations that are less than 5 % of the total GSH pool. Here, the lack of detection of GSSG similarly implied very low levels of GSSG in wolffish tissues.

In the three studied tissues, the activity GR is higher (only marginally in white muscle and heart) following acclimation at cold temperature. These data contrast with previous studies on killifish where Leggatt *et al.* (2007) did not observe compensation of liver GR in response to temperature when measured at a common temperature. This divergence with Leggatt *et al.* (2007) could reflect differences between antioxidant strategies of eurythermal (i.e. *F. heteroclitus*) and stenothermal (*A. minor*) species. In an earlier study comparing the antioxidant enzymes activities in related Arctic and temperate fish species, Speers-Roesch and Ballantyne (2005) observed similar GR activities in the liver of fish inhabiting the two thermal habitats. The Speers-Roesch and Ballantyne (2005) study involved interspecific comparison as opposed to my intraspecific study.

In heart ventricle and white muscle, an increase of CAT is observed at high acclimation temperature while it remains elevated at both temperatures in liver. Overall, the response of CAT activity to acclimation temperature is the opposite of that of GR. In goldfish catalase is also shown to inversely compensate during temperature acclimation, i.e. its activity increases with acclimation temperature (Hazel and Prosser, 1970; Sidell *et al.*, 1973). This relationship of catalase with temperature might be the result of the primary association of catalase with peroxisomes (de Duve, 1996). In winter acclimated rainbow trout, mitochondrial oxidation of palmitoyl carnitine and palmitoyl carnitine transferase activity (CPT)

are compensated compared to summer acclimated trout (St-Pierre et al., 1998). This suggests that at cold temperature, β-oxidation of fatty acids preferentially occurs in mitochondria, hence the reduction of peroxisomal activity (i.e. catalase activity) in the white muscle and ventricle of cold acclimated spotted wolffish. Conversely, the generally observed higher catalase activity in fish acclimated at warmer temperature could be associated with an increase of long chain fatty acid β-oxidation in the peroxisomes.

Acclimation to low temperature did not alter the concentration of TBARS. TBARS are shown to be responsive to conditions leading to oxidative stress such as an acute exposure to high and low temperature in the North Sea eelpout (Heise *et al.*, 2006a; Heise *et al.*, 2006b). North Sea eelpout sampled during winter presented higher TBARS concentration than summer fish (Heise *et al.*, 2007). However, in a second study on the same species, TBARS were higher during summer (Almroth *et al.*, 2005). TBARS concentration was also shown to be higher during summer in the skeletal muscle of Atlantic salmon (*Salmo salar*) (Nordgarden *et al.*, 2003). This indicator of lipid peroxidation is probably more useful in the evaluation of the effects of an acute stressor on lipid peroxidation and the redox state of cells than it is in mid- to long-term acclimation studies, as suggested by the equivocal results obtained in seasonal and temperature acclimation studies. Nevertheless, the absence of a significant difference of TBARS concentration in wolffish acclimated at 4 and 12 °C may be a result of sufficient antioxidant systems at both temperatures.

4.5.3 Relationships between mitochondrial capacity and antioxidant defences

Contrary to expectations, the relationships between antioxidant and mitochondrial enzymes are far from evident. Antioxidant enzymes, in white muscle often show a positive relationship with mitochondrial enzymes while in heart (in which mitochondrial capacity is almost ten times higher) no relationships are detected. In heart, the only positive relationship detected occurs between CS activity and GSH concentration. This suggests that antioxidant strategies may vary between tissues. A positive relationship was also found between CS activity and superoxide dismutase and glutathione peroxidase (two other antioxidant enzymes) in the tissues of various bathy- and mesopelagic fish species (Janssens *et al.* 2000). Leary *et al.* (2003) also found a positive relation between CCO and various antioxidant enzymes in red and white muscle and heart of rainbow trout; however, these authors compared antioxidant and mitochondrial enzymes activities between different fibre types instead of within, as in the present study. On the other hand, the finding by Speers-Roesch and Ballantyne (2005) of a negative relationship between CCO and SOD, CAT and to a lesser extent GR highlights the necessity of further studies to elucidate the relationships between aerobic and antioxidant capacities.

4.5.4 20S proteasome activity in relation to mitochondrial and antioxidant enzymes activities

Again, the relationship between 20S proteasome and mitochondrial enzymes are not consistent in the three tissues studied. The only relationships found are in the

white muscle (Fig. 4.4a,b) while no relationships are observed in liver and heart. In white muscle there is a strong association between the capacity for protein degradation and aerobic metabolism. It is surprising that the only relationship is found in the tissue having the lowest oxidative capacity (in terms of Complex I activity). In white muscle, 20S proteasome also displays positive relationship with antioxidant enzyme CAT and GSH concentrations (only at 12 °C). In contrast, a negative relationship is observed with GSH concentration in the liver while no relationships are observed with antioxidant enzymes in liver and heart. There is no obvious explanation for this discrepancy. This is, to my knowledge the first attempt to link 20S proteasome activity with mitochondrial and antioxidant enzymes activities. The data suggest that the protein degradation in white muscle is more susceptible to inter-individual variation of mitochondrial metabolism than heart and liver. Mitochondrial ROS production is thought to be significant only when respiration is inhibited by high membrane potential, which is observed during state IV conditions (Korshunov *et al.*, 1997). This situation may occur in white muscle more than in heart or liver, especially in a relatively inactive species like the wolffish. The relationship observed between antioxidant enzymes and 20S proteasome activities also suggest a link between mitochondrial ROS production but for now, this link should be regarded as mostly speculative.

4.6 Conclusion

The aim of the present study was to examine the effects of acclimation to cold and warm temperature on mitochondrial oxidative capacity, antioxidant defences and 20S proteasome activity in spotted wolffish. Compensation of mitochondrial ca-

pacity following acclimation to cold temperature was observed in heart and liver but not in the white muscle. There is no consensus in the data concerning the relationships between mitochondrial and antioxidant enzymes activities in the tissues studied unless that they tend to be positively related. Acclimation temperature had a very marginal effect on these relationships, if any. In no case was a negative relationship between activities of antioxidant and mitochondrial enzymes observed. Accordingly, it can be concluded that mitochondrial compensation following acclimation to low temperature is not always accompanied by compensations of antioxidant enzymes as different tissues respond differently to the same treatment. Only two antioxidant systems were studied (CAT and glutathione system) and the inconsistencies observed within the three tissues highlight the need for further studies, including a wider variety of antioxidant systems and tissues. The relationships between 20S proteasome and mitochondrial enzyme activities suggest a tight connection between aerobic capacity and the need for protein degradation, at least in white muscle. Whether or not this connection is mediated by the need to degrade proteins that are oxidatively damaged by the mitochondrial ROS production remains an open question. Alternatively, increased mitochondrial capacity could be a response to sustain the costs of replacing the proteins degraded by the proteasome.

5

Conclusion and perspectives

Temperature has profound effects on physiological processes in ectothermic vertebrates. Protein metabolism is no exception; the effect of temperature has mostly been studied with respect to protein synthesis. Temperature generally has a parabolic effect on protein synthesis with a maximum rate being observed at optimal growth temperature. Results of protein synthesis from the first experiment with juvenile (~5 g) spotted wolffish are in accordance with this general view. The rate of protein synthesis was lower at 4 °C than at 8 °C while it was intermediate at 12 °C. The effect of temperature on protein degradation was and still is poorly understood. 20S proteasome activity is mainly responsible for the degradation of short-lived and oxidatively modified proteins and has been recently identified as a potentially good proxy for protein degradation in fish. Again, the results of the first experiment are in accordance with this concept as, despite the decrease of protein synthesis at low temperature, the activity of 20S proteasome activity was maintained high in fish acclimated at lower temperature (4 °C) and correlated negatively with specific growth rate. To understand if this elevated activity of

20S proteasome was a response to an increased level of protein oxidation at cold temperature, the oxidative stress markers TBARS and carbonyl-protein content were measured, along with the concentration of the antioxidant GSH. TBARS and carbonyl-protein concentrations did not change among temperature groups but reduced glutathione concentration was significantly higher in cold acclimated fish, suggesting a higher antioxidant capacity in this group. The data suggest that lower growth rate in cold temperature results from both high 20S proteasome activity and a reduced rate of protein synthesis.

The second experiment expanded the inquiry to a wider range of fish mass and used a multi-tissue approach to study the relationship between SGR and 20S proteasome activity in heart ventricle, liver and white muscle tissue. Fish were acclimated at 4 and 12 °C in order to determine if protein degradation via the proteasome pathway could be imposing a limitation on somatic growth in fish. The growth data showed a sharp decrease of optimal temperature for growth with increasing mass from approximately 10 to 7 °C for fish of 5 and 150 g, respectively, without any important decrease in fish bigger than 150 g. From the results of the first experiment, it was expected that the activity of 20S proteasome would be higher in fish acclimated at cold temperature but this was not the case. The reduction of the thermal optimum for growth with increasing fish mass suggests that there might be a reduction of the interference from the 20S proteasome on protein accretion as fish grow bigger, which could explain the discrepancies between the results of the two experiments. The white muscle 20S proteasome activity is negatively correlated to SGR (partial Pearson's r = -0.609) in fish acclimated at cold temperature (4 °C) while no relation was ob-

served at 12 °C. *The experimental design did not include the food consumption of the fish as all variables were measured in individual animal and it is virtually impossible to measure food consumption of individuals. McCarthy et al. (1998 and 1999) showed that, in Atlantic wolfish, food consumption increases with temperature. Accordingly, at cold temperature where food consumption and protein synthesis are lower, a lower rate of protein turnover should be expected. It appears not to be the case in the first experiment as the activity of 20S proteasome remained elevated. In the second experiment, the effect of temperature on 20S proteasome activity differs among tissues as only in the liver was an increase of 20S proteasome activity observed with increased temperature. It is very difficult to distinguish the effect of temperature and the effect of food intake as they are intimately related.* Nevertheless, the results from the first two experiments suggest that the action of 20S proteasome impairs the accretion of proteins in white muscle following acclimation to cold temperature.

To test whether the impairment of protein accretion by 20S proteasome is a result of enhanced mitochondrial capacity following acclimation to cold temperature, the third experiment documented the effects of acclimation to low and high temperature (4 and 12 °C) on mitochondrial and antioxidant capacities in white muscle, heart ventricle and liver. The relationships between mitochondrial and antioxidants enzymes and 20S proteasome activities were examined. Following an acclimation period of 51 days, mitochondrial capacities were measured as the activities of Complex I of the mitochondrial electron transport system (CPLXI) and citrate synthase (CS). Glutathione disulfide reductase (GR) and catalase (CAT) activities as well as glutathione content were also measured to

estimate antioxidant capacities. Following acclimation at cold temperature, mitochondrial activities of heart ventricle and liver were positively compensated while no compensation was observed in white muscle. The lack of compensation of the mitochondrial capacities has previously been observed and attributed to various reasons (species, strain or technical considerations). In this case, it is suggested that the lack of compensation in white muscle is a result of the low level of swimming activity of the spotted wolffish.

The pattern of antioxidant enzyme activities and GSH concentrations differed in the three studied tissues following acclimation to high and low temperatures. At cold temperature, GR activity was clearly increased in liver while only a marginal (but still significant) increase was observed in heart and white muscle. At high temperature, CAT activity was increased in heart and white muscle but CAT remained elevated at both acclimation temperatures in liver. GSH concentration was unaffected by acclimation temperature which contrasts with the results of the first experiment. Again, this divergence may be a result of ontogenic changes between fish of 5 g (Chapter 2) and > 150 g (Chapter 3). The relationships between the activity of mitochondrial and antioxidant enzymes were examined in the three tissues. In the liver and white muscle, a number of positive relationships (some marginal) were observed while none were observed in heart. GSH concentration is shown to be positively correlated to CS activity in heart but not in liver or white muscle. These observations led to the conclusion that tissues may use different antioxidant strategies in response to high or low temperature acclimation and call for more work in order to elucidate the relationships between mitochondrial and antioxidant capacities.

The relationships between 20S proteasome and mitochondrial enzyme activities suggest a connection between aerobic capacity and the need for protein degradation, at least in white muscle. Whether or not this connection is mediated by the need to degrade proteins that are oxidatively damaged by the mitochondrial ROS production remains an open question. Alternatively, increased mitochondrial capacity could be a response to sustain the costs of replacing the proteins degraded by the proteasome. Whatever the direction of the relationship, the major finding of this third experiment is that inter-individual variation of oxidative capacities is related to that of the 20S proteasome activity and, by extension, on the protein metabolism in the white muscle of spotted wolffish. Whether this observation can be generalized to other fish species is unknown. As mentioned before because wolffish species display very low spontaneous swimming activity, the energetic requirement of their skeletal muscle for activity is minimal. This phenomenon may exacerbate the relationships between their oxidative capacities and protein metabolism. Accordingly, these species constitute an excellent model for this type of studies but whether these observations hold true in more active fish species remains to be tested. Interestingly, Dobly *et al.* (2004) found a negative relationship between the hepatic activity of 20S proteasome and growth efficiency in rainbow trout. These authors did not observed such a relationship in the muscle; unfortunately they did not mention what type of muscle fibre was studied (red or white). Nevertheless, these results warrant further investigation on the relationship between inter-individual variability of oxidative capacity and that of protein metabolism and food conversion efficiency (FCE). If higher oxidative capacities are associated with a higher rate of protein degradation, this

should necessarily impair protein synthesis retention efficiency and FCE. Such types of studies would contribute to our understanding of the ontogeny of growth processes in cold water fish species and generate valuable information for the field of aquaculture research.

In the first chapter, the rate of incorporation of phenylalanine into proteins was used to compare the rate of protein synthesis between acclimation groups. This methodology had been successfully applied to study the effect of seasonal acclimation/temperature on the rate of protein synthesis in cod (Treberg *et al*, 2005) and cunner (Lewis and Driedzic, 2007). This procedure is reasonably accurate to allow for the comparison of the rate of protein synthesis between groups of fish. However it does not allow for the calculation of the fractional rate of protein synthesis (*Ks* as $\%\cdot day^{-1}$) and the estimation of *Kd* from the difference between *Ks* and *Kg*. This decision was made following practical considerations: 1) it does not require the use of expensive enzymes for the conversion of phenylalanine to phenylethylamine, and 2) the yield of this reaction is generally poor and thus involves a great loss of the label, reducing the overall sensitivity of the method. Furthermore, I have no reason to believe that the protein content of phenylalanine would change in relation to acclimation temperature and accordingly there is no obvious reasons to correct for it. The conversion of phenylalanine to tyrosine is the normal pathway of phenylalanine oxidation and, as such, it is possible that some of the counts obtained in both the free pool and protein-bound pool were actually from tyrosine. However, it must be pointed out that, given the massive concentration of phenylalanine injected in the fish, the concentration of labelled tyrosine in the free pool protein-bound pool should be marginal. In the

future, the use of the method described in the supplement section will allow for the calculation of *Kd* from the difference between *Ks* and *Kg* and allow for the comparison between the activity of 20S proteatome and estimated *Kd*.

For the second and third experiments, it would have been extremely interesting to obtain protein synthesis data along with 20S proteasome activity in order to have a better picture of protein metabolism in the fish acclimated at different temperatures. It was not possible to obtain this information as the facility where the fish were maintained was not certified for radioactive work. Accordingly, to avoid this sort of limitation in the future, a simple method was devised to measure the fractional rate of protein synthesis in fish using the stable isotope labeled tracer (ring-D_5-L-phenylalanine) instead of radioactive phenylalanine. The method presented in the following supplement section takes advantage of the increasingly available technology of liquid chromatography with tandem mass spectrometry detection (LC-MSMS). The technique was used to measure the fractional rate of protein synthesis in the gills of goldfish (*Carrassius auratus*). The obtained *Ks* was validated by comparing it with previously published *Ks* for goldfish under the same conditions. It was not possible to measure *Ks* in the same animals using both the radioactive and stable isotope methods for two reasons: 1) I was not allowed to use radioactive sampled in the LC-MS, and 2) the use of tritiated phenylalanine would introduce a third phenylalanine mass: unlabelled phenylalanine (mass = 165 $g \cdot mol^{-1}$), L-[2,3,4,5,6-^{2}H]phenylalanine (mass = 170 $g \cdot mol^{-1}$) and L-[2,3,4,5,6-^{3}H]phenylalanine (mass = 175 $g \cdot mol^{-1}$), adding an undue complexity to the method. Accordingly, the comparison of the obtain *Ks* with that from the litterature should be considered just as good as the comparison of data

obtained from different animals and different techniques. The modified technique gives results comparable to the radioactive one, requires fewer steps compared to previously available procedures, and allows studies on fish protein metabolism to be carried out in situations where the use of radioactivity is not possible such as in free-living animals.

6

Supplementary material: Measurement of protein synthesis in fish tissues with non-toxic stable isotopes

This material is submitted for publication in the Transactions of the American Fisheries Society.

6.1 Abstract

A simple method was devised to measure the fractional rate of protein synthesis in fish using the stable isotope labelled tracer (ring-D_5 L-phenylalanine) instead of radioactive phenylalanine. The method takes advantage of the increasingly available technology of liquid chromatography with tandem mass spectrometry detection (LC-MSMS). The technique was validated by measuring the fractional rate of protein synthesis in the gills of goldfish (*Carrassius auratus*). The values obtained were within the published range for fish gills. The modified technique requires fewer steps compared to previously available procedures and allows studies on fish protein metabolism to be carried out in situations where the use of radioactivity is not possible such as in free living animals.

6.2 Introduction

Growth is a fundamental process in all living animals. The tissue protein pool is in constant state of renewal with proteins entering the pool via synthesis and being removed via degradation (Fraser *et al.*, 2002). Tissue growth is insured through accumulation of a proportion of newly synthesized proteins. In the process of understanding animal growth, the rate of protein synthesis is, therefore, a key element and many techniques to evaluate it are available. Over the last three decades, the rate of protein synthesis in fish has been mostly evaluated using the flooding dose method of Garlick *et al.* (1980). This procedure involves the injection of a large dose of L-(4-^{3}H)phenylalanine (radioactive phenylalanine) and the subsequent determination of radioactivity incorporated into the protein pool.

This method provided a large advantage over earlier protocols in that it enabled the measurement of the fractional rate of protein synthesis relatively quickly and in a cost-effective way (Garlick *et al.*, 1980; Fraser and Rogers, 2007). However, its major disadvantage is that it requires the use of radiolabeled tracer that limits its use to controlled isotopic laboratories. Methods using stable isotopic tracers in fish have been previously described but most of them are based on a completely different and more complex methodology, such as the stochastic endpoint model (Fraser and Rogers, 2007). In this approach, the animal is fed a known amount of isotopic (^{15}N) amino acid and the excretion of the oxidized amino acid is tracked, mainly in the form of ^{15}N-ammonia in aquatic organisms (Carter *et al.*, 1994; Carter *et al.*, 1998). This method presents several advantages over the flooding dose method in that it uses stable isotopes, it is less invasive, and it uses longer term measurements and tracer dose of labelled amino acid (in opposition to a flooding dose) which probably makes it a more accurate method compared to the flooding dose technique. Nevertheless, the disadvantages are that it only enables the measurement of whole body protein synthesis, it requires burdensome laboratory analysis since the isotopic enrichment of ammonia is measured in the water, it requires the distillation of large volumes of water and, finally, it necessitates the estimation of isotope consumption, usually via X-ray radiography (Fraser and Rogers, 2007). Because this method measures the specific enrichment of excreted ammonia, it also must be used in tightly closed systems to avoid losses of tracer and, as such, is inappropriate for field studies. The flooding dose method has previously been modified for the use of ^{15}N-amino acids (Krawielitzki and Schadereit, 1992; Owen *et al.*, 1999). Despite that this modification of the flooding dose technique allowed its use outside the laboratory, it has rarely been

used because of the general perception of being costly and very complicated. This study describes a flooding dose technique using stable isotope labelled amino acid that is cost effective, simple and accurate. The technique involves the injection of ring-D_5-phenylalanine (D_5-PHE) and, following an incorporation period of a few hours, the detection of the specific enrichment of both the free amino acids and the protein pool using high performance liquid chromatography and detection with tandem mass spectrometry (HPLC-MSMS), without the need for derivatization of the amino acid. The methodology is straightforward and necessitates only a few steps compared to previously described procedures (Krawielitzki and Schadereit, 1992; Owen *et al.*, 1999). The method was validated by a time series experiment on goldfish (*Carassius auratus*) gills and subsequent comparison with the data available from the literature. The choice of gills was motivated by their relatively high rate of protein synthesis (Carter *et al.*, 1998; Fraser and Rogers, 2007). The method fulfilled the assumptions of the flooding dose technique, gave results similar to those published, and may be used for different organs or whole body protein synthesis.

6.3 Materials and methods

6.3.1 Fish injection and sampling

The experiment consisted of a time trial and was designed to validate the use of LC-MSMS to measure the incorporation of deuterium-labelled phenylalanine (D_5-PHE) in the protein pool. Goldfish were obtained from a local pet shop and held in the animal care facility of Université du Québec à Rimouski (Québec,

Canada) for a week prior to the beginning of the experiment. Fish were maintained in a 75 L aquarium, fed daily, temperature was maintained at 21 °C and pH, ammonium, nitrite and nitrate were monitored daily using commercial kits (Hagen Canada). Food was withdrawn 24 hours prior to the experiment. Nine goldfish with an average weight of 3.03 g received an intra-peritoneal injection of a 150 mM solution of phenylalanine containing 50% D5-PHE at a dosage of 1 ml $\cdot$ 100 g^{-1}. The injection solution consisted of (in mM) 75 L-phenylalanine, 75 ring-D_5 L-phenylalanine (98%, Cambridge Isotope Laboratories, Inc. Andover, MA, USA), 150 NaCl, 5 KCl, 5 $NaPO_4$, 2 $CaCl_2$, 10 $NaHCO_3$, 2.0 Na_2HPO_4, 1.0 $MgSO_4$, 5 D-glucose, and 5.0 HEPES at pH 7.6. Fish were randomly assigned to 3 groups of different incorporation time (3 fish per incorporation period, 60, 180 and 320 minutes). Two fish were not injected and used as controls. After the incorporation period, the fish were stunned by a blow to the head and killed by severing the spinal cord. The abdominal cavity was immediately exposed and copiously rinsed with distilled water to wash off the unabsorbed tracer. The two first gill arches of both sides of the fish were dissected, washed in distilled water and immediately frozen on dry ice and stored at -80 °C until further laboratory analysis.

6.3.2 Tissue homogenisation and sample preparation

Approximately 75 mg of frozen gill tissue was homogenised in 1 ml of 0.2 M ice-cold perchloric acid (PCA). The homogenizer was washed with 1 ml of 0.2 M ice-cold PCA that was then combined with the homogenate and following an incubation period of 10 min on ice, the homogenate was centrifuged at 15,000

g for 5 min at 4 °C, to precipitate the protein. The supernatant was saved as it contained the free pool of amino acids. The protein pellet was washed three times by resuspending it in 1 ml of 0.2 M PCA, incubating on ice for 10 min and centrifuging for 10 min at 15,000 g. The protein pellet was then dissolved in 2 ml of 0.3 M NaOH (approximately 1 hour at 37°C) to resolubilize the protein. A 1.1 ml sample was withdrawn in order to measure protein concentration then 0.867 ml of 20 % PCA was added to the remaining solution and centrifuged 10 min at 15,000 g to precipitate the protein once more. The pellet was washed twice as before; then the protein pellet was hydrolysed in 6 ml of 6 N HCl at 110 °C for 18-24 hours to yield the constituent free amino acids. The hydrolysate was freeze dried and stored at -80 °C until further analysis. Immediately before the LC-MSMS analysis, the samples were dissolved in 5 ml methanol (HPLC grade), filtered through a 0.2 µm cellulose acetate syringe filter and subsequently diluted 1/10 in methanol.

6.3.3 Liquid chromatography and quantification

The system consisted of a Surveyor LC pump and Autosampler coupled to a LCQ Advantage mass spectrometer (ion trap) from ThermoFinnigan (Waltham, MA, USA); Xcalibur® software (revision 1.3) was used to control the system, acquisition and process data. Analysis of phenylalanine and D_5-PHE was performed on a Discovery C18 column (50 mm x 2.1 mm, 5 µm) from Supelco (Sigma-Aldrich, St-Louis, USA) with isocratic elution at 0.5 ml min^{-1} with methanol:formic acid in water at 0.1% in 90:10 proportion as mobile phase. The column temperature was maintained at 30 °C and the volume of injection was 10 µl in the full loop mode.

Samples were kept at 20 °C in the autosampler. Quantification was performed with a four point calibration plot and the standard solutions were imbedded in each sequence of analysis: from 0 to 15 µg ml^{-1} for both phenylalanine and from 0 to 0.15 µg ml^{-1} for D_5-PHE.

6.3.4 Mass spectrometry

The ionisation was performed through an atmospheric pressure chemical ionization (APCI) with operating conditions summarised in Table 6.1. The mass spectrometer was operated in positive ion mode with selective reaction monitoring MS-MS detection with the fragmentation transition (mass-to-charge ratios of the parent ion to the product ion) m/z 166 → 120 for phenylalanine and m/z 171 → 125 for D_5-PHE (Figure 6.1), with collision energy at 25V.

Table 6.1: APCI-MS operating conditions.

Parameter	**value**
Vaporizer temperature (°C)	400
Capillary temp. (°C)	160
Sheath gas flow, N_2	76 arbitrary units
Auxiliary gas flow, N_2	4 arbitrary units
Source voltage (kV)	6
Source current (μA)	5
Capillary voltage (V)	3

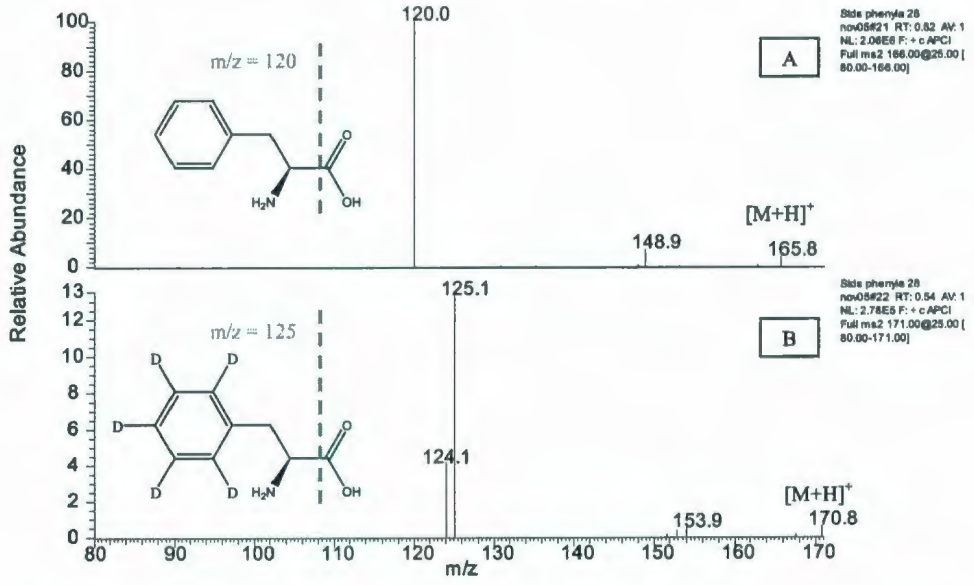

Figure 6.1: Mass spectrum of phenylalanine (A) and ring-D5 phenylalanine (B) obtained from MSMS analysis.

6.3.5 Calculations and statistical analysis

Fractional rate of protein synthesis (*Ks*, % protein synthesized per day) in gill was calculated from D_5-PHE enrichment of the protein pool (*Sb* = D_5-PHE/total phenylalanine in hydrolysed protein pellet) and free amino acid pool (*Sa* = D_5-PHE/total phenylalanine in 0.2 M PCA supernatant) according to the equation of Garlick *et al.* (1980); *Ks* (% day^{-1}) = $(Sb/Sa) \times (1440\ /t) \times 100$, where t was the incorporation time in minutes and 1440 is used to convert from minutes to days (60 min × 24 hrs = 1440). Regression analyses were performed using Systat 11 (Systat inc.) as described in Zar (1999).

6.4 Result and discussion

With an analytical method based on mass spectrometry, detection of the signal of each compound is very specific and complete chromatographic separation of the phenylalanine from other compounds in the matrix is not necessary. The deuterated aromatic ring in the phenylalanine molecule results in a molecular mass 5 units higher than the molecular mass of phenylalanine, 170 vs 165, respectively. In the mass spectrum this translates to a fragmentation pattern, with the ion resulting from the loss of the carboxylic group as the most intense ion, and allows unequivocal identification of phenylalanine (m/z = 120) and D_5-PHE (m/z = 125) with MSMS analysis (Figure 6.1). Higher selectivity is achieved with the selective reaction monitoring mode (SRM) of the mass spectrometer; in this mode the pseudo-molecular ion [M+H]+ of each compound is isolated alternatively and only the product ion of interest is detected. The transition for phenylalanine is m/z 166 $\rightarrow$ 120 and for D_5-PHE is m/z 171 $\rightarrow$ 125. The signal in SRM mode is thus very specific for each compound with very low background from the matrix. As an example, the chromatogram for phenylalanine and D_5-PHE extracted from the protein pool of the gills of a fish following an incorporation period of 325 min is illustrated in Figure 6.2. The retention time of each compound is similar at 0.83 minute, but since the signal is specific to each SRM transition, adequate quantification can be achieved.

Following the flooding dose injection, D_5-PHE enrichment of the free phenylalanine pool (D_5-PHE/total phenylalanine) was 42.49 $\pm$ 4.87 % and remained elevated and stable throughout the time course (Figure 6.3). Accordingly, the

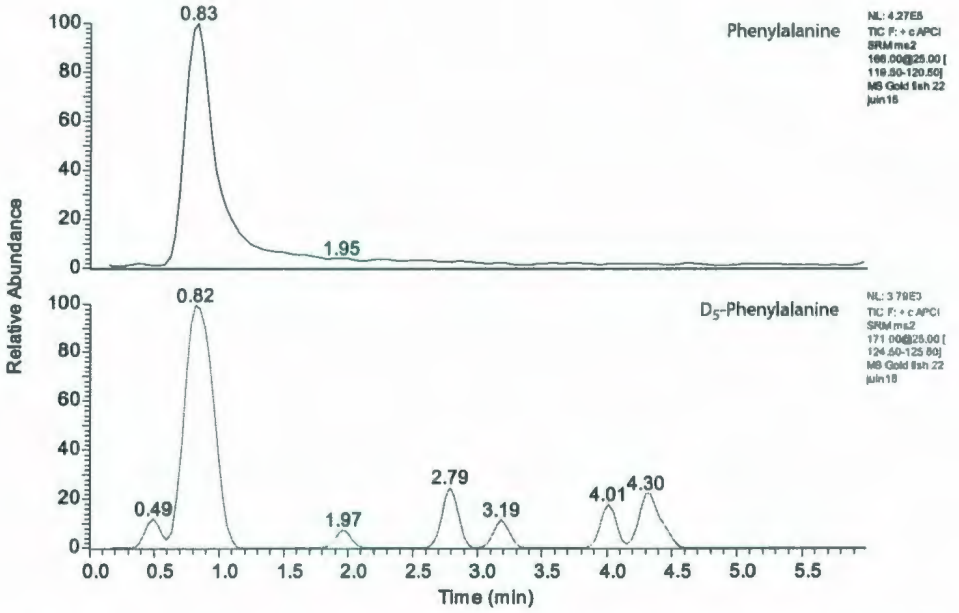

Figure 6.2: Liquid chromatograms showing the abundance of phenylalanine and ring-D_5-phenylalanine in the protein pool of a goldfish after 325 min following the injection an amino acid solution. Upper trace corresponds to the signal from the SRM transition 166 → 120 for phenylalanine; lower trace corresponds to the signal from the SRM transition 171 → 125 for ring-D_5-phenylalanine.

slope of *Sa* in relation to incorporation time was not significantly different from 0 ($r^2 = 0.004$, n = 9, p = 0.87, Figure 6.3). The free phenylalanine pool was on average 11.6 times higher in the fish injected with the tracer solution compared to the control fish (Table 6.2). Finally, the incorporation of the labelled phenylalanine was linear over time. The equation of the linear regression was $sb = 2.3 \times 10^{-5} (\pm 5.0 \times 10^{-6}) \times t + 0.002 (\pm 0.001)$, ($r^2 = 0.78$, n = 7, $p < 0.01$, Figure 6.3). The intercept of the regression was not significantly different from zero indicating that the flooding by the phenylalanine equilibrated rapidly with the free amino acids pool and that labelling of gill proteins started quickly after the injection. After 60 min of incorporation, the specific enrichment of the gill proteins could only be measured in one animal, the two other being below the limit of detection. Nevertheless, D_5-PHE incorporation in the protein pool could be measured in all fish when incorporation time was 3 hours or longer.

The fractional rate of protein synthesis for the three time periods following injection of amino acid were 20.52 (n = 1), 10.39 ± 0.93 (n = 3) and 9.48 ± 2.01 % day^{-1} (n = 3) for the 60, 180 and 325 min incorporation, respectively. There was no difference between mean synthesis rate measured for 180 and 325 min incorporation groups. However, the synthesis rate measured after 60 minutes of incubation was twice as high as compared with the two other groups, and is most likely inaccurate. Since the enrichment of the precursor pool remained stable for at least 5 hours, it is thus advisable and more accurate to use longer incorporation periods (Foster *et al.*, 1992; Owen *et al.*, 1999).

Since phenylalanine is the most commonly used amino acid tracer to measure

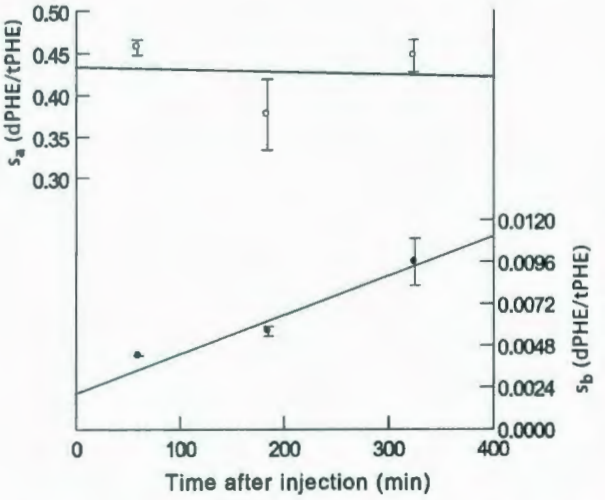

Figure 6.3: Enrichment (D_5-PHE/total phenylalanine) of the gill free pool (Sa ; open circles) and proteins (Sb ; black circles) in goldfish following a single dose injection of 150 mM phenylalanine (containing 75 mM D_5-PHE) to measure in vivo rates of protein synthesis. Each dot represents mean values ± SEM of three fish (except Sb at 60 min where specific enrichment could only be determined in one animal).

the rate of protein synthesis using the flooding dose method in fish, the results of this study could be directly compared with the literature (Owen *et al.*, 1999; Carter and Houlihan, 2001). The rates of protein synthesis measured using D_5-PHE and LC-MSMS are within the range of previously published Ks for fish gills, which vary between 6 and 14% day^{-1} for a wide variety of fish species (Carter *et al.*, 1998). This method is thus suitable and accurate for measuring the fractional rate of protein synthesis in gills but also in different organs. As well, the method could be extended to whole body protein synthesis that varies between 0.9 and 10.3 % day^{-1} in juvenile fish and between 13.5 and 106 % day^{-1} in larval fish (Carter *et al.*, 1998). When working with larval fish, an alternative to injecting the labelled amino acid is bathing the animals in a flooding concentration of amino acid (Houlihan *et al.*, 1992). Again the stable isotope technique reported

here could be applied.

Table 6.2: Data used for the calculation of the fractional rate of protein synthesis (ks) using ring-D_5 phenylalanine and LC-MSMS in gill of goldfish (*Carassius auratus*). Time refers to the time following injection of the amino acid solution into the animal. Amino acid levels are expressed as $\mu g \cdot g^{-1}$.

			Free pool			Protein pool			
Fish	Time	Mass	dphe	phe	*Sa*	dPHE	PHE	*Sb*	*Ks*
	(min)	(g)	($\mu g \cdot g^{-1}$)	($\mu g \cdot g^{-1}$)		($\mu g \cdot g^{-1}$)	($\mu g \cdot g^{-1}$)		(%day^{-1})
1	60	1.3	696.90	858.07	0.45	ND	164.14	ND	ND
2	63	4.2	580.88	665.52	0.47	0.93	221.66	0.0042	20.52
3	66	1.4	906.42	1117.64	0.45	ND	301.63	ND	ND
4	186	4.8	505.02	622.56	0.38	1.09	207.38	0.0052	10.71
5	187	1.4	732.52	977.67	0.43	1.46	256.05	0.0057	10.18
6	190	3.3	416.84	538.94	0.32	1.45	243.98	0.0059	10.28
7	321	4.3	819.74	924.55	0.47	2.50	283.60	0.0088	8.35
8	325	1.3	798.59	983.40	0.45	2.97	252.19	0.0116	11.49
9	329	5.3	332.97	458.34	0.42	1.51	181.51	0.0083	8.58
10	control	ND	ND	120.16	ND	ND	231.97	ND	ND
11	control	ND	ND	125.73	ND	ND	250.32	ND	ND

In summary, the aim of this study was to measure *in vivo* rate of protein synthesis in fish using the flooding dose technique and a stable isotope labelled amino acid (D_5-PHE). The methodology described allowed for the determination of the specific enrichment in both the intracellular free amino acid pool and protein-bounded pool. The new method fulfilled the assumptions of the flooding

dose technique as shown by the rapid and maintained enrichment of the free amino acid pool and the linear incorporation of deuterated phenylalanine into protein over the incorporation period. An incorporation period greater than 1 h is recommended as the concentration of D_5-PHE in the protein pool after 60 min incorporation was below the limits of the presented methodology. The method gave results similar to those available in the literature based on studies with radioisotopes. It requires fewer steps compared to previously reported methods and may be used for a variety of organs or whole body protein synthesis. The use of stable isotope instead of radioactive tracer is advantageous as it is portable in situations where radioactivity is not an option such as in teaching laboratories and field studies. Indeed, it should be possible to measure protein synthesis in free-swimming fish in situations where the sample animal may be tagged and recaptured such is in aquaculture cages, tidal pools, or in species with limited movement over a period of approximately 3 hours.

7

List of abbreviations

ANCOVA	analysis of covariance
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
ATP	adenosine triphosphate
CAT	catalase
CI	confidence interval
COX	cytochrome oxydase
CPLXI	complex I
CPT	palmitoyl carnitine transferase
CS	citrate synthase
D_5-PHE	deuterated phenylalanine
DNA	dehydroxyribonucleic acid
DTNB	dithionitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
FQRNT	Fond Quebecois pour la Recherche en Nature et Technologies
GLM	general linear model
G_max	maximal growth rate
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione dissulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC-MSMS	see LC-MSMS
Kd	fractional rate of protein degradation
Kg	fractional rate of protein growth
Ks	fractional rate of protein synthesis
LC-MSMS	liquid chromatography with tandem mass spectrometry detection
LLVY-AMC	N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin
mCB	monochlorobimane

MDA	malonedialdehyde
mRNA	messenger RNA
PBS	phosphate buffer saline
PCA	perchloric acid
PFTE	Teflon
PSRE	protein synthesis retention efficiency
Q_{10}	temperature coefficient
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	regulatory particle
Sa	free amino acid pool enrichment
Sb	protein pool enrichment
SD	standard deviation
SDS	sodium dodecyl sulfate
SGRl	specific growth rate (in length)
SGRw	specific growth rate (in weight)
SRM	selective reaction monitoring
SSA	spontaneous swimming activity
TBARS	thiobarbituric acid reactive substance
TCA	trichloroacetic acid
TEA	triethanolamine
T_{optsgr}	optimal temperature for specific growth rate
UPP	ubiquitin proteasome pathway

8

References

Almroth, B. C., Sturve, J., Berglund, A. & Forlin, L. (2005). Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Aquatic Toxicology* **73**, 171-180.

Aoki, T., Yamashita, T. & Ueno, R. (2000). Distribution of cathepsins in red and white muscles among fish species. *Fisheries Science* **66**, 776-782.

Attaix, D., Combaret, L., Pouch, M. N. and Taillandier, D. (2001). Regulation of proteolysis. *Current Opinion in Clinical Nutrition and Metabolic Care* **4**, 45-49.

Barsukov, V. V. (1959). *The wolffish (Anarhichadidae); Translated for the Smithsonian Institution.* New Delhi: Indian National Scientific Documentation Centre.

Bjornsson, B., Steinarsson, A. & Arnason, T. (2007). Growth model for Atlantic cod (*Gadus morhua*): Effects of temperature and body weight on growth rate. *Aquaculture* **271**, 216-226.

Bjornsson, B., Steinarsson, A. & Oddgeirsson, M. (2001). Optimal temperature for growth and feed conversion of immature cod (*Gadus morhua* L.). *Ices Journal of Marine Science* **58**, 29-38.

Bjornsson, B. & Tryggvadottir, S. V. (1996). Effects of size on optimal temperature for growth and growth efficiency of immature Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture* **142**, 33-42.

Blier, P. U. and Gugerley, H. (1988). Metabolic responses to cold-acclimation in the swimming musculature of lake whitefish, *Coregonus-clupeaformis*. Journal of Experimental Zoology **246**, 244-252.

Blier, P. U. & Lemieux, H. (2001). The impact of the thermal sensitivity of cytochrome c oxidase on the respiration rate of Arctic charr red muscle mitochondria. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **171**, 247-253.

Boveris, A. & Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal* **134**, 707-716.

Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P. M., Finley, D. and Schmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nature Cell Biology* **1**, 221-226.

Brett, J. R. (1979). Environmental factors and growth. In *Fish physiology and growth* (Hoar, W. S., Randall, D. J. & Brett, J. R., eds.). New York: Academic Press.

Cadenas, E., Boveris, A., Ragan, C. I. & Stoppani, A. O. M. (1977). Production of superoxide radicals and hydrogen-peroxide by nadh-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Archives of Biochemistry and Biophysics* **180**, 248-257.

Carlberg, I. & Mannervik, B. (1985). Glutathione reductase. In *Methods in Enzymology* (Meister, A., ed.), pp. 484-490.

Carter, C. G. & Houlihan, D. F. (2001). Protein synthesis. In *Fish physiology: Nitrogen excretion* (Wright, P. A. & Andersen, P. M., eds.), pp. 31-75. London: Academic Press.

Carter, C. G., Houlihan, D. F. & Owen, S. F. (1998). Protein synthesis, nitrogen excretion and long-term growth of juvenile *Pleuronectes flesus*. *Journal of Fish Biology* **53**, 272-284.

Carter, C. G., Owen, S. F., He, Z. Y., Watt, P. W., Scrimgeour, C., Houlihan, D. F. & Rennie, M. J. (1994). Determination of protein-synthesis in rainbow-trout, *Oncorhynchus mykiss*, using a stable-isotope. *Journal of Experimental Biology* **189**, 279-284.

Chance, B. & Maehly, A. C. (1955). Assay of catalases and peroxidases. In *Methods in Enzymology* (Colowick, S. P. & Kaplan, N. O., eds.), pp. 764-775.

Chapman, K. E., Sinclair, S. E., Zhuang, D. M., Hassid, A., Desai, L. P. & Waters, C. M. (2005). Cyclic mechanical strain increases reactive oxygen species production in pulmonary epithelial cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **289**, L834-L841.

Conconi, M., Szweda, L. I., Levine, R. L., Stadtman, E. R. & Friguet, B. (1996). Age-related decline of rat liver multicatalytic proteinase activity and protection from oxidative inactivation by heat-shock protein 90. *Archives of Biochemistry and Biophysics* **331**, 232-240.

Couture, P., Dutil, J. D. & Guderley, H. (1998). Biochemical correlates of growth and condition in juvenile Atlantic cod (*Gadus morhua*) from Newfoundland. *Canadian Journal of Fisheries and Aquatic Sciences* **55**, 1591-1598.

Coux, O., Tanaka, K. & Goldberg, A. L. (1996). Structure and functions of the 20S and 26S proteasomes. *Annual Review of Biochemistry* **65**, 801-847.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta* **329**, 23-38.

de Duve, C. (1996). The peroxisome in retrospect. *Ann N Y Acad Sci* **804**, 1-10.

Delbarre-Ladrat, C., Verrez-Bagnis, V., Noel, J. & Fleurence, J. (2004). Relative contribution of calpain and cathepsins to protein degradation in muscle of sea bass (*Dicentrarchus labrax* L.). *Food Chemistry* **88**, 389-395.

Dobly, A., Martin, S. A. M., Blaney, S. C. & Houlihan, D. F. (2004). Protein growth rate in rainbow trout (*Oncorhynchus mykiss*) is negatively correlated to liver 20S proteasome activity. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **137**, 75-85.

Eckerle, L. G., Lucassen, M., Hirse, T. & Portner, H. O. (2008). Cold induced changes of adenosine levels in common eelpout (*Zoarces viviparus*): a role in modulating cytochrome c oxidase expression. *Journal of Experimental Biology* **211**, 1262-1269.

Falk-Petersen, I. B., Hansen, T. K., Fieler, R. & Sunde, L. M. (1999). Cultivation of the spotted wolffish *Anarhichas minor* (Olafsen) - a new candidate for cold-water fish farming. *Aquaculture Research* **30**, 711-718.

Finkel, T. & Holbrook, J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Foss, A., Imsland, A. K., Falk-Petersen, I. B. & Oiestad, V. (2004). A review of the culture potential of spotted wolffish *Anarhichas minor* Olafsen. *Reviews in Fish Biology and Fisheries* **14**, 277-294.

Foster, A. R., Houlihan, D. F., Hall, S. J. & Burren, L. J. (1992). The effects of temperature acclimation on protein synthesis rates and nucleic acid content of juvenile cod (*Gadus morhua* L.). *Canadian Journal of Zoology* **70**, 2095-2102.

Fraser, K. P. P., Clarke, A. & Peck, L. S. (2002). Low-temperature protein metabolism: seasonal changes in protein synthesis and RNA dynamics in the Antarctic limpet *Nacella concinna* Strebel 1908. *Journal of Experimental Biology* **205**, 3077-3086.

Fraser, K. P. P. & Rogers, A. D. (2007). Protein metabolism in marine animals: The underlying mechanism of growth. In *Advances in Marine Biology, Vol 52*, pp. 267-362.

Friguet, B. (2006). Oxidized protein degradation and repair in ageing and oxidative stress. *Febs Letters* **580**, 2910-2916.

Garlick, P. J., McNurlan, M. A. & Preedy, V. R. (1980). A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3H]phenylalanine. *Biochemical Journal* **192**, 719-723.

Glickman, M. H. & Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. *Physiological Reviews* **82**, 373-428.

Goll, D. E., Thompson, V. F., Li, H. Q., Wei, W. & Cong, J. Y. (2003). The calpain system. *Physiological Reviews* **83**, 731-801.

Goll, D. E., Thompson, V. F., Taylor, R. G. & Ouali, A. (1998). The calpain system and skeletal muscle growth. *Canadian Journal of Animal Science* **78**, 503-512.

Gracey, A. Y., Fraser, E. J., Li, W. Z., Fang, Y. X., Taylor, R. R., Rogers, J., Brass, A. & Cossins, A. R. (2004). Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16970-16975.

Guderley, H. (1990). Functional significance of metabolic responses to thermal acclimation in fish muscle. *AJP - Regulatory, Integrative and Comparative Physiology Am J Physiol Regul Integr Comp Physiol* **259**, R245-252.

Guderley, H. (2004a). Locomotor performance and muscle metabolic capacities: impact of temperature and energetic status. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **139**, 371-382.

Guderley, H. (2004b). Metabolic responses to low temperature in fish muscle. *Biological Reviews* **79**, 409-427.

Guderley, H. and Gawlicka, A. (1992). Qualitative modification of muscle metabolic organization with thermal-acclimation of rainbow-trout, *Oncorhynchus mykiss*.

Fish Physiology and Biochemistry **10** 123-132.

Guderley, H., Lapointe, D., Bedard, M. & Dutil, J. D. (2003). Metabolic priorities during starvation: enzyme sparing in liver and white muscle of Atlantic cod, *Gadus morhua* L. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **135**, 347-356.

Guderley, H. & St-Pierre, J. S. (2002). Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *Journal of Experimental Biology* **205**, 2237-2249.

Hansen, T. K. & Falk-Petersen, I. B. (2002). Growth and survival of first-feeding spotted wolffish (*Anarhichas minor* Olafsen) at various temperature regimes. *Aquaculture Research* **33**, 1119-1127.

Hawkins, A. J. S. (1991). Protein turnover: a functional appraisal. *Functional Ecology* **5**, 222-233.

Hazel, J. & Prosser, C. (1970). Interpretation of inverse acclimation to temperature. *Journal of Comparative Physiology* **67**, 217-228.

Hazel, J. R. (1995). Thermal adaptation in biological-membranes - is homeoviscous adaptation the explanation. *Annual Review of Physiology* **57**, 19-42.

Heise, K., Estevez, M. S., Puntarulo, S., Galleano, M., Nikinmaa, M., Portner,

H. O. & Abele, D. (2007). Effects of seasonal and latitudinal cold on oxidative stress parameters and activation of hypoxia inducible factor (HIF-1) in zoarcid fish. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **177**, 765-777.

Heise, K., Puntarulo, S., Nikinmaa, M., Abele, D. & Portner, H. O. (2006). Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout *Zoarces viviparus* L. *Journal of Experimental Biology* **209**, 353-363.

Heise, K., Puntarulo, S., Nikinmaa, M., Lucassen, M., Portner, H. O. & Abele, D. (2006). Oxidative stress and HIF-1 DNA binding during stressful cold exposure and recovery in the North Sea eelpout (*Zoarces viviparus*). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **143**, 494-503.

Hochachka, P. & Somero, G. (2002). *Biochemical adaptation. Mechanism and process in physiological evolution.* New York: Oxford University Press.

Holmgren, A., Johansson, C., Berndt, C., Lonn, M. E., Hudemann, C. & Lillig, C. H. (2005). Thiol redox control via thioredoxin and glutaredoxin systems. *Biochemical Society Transactions* **33**, 1375-1377.

Houlihan, D., McMillan, D. & Laurent, P. (1986). Growth rates, protein synthesis, and protein degradation rates in rainbow trout: Effects of body size. *Physiological Zoology* **59**, 482-493.

Houlihan, D. F., Carter, C. G. & McCarthy, I. D. (1995). Protein turnover in animals. In *Nitrogen metabolism and excretion* (Walsh, P. J. & Wright, P., eds.). New York: CRC Press.

Houlihan, D. F. & Martin, S. A. M. (2002). Protein metabolism in fish; gambling on proteomics. *Integrative and Comparative Biology* **42**, 1245-1246.

Houlihan, D. F., Wieser, W., Foster, A. & Brechin, J. (1992). *In vivo* protein-synthesis rates in larval nase (*Chondrostoma nasus* L). *Canadian Journal of Zoology-Revue Canadienne De Zoologie* **70**, 2436-2440.

Imsland, A. K., Foss, A., Sparboe, L. O. & Sigurdsson, S. (2006). The effect of temperature and fish size on growth and feed efficiency ratio of juvenile spotted wolffish *Anarhichas minor*. *Journal of Fish Biology* **68**, 1107-1122.

Imsland, A. K., Le François, N. R., Lamarre, S. G., Ditlecadet, D., Sigurosson, S. & Foss, A. (2006). Myosin expression levels and enzyme activity in juvenile spotted wolffish (*Anarhichas minor*) muscle: a method for monitoring growth rates. *Canadian Journal of Fisheries and Aquatic Sciences* **63**, 1959-1967.

Imsland, A. K., Sunde, L. M., Folkvord, A. & Stefansson, S. O. (1996). The interaction of temperature and fish size on growth of juvenile turbot. *Journal of Fish Biology* **49**, 926-940.

Janssen, A. J. M., Trijbels, F. J. M., Sengers, R. C. A., Smeitink, J. A. M., Van

den Heuvel, L. P., Wintjes, L. T. M., Stoltenborg-Hogenkamp, B. J. M. & Rodenburg, R. J. T. (2007). Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. *Clinical Chemistry* **53**, 729-734.

Janssens B. J., Childress J. J., Baguet F. and Rees J. F. (2000). Reduced enzymatic antioxidative defense in deep-sea fish. *Journal of Experimental Biology* **203**,3717-3725

Jobling, M. (1985). Growth. In *Fish energetics; New perspectives* (Tyler, P. & Calow, P., eds.), pp. 213-231. Beckenham: Croom Helm Ltd.

Jobling, M. (1994). *Fish bioenergetics.* London: Chapman & Hall.

Katersky, R. S. and Carter, C. G. (2007). A preliminary study on growth and protein synthesis of juvenile barramundi, *Lates calcarifer* at different temperatures. *Aquaculture* **267**, 157-164.

Kamencic, H., Lyon, A., Paterson, P. G. & Juurlink, B. H. J. (2000). Monochlorobimane fluorometric method to measure tissue glutathione. *Analytical Biochemistry* **286**, 35-37.

Korshunov S. S., Skulachev V. P. and Starkov A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Letters* **416** 15-18.

Krawielitzki, K. & Schadereit, R. (1992). Estimation of protein-synthesis rates using the flooding method and [N-15] lysine. pp. 8-12.

Lamarre, S. G., Driedzic, W. R., Le François, N. R. & Blier, P. U. (20XX, accepted subject to minor revision) . White muscle 20S proteasome activity is negatively correlated to growth rate at low but not high acclimation temperature in the spotted wolffish (*Anarhichas minor; Olafsen*). *Journal of Fish Biology.*

Lamarre, S. G., Le François, N. R., Driedzic, W. R. & Blier, P. U. (2009). Protein synthesis is lowered while 20S proteasome activity is maintained following acclimation to low temperature in juvenile spotted wolffish (*Anarhichas minor*, Olafsen). *Journal of Experimental Biology.* **212**, 1294-1301.

Leary S. C., Lyons C. N., Rosenberger A. G., Ballantyne J. S., Stillman J. and Moyes C. D. (2003). Fiber-type differences in muscle mitochondrial profiles. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **285**, R817-R826.

Le François, N. R., Lamarre, S. G. & Blier, P. U. (2004). Tolerance, growth and haloplasticity of the Atlantic wolffish (*Anarhichas lupus*) exposed to various salinities. *Aquaculture* **236**, 659-675.

Le François, N. R., Lemieux, H. & Blier, P. U. (2002). Biological and technical evaluation of the potential of marine and anadromous fish species for cold-water mariculture. *Aquaculture Research* **33**, 95-108.

Leggatt, R. A., Brauner, C. J., Schulte, P. M. and Iwama, G. K. (2007). Effects of acclimation and incubation temperature on the glutathione antioxidant system in killifish and RTH-149 cells. *Comparative Biochemistry and Physiology A* ***146***, *317-326.*

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S. & Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* **186**, 464-478.

Lewis, J. M., Costa, I., A.L, V., Almeida-Val, V. M. F., Gamperl, A. K. & Driedzic, W. R. (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.

Lewis, J. M. & Driedzic, W. R. (2007). Tissue-specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperate labrid, *Tautogolabrus adspersus. American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **293**, R474-R481.

Livingstone, D. R., Lips, F., Martinez, P. G. & Pipe, R. K. (1992). Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis. Marine Biology* **112**, 265-276.

Loughna, P. T. & Goldspink, G. (1985). Muscle protein synthesis rates during

temperature acclimation in a eurythermal (*Cyprinus carpio*) and a stenothermal (Salmo gairdneri) species of teleost. *Journal of Experimental Biology* **118**, 267-276.

Lucassen, M., Schmidt, A., Eckerle, L. G. & Portner, H. O. (2003). Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **285**, R1410-R1420.

Martin, S. A. M., Blaney, S., Bowman, A. S. & Houlihan, D. F. (2002). Ubiquitin-proteasome-dependent proteolysis in rainbow trout (*Oncorhynchus mykiss*): effect of food deprivation. *Pflugers Archiv-European Journal of Physiology* **445**, 257-266.

Martin, S. A. M., Cash, P., Blaney, S. & Houlihan, D. F. (2001). Proteome analysis of rainbow trout (*Oncorhynchus mykiss*) liver proteins during short term starvation. *Fish Physiology and Biochemistry* **24**, 259-270.

McCaman, M. W. & Robins, E. (1962). Fluorimetric method for the determination of phenylalanine in serum. *Journal of Laboratory and Clinical Medicine.* 885-890.

McCarthy, I. D., Moksness, E. & Pavlov, D. A. (1998). The effect of temperature on growth rate and growth effiency of juvenil common wolffish. *Aquaculture International* **6**, 207-218.

McCarthy, I. D., Moksness, E., Pavlov, D. A. & Houlihan, D. F. (1999). Effects of water temperature on protein synthesis and protein growth in juvenile Atlantic wolffish (*Anarhichas lupus*). *Canadian Journal of Fisheries and Aquatic Sciences* **56**, 231-241.

McLean, L., Young, I. S., Doherty, M. K., Robertson, D. H. L., Cossins, A. R., Gracey, A. Y., Beynon, R. J. & Whitfield, P. D. (2007). Global cooling: Cold acclimation and the expression of soluble proteins in carp skeletal muscle. *Proteomics* **7**, 2667-2681.

Meister, A. & Anderson, M. E. (1983). Glutathione. *Annual Review of Biochemistry* **52**, 711-760.

Mommsen, T. P. (2004). Salmon spawning migration and muscle protein metabolism: the August Krogh principle at work. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **139**, 383-400.

Nelson, D. & Cox, M. (2005). *Lehninger principles of biochemistry.* New York: WH Freeman.

Nordgarden, U., Ornsrud, R., Hansen, T. & Hemre, G. I. (2003). Seasonal changes in selected muscle quality parameters in Atlantic salmon (*Salmo salar* L.) reared under natural and continuous light. *Aquaculture Nutrition* **9**, 161-168.

Owen, S. F., McCarthy, I. D., Watt, P. W., Ladero, V., Sanchez, J. A., Houlihan, D. F. & Rennie, M. J. (1999). In vivo rates of protein synthesis in Atlantic salmon (*Salmo salar* L.) smolts determined using a stable isotope flooding dose technique. *Fish Physiology and Biochemistry* **20**, 87-94.

Peragon, J., Barroso, J. B., Garcia-Salguero, L., de la Higuerala Higuera, M. & Lupianez, J. A. (2001). Growth, protein-turnover rates and nucleic-acid concentrations in the white muscle of rainbow trout during development. *International Journal of Biochemistry & Cell Biology* **33**, 1227-1238.

Perepechaeva, M. L., Kolosova, N. G. & Grishanova, A. Y. (2006). Activity of 20S proteosomes and content of oxidized proteins in rat liver after long-term cold exposure. *Bulletin of Experimental Biology and Medicine* **142**, 182-185.

Podrabsky, J. E. & Somero, G. N. (2004). Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. *Journal of Experimental Biology* **207**, 2237-2254.

Poppek, D. & Grune, T. (2006). Proteasomal defense of oxidative protein modifications. *Antioxidants & Redox Signaling* **8**, 173-184.

Portner, H. O. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88**, 137-146.

Ricker, W. E. (1975). Computation and interpretation of biological statistics of fish populations. *Bulletin of the Fisheries Research Board of Canada* **191**, 1-382.

Ritola, O., Livingstone, D. R., Peters, L. D. & Lindstrom-Seppa, P. (2002). Antioxidant processes are affected in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ozone and oxygen-supersaturated water. *Aquaculture* **210**, 1-19.

Ritola, O., Tossavainen, K., Kiuru, T., Lindstrom-Seppa, P. & Molsa, H. (2002). Effects of continuous and episodic hyperoxia on stress and hepatic glutathione levels in one-summer-old rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Ichthyology* **18**, 159-164.

Salem, M., Levesque, H., Moon, T. W., Rexroad, C. E. & Yao, J. B. (2006). Anabolic effects of feeding beta(2)-adrenergic agonists on rainbow trout muscle proteases and proteins. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **144**, 145-154.

Salem, M., Silverstein, J., Iii, C. E. R. & Yao, J. (2007). Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*). *Bmc Genomics* **8**, 16.

Savoie, A., Le François, N. R., Cahu, C. & Blier, P. U. (2008). Metabolic and digestive activity profiles of newly hatched spotted wolffish (*Anarhichas minor* Olafsen): effect of temperature. *Aquaculture Research* **39**, 382-389.

Savoie, A., Le François, N. R., Cahu, C., Blier, P. U. & Andreassen, I. (2006). Do protein hydrolysates improve survival and growth of newly-hatched spotted, wolffish (*Anarhichas minor*), a non-metamorphic aquaculture fish species? *Aquaculture* **261**, 782-788.

Shacter, E. (2000). Quantification and significance of protein oxidation in biological samples. *Drug Metabolism Reviews* **32**, 307-326.

Shibatani, T., Nazir, M. & Ward, W. F. (1996). Alteration of rat liver 20S proteasome activities by age and food restriction. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences* **51**, B316-B322.

Shibatani, T. & Ward, W. F. (1995). Sodium Dodecyl-Sulfate (Sds) Activation of the 20s Proteasome in Rat-Liver. *Archives of Biochemistry and Biophysics* **321**, 160-166.

Sidell, B., Wilson, F., Hazel, J. & CL, P. (1973). Time Course of Thermal Acclimation in Goldfish. *Journal of Comparative Physiology* **84**, 119-127.

Somero, G. N. (1995). Proteins and temperature. *Annual Review of Physiology* **57**, 43-68.

Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic "strategies". *Comparative Biochemistry and Physiology B-Biochemistry &*

Molecular Biology **139**, 321-333.

Speers-Roesch B. and Ballantyne J. S. (2005). Activities of antioxidant enzymes and cytochrome c oxidase in liver of Arctic and temperate teleosts. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology* **4**, 487-494.

Srere, P. (1969). Citrate synthase. In *Methods in Enzymology* (Lowenstein, J., ed.), pp. 3-11.

St-Pierre, J., Charest, P. M. & Guderley, H. (1998). Relative contribution of quantitative and qualitative changes in mitochondria to metabolic compensation during seasonal acclimatisation of rainbow trout *Oncorhynchus mykiss*. *Journal of Experimental Biology* **201**, 2961-2970.

Strand, H. K., Hansen, T. K., Pedersen, A., Falk-Petersen, I. B. & Oiestad, V. (1995). First feeding of common wolffish on formulated dry feed diets in a low water-level raceway system. *Aquaculture International* **3**, 1-10.

Sugden, H. & Fuller, J. (1991). Regulation of protein turnover in skeletal and cardiac striated muscle. *Biochemical Journal* **273**, 21-37.

Todgham, A. E., Hoaglund, E. A. & Hofmann, G. E. (2007). Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **177**, 857-866.

Treberg, J. R., Hall, J. R. & Driedzic, W. R. (2005). Enhanced protein synthetic capacity in Atlantic cod (*Gadus morhua*) is associated with temperature-induced compensatory growth. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **288**, R205-R211.

Turrens, J. F. (1997). Superoxide production by the mitochondrial respiratory chain. *Bioscience Reports* **17**, 38.

Velickovska, V., Lloyd, B. P., Qureshi, S. & van Breukelen, F. (2005). Proteolysis is depressed during torpor in hibernators at the level of the 20S core protease. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **175**, 329-335.

Winberg, G. G. (1960). Rate of metabolism and food requirement of fishes. *Fisheries Research Board of Canada* **194**, 202-211.

Woods, A. K. & Storey, K. B. (2005). Effects of hibernation on multicatalytic proteinase complex in thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*. *Molecular and Cellular Biochemistry* **271**, 205-213.

Zar, J. H. (1999). *Biostatistical analysis.* Upper Saddle River: Prentice Hall. 663p.